Bisphenol A affects axonal growth, musculature and motor behavior in developing zebrafish

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A B S T R A C T

Bisphenol A (BPA) is a ubiquitous contaminant in environment and human body. The reproductive and developmental effects of BPA exposure in aquatic and laboratory animals have been extensively studied. However, BPA exposure on the nervous system and motor behavior development are not well understood. In this study, we utilized zebrafish embryo as a model system to investigate the effect of developmental BPA exposure on larval teratology, motor behaviors, axonal growth of spinal motoneurons and muscle structure at various developmental stages. Our findings revealed that BPA exposure altered spontaneous movement, significantly decreased touch response and swimming speed in response to light stimulation in developing zebrafish. These effects were observed at the concentrations that did not yield any significant teratogenic effects. Correlated with those changes in swimming activity, BPA-induced axonal muscle damage occurred at the same concentration range (1–15 μM), but disruption of axonal growth of primary and secondary motoneuron occurred only at higher concentration (15 μM). BPA-induced apoptotic cell death subsequent to initial ROS formation and oxidative DNA damage may be the underlying mechanism for axonal muscle damage, suggesting the functional relevance of muscle structural changes and the observed deficits in swimming activity.

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1. Introduction

Bisphenol A (BPA) is a high production volume chemical widely used as an intermediate in the production of numerous consumer products including polycarbonate plastic and epoxy resins. Incomplete polymerization or gradual breakdown of BPA-containing products results in potential leaching of BPA into food or water stored in polycarbonate bottle or resin-lined cans. In human, widespread exposure to BPA is thought to occur primarily through ingestion food or water in contact use of these materials, resulting in an average circulating BPA level up to 3 ng/ml (Vanden Berg et al., 2010).

BPA is composed of two phenol rings and has structural homology with 17β-estradiol. BPA mimics estrogen actions in vivo and in vitro, and therefore recognized as an endocrine-disrupting chemical. The endocrine-disrupting effects of BPA and reproductive toxicities have been well documented in various aquatic and laboratory animal studies (Crain et al., 2007; Sohani et al., 2001). Most of those reported reproductive and developmental effects are attributed to BPA either acting as an estrogen receptor agonist or disrupting thyroid hormone system (Golub et al., 2010; Rubin, 2011; Taylor et al., 2011). While the effects of BPA on endocrine system have been extensively studied, its action on the nervous system is not well understood. Recent animal studies showed that BPA exposure impairs hippocampal neurogenesis and spatial learning and memory (Kim et al., 2011) and disrupts neocortical development by accelerating neuronal differentiation and migration in mice (Nakamura et al., 2006). Deficits in the development of synaptic plasticity (Zhou et al., 2009) and decreases in gene expression of dopamine transporters were observed in rats following developmental BPA exposure (Ishido et al., 2007). Perinatal BPA exposure affects nonsocial behavior (Farabollini et al., 1999), impairs sexual differentiation of exploratory behavior and increases depression-like behavior in rats (Fujimoto et al., 2006). In vitro, BPA affects neuronal differentiation and suppresses neurite extension in PC12 cells (Seki et al., 2011) and enhances motility and density of dendritic filopodia in the cultured hippocampal neurons with activation of NMDA receptor subunit NR2B via an ER-mediated signaling pathway (Xu et al., 2010).

BPA has been found in lakes, rivers and the ocean, as well as in sediments and soils. BPA in water bodies is most frequently the result of its presence in municipal wastewater discharges and in leachate from landfills (Huang et al., 2012). While most reported
levels in fresh water are low (<1 μg/L), some of the higher levels reported in the environment have caused adverse effects in laboratory experiments (Keiter et al., 2012; Lam et al., 2011; Staples et al., 2011; Wu et al., 2011). So far, a number of laboratory studies have shown BPA caused developmental and reproductive effects in aquatic animals including fish and shellfish (Chan and Chan, 2012; Molina et al., 2013). However, the neurotoxic effects following BPA developmental exposure on aquatic life have not been well studied. Zebrafish (Danio rerio) is emerging as an important vertebrate animal model in pharmacology, toxicology and ecotoxicology. The use of zebrafish for investigating the neurotoxicity of various chemicals has received increased popularity in recent years (Chen et al., 2012; Svboda et al., 2002; Yang et al., 2011), but its use for exploring BPA developmental neurotoxicity has just started. One recent study reported that developmental BPA exposure lead to early life-stage hyperactivity and learning deficits in adult zebrafish (Saili et al., 2012). However, the possible mechanisms underlying the observed neurobehavioral deficits following BPA exposure have not been explored. Compared with other model organisms, development of motor behavior in larvae zebrafish and its locomotion network has been well characterized. Embryonic motor behaviors appear in sequence and consist of an early period of transient spontaneous movement, followed by the emergence of twitching response to touch, and later by the ability to swim (Drapeau et al., 2002). Several types of neuronal cells within spinal cord as well as muscle cells were thought to be involved in the control of locomotion behavior in zebrafish (Brustein et al., 2003). In the present study, we utilized zebrafish embryo as a model and explored the effects of BPA developmental exposure on motor behavior, axonal growth of spinal motoneuron, and musculature morphology. At the molecular level, we further measured the level of reactive oxygen species (ROS), DNA damage, caspase-3 activity, and apoptotic cell death in whole zebrafish embryos since various pathologies in muscle or neuronal cells may result from oxidative stress-induced apoptotic signaling that is consequent to ROS increase and disruption of intracellular redox homeostasis, and irreversible oxidative modifications of lipid, protein, or DNA. The changes of these morphological and physiological endpoints during the early developmental stage may contribute to the abnormal development of neuronal networks and motor behavior in zebrafish. The objective of the present study was to investigate whether BPA developmental exposure induces motor behavior deficits and elucidate the potential cellular mechanism underlying the neurobehavioral deficits.

2. Materials and methods

2.1. Fish husbandry and embryo collection

Wild-type strain (AB) of zebrafish (Danio rerio) were raised and kept at standard laboratory conditions of 28 °C with a 14:10 light/dark photoperiod (lights on at 8:00 a.m.) in a recirculation system according to standard zebrafish breeding protocols (Westferferd, 1995). Water supplied to the system was filtered by reverse osmosis (pH 7.0–7.5) and Instant Ocean® salt was added to the water to raise conductivity to 450–1000 μS/cm. The adult fish were fed twice daily with live artemia (Jiaohong Feed Co., Tianjin, China) and dry flake diet (Zeigler, Aquatic Habitats, Apopka Florida, USA). Zebrafish embryos were obtained from spawning adults in tanks overnight with a sex ratio of 1:1. Embryos were collected within 1 h after the light was switched on and rinsed in embryo medium (Westferferd, 1995). The fertilized and normal embryos were inspected and staged using a stereomicroscope (Nikon, Japan) according to the previously described method (Kimmel et al., 1995). Fish care was in accordance with the approved protocol by the Institutional Animal Care and Use Committee at Wenzhou Medical College.

2.2. Chemical exposure and teratology screening

BPA [2,2-bis(4-hydroxyphenyl)propane, CAS#80-05-7, purity > 99%, Sigma] stock solution were prepared in 100% dimethyl sulfoxide (DMSO) and stored at −20 °C. Working solution was prepared by dilution of the stock solution immediately prior to experimental use. Selected embryos at 6 h post fertilization (hpf) were randomly distributed into 6-well plate (15 embryos in 5 ml solution per well), and exposed to BPA at 1, 5, 15 μM. The BPA concentrations used in this study were chosen in light of measured concentrations in aquatic systems (Huang et al., 2012), along with previously tested concentrations of BPA in fish (Keiter et al., 2012; Lam et al., 2011; Staples et al., 2011; Wu et al., 2011). Unless otherwise stated, all experimental exposure in this study were started at 6 hpf, but terminated at different time points with no media change during whole exposure period. The final concentration of DMSO was 0.1% across all treatment groups. Gross teratogenic response in zebrafish following BPA exposure was screened from 8 to 96 hpf based on previously published methods (Chen et al., 2012).

2.3. Locomotion analysis in larval zebrafish

Four types of motor behaviors were analyzed: spontaneous movement, touch response, free swimming activity and swimming in response to alternating light-to-dark photoperiod stimulation. To determine whether BPA exposure affect spontaneous movement, embryos were treated with BPA beginning at 6 hpf. Starting from 18 hpf, spontaneous movement of embryos (alternating tail bending or coiling) was videotaped for 1 min via a CCD camera (Nikon, Japan) mounted on a dissection microscope hourly until 30 hpf. All spontaneous movement recordings started 5 min after adaptation on the recording station. A total of 45 embryos in each treatment group from three replicate experiments were used for final spontaneous movement data analysis.

For touch response at 27 and 48 hpf, embryos were dechorionated with 0.1 mg/ml protease E (Roche, Germany) immediately at the end of BPA exposure. Larvae were allowed to adapt for 10 min in 96-well plate before touching starts. Fish response was evoked by touching the dorsal tail region with an eyelash probe and touch response was recorded using camcorder at a rate of 25 frames per second (fps). The time and the distance of tail bending (27 hpf) or swimming (48 hpf) after the initial touch was analyzed using Image-Pro Plus 6 based on previously described methods (Downes and Granato, 2004; Tallafuss and Eisen, 2008). A total of 45 embryos in each treatment group from three replicate experiments were used for touch response assessment.

Free swimming activity during 20 min visible light and larval swimming in response to a 70 min light-to-dark photoperiod stimulation were further assessed as detailed in our previous study (Chen et al., 2012; Huang et al., 2010). Briefly, a total of 90 embryos for each treatment group from three replicate experiments were used for these assays. Embryos were exposed to BPA in a 6-well plate from 6 to 96 hpf, then fish water was removed and wells rinsed three times. At 120 hpf, larvae were transferred to a new 24-well plate (one fish per well) for behavioral testing. The test was monitored with the Zebralab Video-Track system (Videotrack, version 3.5, ViewPoint Life Science, France) equipped with a Sony one-third inch Monochrome camera (Model DR2-HIBW-CSBOX, 30 fps) and an infrared filter. The entire record hardware is linked to the computer program and kept insulated from laboratory
environment in a sealed opaque plastic box (Zebribox, ViewPoint Life Science) during testing.

2.4. Immunohistochemistry

To facilitate the imaging of motoneuron axon at later developmental stage, embryos were raised in a 6-well plate with 0.0045% 1-phenyl-2-thiourea (PTU, Sigma) added to inhibit pigmentation development. Larvae were fixed with 4% paraformaldehyde (PFA) at different development time points and whole-mount immunohistochemistry staining was performed using well-characterized antibodies to visualize specific subsets of neurons and their axons based on previously described methods (Chen et al., 2012; He et al., 2011). Primary motoneurons were immunolabeled using znp-1 at 27 hpf (1:250, Developmental Studies Hybridoma Bank, University of Iowa); secondary motoneurons were immunolabeled using zn-5 at 48 hpf and 72 hpf (1:500, Zebrafish International Resource Center, University of Oregon). Briefly, embryos were fixed overnight at 4 °C and then washed three times (5 min each) with PBS containing 0.1% Tween-20 (PBST). The embryos were then incubated on ice with 0.005% trypsin in PBS for various lengths of time depending on the age of the embryos. The trypsin-treated embryos were washed 3 times with PBST and post-fixed in 4% PFA for 10 min at room temperature (RT). After washing twice (5 min each) in PBST, larvae were blocked with 10% normal goat serum (NGS) in PBS with 0.5% Triton X-100 for at least 1 h at RT. Primary antibody was incubated overnight at 4 °C in 1% NGS-PBST. Alexa Fluor 488 conjugated secondary antibody (Molecular Probes) was used to reveal the fluorescence signals. Samples were imaged with fluorescent microscope (Nikon, TE2000; Japan). A minimum of four segments in the trunk region spanning the vent were analyzed in each fish. Average axonal length from a total of 12 fish in each treatment group was quantified using ImagePro Plus software program (Media Cybernetics, Inc., Silver Spring, MD).

2.5. Transmission electron microscopy (TEM)

For ultrastructural analysis of axial muscle, zebrafish larvae were removed from 6-well plate at 96 hpf and fixed as previously described (Chen et al., 2012). Ultrathin cross-sections (80 nm) were mounted on copper grids and stained with uranyl acetate and lead citrate and examined under transmission electron microscopy (Hitachi H-7500, Tokyo, Japan) using an accelerating voltage of 70 keV.

2.6. Cellular death assays

To determine if BPA exposure induced inappropriate cellular death in zebrafish, two independent cellular death assays were exploited. Cell death was detected in live embryos using Acridine Orange (AO) staining based on the published method (Parneg et al., 2004). Briefly, zebrafish were exposed to BPA until 96 hpf and then stained with AO dye (5 μg/l) for 30 min. After washed 3 times (10 min each), larvae were checked for the distribution of necrotic cells under fluorescence microscope before transferring to 96-well plates for AO dye extraction. For quantitative reading, 50 μl 100% ethanol was added into each well and incubated at −20 °C for 30 min. 50 μl H2O were then added into each well for 10 min at RT. The relative fluorescent unit (RFU) of each sample was measured at 490 nm excitation and 530 nm emission with a microplate reader (Varioskan Flash, USA).

AO stains cells with disturbed membrane permeability, so it preferentially stains necrotic or very late apoptotic cells. To more specifically quantify cellular death due to apoptosis, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was performed on whole-mounted 96 hpf larvae with In Situ Cell Death Detection Kit (Roche Diagnostics Corp., Indianapolis). Embryos were exposed to various concentrations of BPA from 6 to 96 hpf. At the end of the exposure, embryos were fixed with 4% PFA, permeabilized and incubated with terminal transferase and fluorescein-dUTPs to specifically label cells undergoing apoptosis. Apoptotic cells were visualized and number of cells were quantified according to the previously described method (Usenko et al., 2007).

2.7. The activity of caspase-3 and ROS formation

The activity of caspase-3 and ROS concentration were measured based on the previous method with minor modification (Deng et al., 2009). Briefly, at the end of the exposure (96 hpf), 15 larvae from each group were collected and rinsed with PBS (pH 7.4) and homogenized in lysis buffer on ice for 20 min. Homogenates were centrifuged at 2000 × g at 4 °C for 15 min and supernatants were collected for subsequent measurements of caspase-3 activity (Beyotime Institute of Biotechnology, Haimen, China) and ROS concentration. For ROS measurements, 20 μl of homogenate was added to a 96-well plate and incubated at RT for 5 min. Then 100 μl of 10 μg/l dichlorofluorescein-diacetate (DCFH-DA, dissolved in DMDO) were added to each well and incubated at 37 °C for 30 min. The fluorescence intensity was measured using a microplate reader (Varioskan Flash, USA) with excitation at 485 nm and emission at 530 nm. The ROS concentration was expressed in arbitrary units (DCF/mg protein). For both caspase-3 activity and ROS concentration, six replicate samples from each treatment group were collected and measured.

2.8. Measurement of DNA damage by comet assay

DNA single-strand break in larvae was determined at 96 hpf by alkali single-cell gel electrophoresis (comet assay) according to the previous methods with minor modifications (Belpaeme et al., 1998; Erel et al., 2009; Jarvis and Knowles, 2003; Lee and Steinert, 2003). DNA damage from 15 samples in each group was automatically scored based on following parameters: head DNA %, tail DNA %, tail moment, olive tail moment using imaging analysis software Casp.

2.9. Quantitative real-time PCR

A set of 45 larvae from each treatment group were collected at 96 hpf in each replicate experiment (total three replicates). Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) per the manufacturer’s instructions. Genomic DNA contamination of RNA samples was removed by digestion with RNase-free DNase I (Promega, Madison, WI, USA). The quantity of total RNA was determined at 260 nm by Nanodrop and 0.5 μg of RNA was used to synthesize cDNA using PrimeScript® RT reagent kit (Takara, Japan). The primers for Bcl-2, Bax and β-actin were used as previously described (Deng et al., 2009). The sequences for Bcl-2: forward-TCCTCGTTCACAGCTCTCT, reverse-ACGGTTTCCAGCCCATC. The primer sequences for Bax: forward-GGCTATTCCAACAGGGTTCC, reverse-TGGCAATCCAAAGTGGT. The primer sequences for β-actin: forward-AAGCGAGGATCGACTCAC, reverse-TGGAGTCTCCAGTGCATT. Real-time PCR was carried out with Mastercycler® ep Realpore (Eppendorf, Germany) in sterile, 96-well PCR plates. The reaction mixtures included 10 μl of SsoFast™ EvaGreen® supermix with low ROX, 0.4 μM of primers, 4.2 μl of Milli-Q water, and 5 μl of cDNA. The thermal cycling program was set as follows: 30 s at 95 °C and followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The housekeeping gene β-actin was used as the internal reference and the comparative CT (∆∆CT)
method was used for the quantification of gene expression against control group as previously described (Chen et al., 2012).

2.10. Statistical analysis

One-way analysis of variance (ANOVA) was applied to determine the statistical significance followed by Tukey test to compare the differences among treatment groups using SPSS 16.0 (SPSS, Chicago, IL, USA). The value $p < 0.05$ was used as the criterion for statistical significance. The data for all groups were expressed as mean ± standard error (SEM). If data do not meet the criteria for one-way ANOVA, nonparametric tests were applied to data analysis or logarithmic transformation of data was performed.

3. Results

3.1. Teratogenic effects of BPA exposure

To screen for gross teratogenic effects resulting from BPA exposure, wild-type zebrafish exposed to BPA at various concentrations from 8 to 96 $\mu$g L$^{-1}$ were scored for teratogenic endpoints, including structural malformation (snout, jaw, otic vesicles, notochord, somite, fin or swim bladder), edema (heart, brain, pericardial and yolk sac), curvature of body axis, reduced pigmentation, hatch rate and mortality. However, we did not observed any significant differences among treatment groups under current exposure conditions as all groups exhibited 0–4% developmental abnormalities (data not shown).

3.2. BPA alters larval motor behaviors

Zebrafish motor behavior appears sequentially during development, which include an early, transient period of spontaneous tail coiling, followed by escape response to touch, and free swimming (Brustein et al., 2003). To determine whether BPA developmental exposures alter the development of motor behavior, zebrafish embryos were exposed to BPA at concentrations ranging from 1 to 15 $\mu$M and evaluated at different time points (from 18 to 120 hpf) for four types of locomotion behavior: spontaneous movement, touch response, free swimming activity and swimming in response to alternating light stimulation.

Spontaneous movement, also called spontaneous tail coiling, appears around 18 hpf in our fish rearing system. The alternating side-to-side contractions of the tail gradually reached a peak of 5 bends/min at 22 hpf in untreated embryos, and then decreased slowly to a low frequency of 2 bend/min at 29 hpf. We found that developmental exposure to BPA did not change the onset of spontaneous movement, but significantly decreased the frequency of bending and coiling from 27 hpf to 29 hpf when exposed to BPA at 15 $\mu$M (Fig. 1A). BPA exposure at 1 or 5 $\mu$M was not statistically different from controls.

The effect of BPA on the touch response was evaluated at 27 and 48 hpf. Touch response occurs in response to a tactile stimulus and serves to move fish in a direction away from the stimulus. Our results demonstrated that the movement of tail (27 hpf) and distance or time of swimming (48 hpf) in response to tail touch were significantly decreased when embryos exposed to 15 $\mu$g BPA (Fig. 1B–D). BPA exposure at 1 or 5 $\mu$M did not cause a significant change in both swimming time and distance when compared with controls (Fig. 1B–D).

Touch response escape swimming behavior involves sensory neurons as well as motor neurons networks. To distinguish the potential effect of BPA on sensory neurons versus general motor problems in embryos, we further tested free swimming activity independent of any touch stimuli in larvae at 120 hpf. For this test, embryos were exposed to BPA from 6 to 96 hpf, and raised in BPA-free fish water till 120 hpf before subjected to a 20-min light period swimming test. We observed a significant decrease in swimming speed following BPA exposure at all concentration tested (Fig. 1E), indicating the existing of motor problem.

Locomotion activities of larvae at 120 hpf were further assessed by a 70-min alternating light–dark photoperiod stimulation protocol (Chen et al., 2012). For normal larvae, a rapid transition from light to dark results in an initial burst of swimming and then activity in continuous dark period decreases gradually. When switched back to light, activity continues to drop and then increases slowly over next 10 min. Return to darkness produces another rapid and substantial increase in swimming activity. In this study, we observed a similar response pattern upon light–to-dark stimulation across all groups, indicating no significant visual effect. However, BPA exposure at concentration ranging from 1 to 15 $\mu$M significantly decreased swimming speed in response to alternating light stimulation (Fig. 1F). This result is consistent with the findings from 20-min free swimming test (Fig. 1E).

3.3. BPA inhibits axonal growth in zebrafish

The decreased motor activity during touch response, free swimming or in response to alternating light stimulation suggested a potential problem with spinal motoneuron, musculature or neuromuscular junction in zebrafish treated with BPA. Since primary and secondary motoneurons are two major cell types that mediate swimming behavior in zebrafish during early development, we first examined whether exposure to BPA altered patterns of axonal growth in primary and secondary motoneurons, and attempted to determine which cell type is more sensitive to the effect of BPA. Corresponding to the window of active growth for these cell types, embryos were sampled at 27, 48 and 72 hpf for the morphometric analysis of axonal growth. Embryos were immunostained with antibody zn-5, a marker for primary motoneuron at early developmental stage (27 hpf) and zn-5, a specific marker of secondary motoneurons and its axons at 48 and 72 hpf. Our results showed that BPA exposure significantly decreased ventral and dorsal axons from secondary motoneurons only at the highest concentration (15 $\mu$M). No significant effects on axonal growth of secondary motoneurons were observed at 1 and 5 $\mu$M (Table 1). However, primary motoneuron seems more sensitive to the inhibitory effects of BPA than secondary motoneuron. Significant decrease in the length of dorsal axons from primary motoneurons was observed at 5 and 15 $\mu$M. These results demonstrated that the altered axonal growth of primary or secondary motoneuron following BPA exposure is not closely correlated with the changes of swimming activity, where significant effects were observed at 1 $\mu$M.

3.4. Effect of BPA on musculature and neuromuscular junction formation

Normal motor behavior not only depends on the neuronal innervations, but also relies on the integrity of muscle structure and neuromuscular function. To investigate the potential effects of BPA on muscle, overall cell death of whole zebrafish was measured by acridine orange staining and TUNEL assay at 96 hpf. Our results demonstrated that BPA exposure ranging from 1 to 15 $\mu$M significantly induced both necrotic (Fig. 2A) and apoptotic cell death (Fig. 2B and C) in the musculature of trunk by a dose-dependent manner. To confirm the potential muscular damage, the structure of axial muscles in the trunk region was further examined under transmission electron microscopy (TEM). Apparent muscle fiber dissolving, mitochondrial swelling and nucleus condensation were noted in embryos exposed to BPA at all concentrations tested (Fig. 2D). In this study, we also examined the formation of
Fig. 1. BPA exposure altered motor behavior development in zebrafish. (A) Spontaneous movement (alternating tail bending and coiling) was recorded hourly starting from 18 hpf until 30 hpf (n = 45 fish per group). (B–D) Touch response was tested at 27 hpf and 48 hpf (n = 45 fish per group). (E) Free swimming activity and (F) locomotion in response to a 70-min alternating light stimulation was measured at 120 hpf after exposure terminated at 96 hpf (n = 90 fish per group). *p < 0.05, **p < 0.01, ***p < 0.001 in comparison with vehicle control.

neuromuscular junction (NMJ) in larvae at 96 hpf by fluorescence conjugated α-bungarotoxin, which specifically labels the postsynaptic nAChR at the NMJ. However, no significant changes in the distribution and formation of NMJ were observed in larvae following BPA exposure (data not shown).

3.5. BPA alters caspase-3 activity and gene expression related to apoptosis

Caspase-3 activity and mRNA expression of Bax and Bcl-2 were also evaluated in zebrafish at 96 hpf following BPA exposure. We
observed a significant increase in caspase-3 activity when embryos exposed to 5 and 15 μM of BPA (Fig. 3A). The change of caspase-3 activity was in general agreement with changes in TUNEL assay (Fig. 2B). Corresponding to the apoptosis observed at the protein level, mRNA expression of Bax was also significantly increased at the concentrations of 5 and 15 μM, with no change observed for Bcl-2 (Fig. 3C and D).

3.6. BPA induces ROS production and DNA damage

Since BPA exposure caused a significant necrotic or apoptotic cell death in whole zebrafish, we attempt to investigate whether this cellular effect was associated with ROS formation or DNA damage. We observed significant increase of ROS production in BPA exposed groups at 5 and 15 μM (Fig. 3B). Comet assay (single-cell gel electrophoresis) was used to evaluate DNA damage in zebrafish at 96 hpf. We found that BPA exposure from 6 to 96 hpf at all tested concentrations (1, 5 or 15 μM) significantly induced DNA damage as measured by increase in percentage of tail DNA, tail moment and Olive tail moment when compared with the controls (Fig. 4B–D). Further statistical analysis indicates a strong correlation between DNA damage and decreased swimming activity ($r = -0.86$, Figs. 1E and 4).

4. Discussion

In this study, we utilized zebrafish embryo as a model system to investigate the effect of developmental BPA exposure on the larval teratology, motor behavior development, axonal growth of spinal motoneurons and muscle structure. Our findings demonstrated that BPA exposure altered spontaneous movement, significantly decreased touch response and swimming speed in response to light stimulation in developing zebrafish. These effects were observed at the BPA concentrations that did not yield any significant teratogenic effects. Correlated with those changes in swimming activity, BPA exposure significantly induced axial muscle damage at same concentration ranges (1–15 μM) in addition to the inhibition of axonal growth at higher level, suggesting the functional relevance of these structural changes. Muscle structural damage may significantly contribute to the observed deficits in swimming activity.

Spontaneous movement is the first motor activity during the early life stage of zebrafish development. This behavior is not myogenic and originates from spinal cord (Brustein et al., 2003). Some studies speculated that spontaneous movement may correlate with the time at which primary motoneurons start innervating the muscle (Saint-Amant and Drapeau, 1998). Our data demonstrated that BPA exposure did not change the onset of spontaneous movement, but decreased the frequency of movement in embryos from 27 to 29 hpf at the level of 15 μM. Consistent with changes in spontaneous movement, morphometric analysis at 27 hpf indicated a decreased length of both ventral and dorsal axons from primary motoneurons when exposed to BPA at 15 μM, suggesting that primary motoneuron may be the major target of BPA in mediating spontaneous movement. Since there are several spinal neurons within spinal cord at this stage, BPA may also interfere with other cell types. Further studies are needed to address this speculation.

Zebrafish embryos normally respond to touch by 21 hpf. The response further evolves at 27 hpf when touching the tail results in a partial coil that elicits brief swimming episodes in dechorionated embryos (Brustein et al., 2003). Touch response behavior involves integration of local spinal motoneurons and their targeted muscle fibers following immediate activation of spinal Rohon-Beard sensory neurons in response to tail touch. In this study, the effect of BPA on touch response was evaluated at 27 and 48 hpf. Our results demonstrated that the movement of tail and distance of swimming in response to touch tail were significantly decreased following BPA exposure at 15 μM. Decrease in touch response may result from a specific effect of BPA on sensory neurons without any effects on spinal motoneuron and muscle network. To distinguish this possibility, we further tested free swimming behavior at 120 hpf in absence of touch stimuli. We observed a significant decrease in swimming activity at all concentrations tested, indicating the existing of motor problem. This motor problem was further confirmed in larvae at 120 hpf under a commonly used alternating light–dark stimulation protocol (Chen et al., 2012; He et al., 2011; MacPhail et al., 2009). BPA exposure ranging from 1 to 15 μM significantly decreased swimming speed in response to light stimulation. A similar response pattern upon light-to-dark stimulation was observed across all treatment groups, suggesting no adverse effect of BPA on the visual acuity of the larvae.

The deficits in touch response, free swimming and locomotion in response to alternating light stimulation suggested a potential structural or functional problem with spinal motoneuron, musculature or neuromuscular junction in zebrafish treated with BPA. Since primary and secondary motoneurons are two major cell types that mediate swimming behavior in zebrafish during early development (Brustein et al., 2003), we first examined whether exposure to BPA altered patterns of axonal growth in primary and secondary motoneurons, and attempted to determine which cell type is more sensitive to the effect of BPA. Corresponding to the window of active growth for these cell types, embryos were sampled at 27, 48 and 72 hpf for the morphometric analysis of axonal growth. Our results showed that BPA exposure significantly decreased ventral and dorsal axons from secondary motoneuron only at 15 μM. No significant effects on axonal growth of secondary motoneuron were observed at 1 and 5 μM. Primary motoneuron seems more sensitive to the inhibitory effects of BPA than secondary motoneuron since significant decrease in the dorsal axon length of primary motoneuron was observed at lower level (5 μM). Our results demonstrated that altered axonal growth of primary or secondary motoneuron are coincident with the changes in touch response measured at 27 hpf and 48 hpf following BPA exposure, suggesting a primary role for motoneuron in regulating touch response behavior. However, the altered axonal growth during early development is not correlated with the changes of free swimming activity measured at 120 hpf, where significant deficit in swimming activity were observed at lower concentration (1 μM), indicating that factors other than motor neuron might contribute to the behavior deficit as well.

### Table 1

Effects of BPA exposure on axonal growth of primary and secondary motoneurons in zebrafish.

<table>
<thead>
<tr>
<th>BPA (μM)</th>
<th>znp-1 positive axon (μm)</th>
<th>znp positive axon (μm)</th>
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<tr>
<td></td>
<td>Ventral (27 hpf)</td>
<td>Dorsal (27 hpf)</td>
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<td>28.2 ± 4.9</td>
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<tr>
<td>15</td>
<td>44.6 ± 5.3</td>
<td>5.7 ± 3.2***</td>
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*p < 0.05, **p < 0.001 in comparison with vehicle control (n = 12 fish per group).
Motor behavior not only depends on neuronal innervations, but also relies on the integrity of muscle structure and neuromuscular function. Furthermore, signals derived from somatic muscle can also influence axonal growth of motoneuron during embryonic development (Beattie, 2000; Zeller et al., 2002). Therefore, we further evaluated how BPA exposure may affect the axial muscle development in zebrafish. The axial muscle structure in the trunk region was examined under transmission electron microscopy. Apparent muscle fibers dissolving, mitochondrial swelling and nucleus condensation were noted in embryos exposed to BPA at
all concentrations tested (Fig. 2D). However, no significant changes in the distribution and formation of NMJ were observed in larvae following BPA exposure.

Both muscular damage and swimming behavior deficits are observed at the same concentration range tested in this study, suggesting the functional relevance of muscle structural changes. However, the cellular mechanism underlying the muscular damage is largely unknown. Reactive oxygen species (ROS) are products of normal metabolism and xenobiotic exposure, and depending on their concentrations, ROS can be harmful to cells and tissues. At physiological low levels, ROS function as “redox messengers” in intracellular signaling and regulation, whereas excess ROS induce oxidative damage of cellular macromolecules, inhibit protein function, cause DNA damage and promote cell death (Circu and Aw, 2010). Consistent with previous report (Wu et al., 2011), a significant increase of ROS formation in zebrafish was detected at 96 hpf following BPA exposure in our study. Corresponding to the trend of ROS formation, we also detected a significant DNA damage in all treatment groups by comet assay, as suggested by increase in percentage of tail DNA, tail moment and Olive tail moment (Fig. 4). In this study, cellular death was examined both qualitatively and quantitatively by AO staining and TUNEL assay. We observed a significant distribution of both necrotic and apoptotic cell in zebrafish, including the musculature of trunk region. Quantitative data demonstrated that BPA exposure ranging from 1 to 15 μM significantly induced both necrotic and apoptotic cell death in whole-mount zebrafish by a dose-dependent manner (Fig. 2).

Cell apoptosis is initiated by extracellular and intracellular signals via two main pathways: the death receptor-mediated and the mitochondria-mediated pathways (Circu and Aw, 2010).

Stimulation of the intrinsic mitochondrial apoptotic pathway by ROS and mitochondrial DNA damage promote outer membrane permeabilization and translocation of cytochrome c, AIF, or Smac, which triggers caspase-dependent or caspase-independent cytosolic signaling events. Various pathologies can result from ROS-induced apoptotic signaling that is consequent to ROS increases and/or antioxidant decreases, disruption of intracellular redox homeostasis, and irreversible oxidative modifications of lipid, protein, or DNA (Circu and Aw, 2010). A recent study demonstrated that the content of total glutathione, reduced glutathione (GSH), and oxidized glutathione (GSSG), as well as the activity of antioxidant enzymes including catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase were all significantly inhibited after exposure to BPA (0.004–4 μM) from 4 to 168 hpf, indicating the occurrence of oxidative stress (Wu et al., 2011). Consistent with this study, we detected a concurrent increase of ROS formation, DNA damage, mRNA expression of Bax and caspase-3 activity at 96 hpf following BPA exposure in zebrafish. Since our study did not measure these endpoints at different time points during zebrafish development, an interpretation of cause–effect relationship of these parameters cannot be derived. However, our data strongly suggest BPA exposure may induce apoptotic cell death via a mitochondria-mediated pathway.

In contrast to our study, one recent report demonstrated a non-monotonic dose–response in larvae (5 dpf) following developmental BPA exposure, where exposure to low level (0.01, 0.1 μM) lead to early life-stage hyperactivity and no hyperactivity when exposure level higher than 1 μM (Saili et al., 2012). This non-monotonic dose response in larvae locomotor behavior was not observed in our study. Certain discrepancies between these two
studies may explain the difference in the observed neurobehavioral changes, including zebrafish strain (AB in this study vs. tropical 5D in Saili’s study), exposure time (90 h in our study vs. 48 h in Saili’s study) and exposure concentration range (1–15 μM in this study vs. 0.001–10 μM in Saili’s study).

In summary, our study demonstrates that BPA altered motor behavior, decreased axonal growth of primary or secondary motoneuron, and induced muscle damage at the concentrations without significant teratogenic effect in zebrafish. BPA-induced apoptotic muscle cell death subsequent to initial ROS formation and oxidative DNA damage may be the underlying mechanism for axonal structural damage, which is closely correlated with decreased free swimming activity. However, because of the complex interplay between endocrine disruptions, neuronal function and motor behavior, further investigation into effects of BPA on the disruption of thyroid hormone, estrogen and neuronal function are needed, which may provide insight on the molecular mechanisms of BPA induced developmental neurotoxicity.

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