Early embryogenesis in zebrafish is affected by bisphenol A exposure

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Summary

Exposure of a developing embryo or fetus to endocrine disrupting chemicals (EDCs) has been hypothesized to increase the propensity of an individual to develop a disease or dysfunction in his/her later life. Although it is important to understand the effects of EDCs on early development in animals, sufficient information about these effects is not available thus far. This is probably because of the technical difficulties in tracing the continuous developmental changes at different stages of mammalian embryos. The zebrafish, an excellent model currently used in developmental biology, provides new insights to the field of toxicological studies. We used the standard whole-mount *in situ* hybridization screening protocol to determine the early developmental defects in zebrafish embryos exposed to the ubiquitous pollutant, bisphenol A (BPA). Three stages (60–75% epiboly, 8–10 somite, and prim-5) were selected for *in situ* screening of different molecular markers, whereas BPA exposure altered early dorsoventral (DV) patterning, segmentation, and brain development in zebrafish embryos within 24 hours of exposure.

Key words: Bisphenol A, Embryogenesis, Zebrafish

Introduction

The drastic advancement in industrialization and technology and the growth in human population in the past century have resulted in unprecedented environmental changes in the human history. The production of large amounts of synthetic industrial and biomedical chemicals as well as pollutants poses a risk to our ecosystem and induces negative effects on the health of wildlife and human beings. Some of the more damaging chemical contaminants are classified as endocrine disrupting chemicals (EDCs) because they can interfere with the synthesis, metabolism, and action of endogenous hormones (Phillips et al., 2008; Phillips and Foster, 2008). EDCs exert different biological effects via diverse mechanisms of actions (Judson et al., 2009; Rhind, 2008; Wigle et al., 2008). EDCs are believed to cause damages to human health and the ecological systems. With the emergence of the global problem of chemical contamination, the adverse biological effects of EDCs are gaining attention among the scientific communities, industry, governments, non-governmental organizations, and the public. There is an increasing need for the identification and quantification of all these ubiquitous chemical contaminants. The possible routes of exposure of humans to the EDCs are through the environments, consumer products, and foods (Feron et al., 2002; Mantovani et al., 2006; Poppenga, 2000; Wigle et al., 2008). To safeguard the public health, instrumental chemical analysis has been adopted globally for assessing the risk of human exposure to EDCs and their metabolites (Hotchkiss et al., 2008). Considering the severe long-term impact of EDCs on public health, a sensitive animal model is required to assess the risks of the EDCs for protecting human and ecological health.

Rapid structural and functional changes occur during the fetal life making it a vulnerable period of development. The process of development is not a simple process of unfolding the inherited genetic program, followed by the commitment of cells to specific lineages, and structural and functional differentiation in respective organs/tissues. Developmental plasticity in animals can be influenced by both genomic (epigenetic and genetic) and environmental factors, which leads to considerable changes in the developmental path for adaptive responses in the fetus (Bateson et al., 2004; Gluckman et al., 2009; Gluckman et al., 2008; Gluckman and Hanson, 2007; Gluckman et al., 2007; Gluckman et al., 2005a; Gluckman et al., 2005b; Hanson and Gluckman, 2008). To fill the information gap between exposures to EDCs and the outcomes of developmental failure, an experimental model that enables us to investigate the early developmental stages is essential. Zebrafish has been extensively used in developmental biology and has become an attractive model for chemical screening. This is a highly scalable model with a well-established genome database (Barros et al., 2008; Yeh et al., 2009; Zon and Peterson, 2005). This model has been used in general toxicology studies for decades. General toxicology studies such as identification of the median lethal concentration (LC_{50}) and end-point phenotype have been performed in zebrafish after bisphenol A (BPA) exposure (Duan et al., 2008; McCormick et al., 2011; Saili et al., 2012). Recently, next-generation sequencing technology was used to identify potential

genes that are altered after BPA exposure in zebrafish embryos (Lam et al., 2011). However, the unique developmental features of zebrafish have not been used in many studies for characterization of exposure to effect. It is difficult to monitor the effects of EDCs on early development by using mammalian embryos; therefore, zebrafish, which has the ability of external fertilization, is used as an alternative model. Gibert and his colleagues used different *in situ* molecular markers to examine the developmental stage of otolith formation in zebrafish after BPA exposure (Gibert et al., 2011). Here, we hypothesize that the primary action of EDCs is to prevent normal development during early embryogenesis and cell fate determination (i.e. cell signaling and epigenetic modification) and thus affect normal development (i.e. cell fate determination and organogenesis), which leads to organ dysfunction. In this study, we used the zebrafish model to show that exposure of zebrafish embryos to low doses of BPA caused disturbance in dorsal/ventral patterning and segmentation, which provides a new insight on developmental toxicology of environmental pollutants.

Materials and Methods

Fish strains and maintenance

We used the AB wild-type line in this study. The zebrafish were raised and staged as described previously (Kimmel et al., 1995). All experimental procedures on zebrafish embryos were approved by the Hong Kong Baptist University, Hong Kong Special Administrative Region.

BPA exposure in zebrafish embryos

BPA (Sigma–Aldrich, USA) was dissolved in DMSO and diluted in egg medium (E3 medium). BPA was used at a final concentration of 50 µM in all experiments, which is comparable to the concentrations used in other studies (Lam et al., 2011; Sun et al., 2009). Embryos at 1–4 cell stage were directly exposed to BPA in 2 ml of E3 medium in a 6-well plate. The embryos were grown at 28℃ for the selected time points (stages), 8 hours post-fertilization (hpf) (60–75% epiboly), 14 hpf (8–10 somite), 24 hpf (prim-5), and 72 hpf (protruding mouth). Control embryos were treated with equal volume of DMSO as that in the BPA-exposed embryos.

Screening procedure and whole-mount *in situ* hybridization

We used the whole-mount *in situ* hybridization (WISH) procedure for screening on the basis of our previous study (Tse and Jiang, 2012). Briefly, BPA-exposed embryos were collected at 3 stages 60–75% epiboly, 8–10 somite (ss), and prim-5 and were fixed in 4% paraformaldehyde (PFA). Standard WISH procedure was applied using zebrafish embryos. Plasmids that were used to make antisense mRNA probes have been published previously: *chd* (Miller-Bertoglio et al., 1997), *eng2* (Schier et al., 1996), *eve1* (Joly et al., 1993), *gata2* (Detrich et al., 1995), *gsc* (Stachel et al., 1993), *krox20* (Strähle et al., 1993), *myoD* (Weinberg et al., 1996), *pax2a* (Krauss et al., 1991), and *otx2* (Heisenberg et al., 1996).

Results and Discussion

BPA is one of the most common EDCs, and the chemical properties and toxicities of BPA have been reported. BPA is a selective estrogen receptor modulator (Richter et al., 2007) and can interact with thyroid hormone receptors (Moriyama et al., 2002; Zoeller et al., 2005) and peroxisome proliferator-activated receptors (Riu et al., 2011). At the physiological levels, BPA is suggested to be a factor attributed to the development of metabolic disorders in humans, such as cardiovascular diseases, obesity, and insulin resistance (Polyzos et al., 2012; vom Saal et al., 2012). A considerable number of studies in rodents have reported the negative effects of BPA on the function and development of reproductive and neuronal systems (Jašarević et al., 2011; Wolstenholme et al., 2011; Xi et al., 2011). More importantly, female mice prenatally exposed to BPA showed a decrease in fertility and fecundity (Cabaton et al., 2011) and had an adverse effect on the fertility of the male offspring (Salian et al., 2009). Furthermore, BPA administration in rodents could disturb neurons in the substantia nigra (Tando et al., 2007) and in the hippocampus (Kunz et al., 2011). In previous studies, zebrafish embryos have been exposed to BPA at concentrations similar to those used in this study, and otolith malformations (70 µM) and cardiac edema (65 µM) have been reported (Duan et al., 2008; Gibert et al., 2011). Although the general effects and the effects of BPA on development in rodents and zebrafish have been reported, important information about the effects of BPA in the initial stages of cell development remains to be addressed. To understand the mechanism underlying these effects is important because these data could reveal the fundamental cause of the observed effects; further, the data can be utilized to predict and evaluate the impact of *in utero* EDC exposure. In this study, we performed screening in the early stage of embryogenesis; we selected 3 critical stages, including dorsoventral (DV) patterning (60–75% epiboly), segmentation (8–10 ss), and brain development (prim-5), within 24 hpf (Tse et al., 2009; Tse et al., 2011).

BPA exposure of embryos at 60–75% epiboly stage disturbs DV patterning

DV patterning is an important developmental process in zebrafish (Schmitz and Campos-Ortega, 1994). Several zebrafish mutants have been identified on the basis of their dorsal or ventral phenotypes, which range from C5 (dorsal) to V4 (ventral) (Mullins et al., 1996; Kishimoto et al., 1997). In this study, we targeted on exposure to BPA at the early development period (within 24 hours). Follow-up examination of the effects of the exposure was performed up to 3 days after fertilization while mild dorsalization (mainly C1–C3) was observed. Dorsalization was characterized by their phenotype of shortened posterior parts during the development (Fig. 1). Because the DV patterning

C

Toxicant	µM	n	Normal %	C1 %	C2 %	C3 %
Ctrl	-	78	100	-	-	-
BPA	10	120	68	32	-	-
BPA	25	86	45	31	23	-
BPA	50	142	10	44	33	13
BPA	75	103	-	29	41	30

Fig. 1. Morphology and phenotypic frequency of 3-day post-fertilized embryos exposed to bisphenol A. Bisphenol A (BPA)-exposed embryos in an AB wild-type zebrafish showed mild dorsalized phenotypes at 3 days post-fertilization. The control embryos were treated with DMSO (**A**). BPA exposed embryos showed C1 to C3 mild dorsalization phenotypes (**B**). Scale bar: 650 µm. Phenotypic frequency is indicated in panel **C**. C1–C3 phenotypes represent dorsalized phenotype as described (Tse et al., 2009; Mullins et al., 1996). n, number of scored embryos.

occurs in the early stage of embryogenesis, the effects of the BPA action can be observed by using selected *in situ* molecular markers (ventral markers, *eve1* and *gata2*; dorsal markers, *chd* and *gsc*) at stage of 60–75% epiboly (Tse et al., 2009). Among various validated markers, *eve1* is a zebrafish homeobox gene similar to *even-skipped* in *Drosophila* (Joly et al., 1993). *eve1* is strongly expressed in the ventrolateral marginal cells. The other gene marker *gata2* is a hematopoietic transcription factor gene (Detrich et al., 1995) for ventral ectoderm and hematopoietic cells in the ventral mesoderm. To trace the dorsal patterning, we used 2 dorsally expressed markers *chordin* (*chd*) and *goosecoid* (*gsc*) (Sasai et al., 1995; Stachel et al., 1993). The expression patterns of *eve1* and *gata2* in embryos exposed to BPA were more restricted in the ventral half of the marginal and the animal zone than that in the controls (Fig. 2A–D). On the other hand, the expression of dorsal markers *chd* and *gsc* was greater in the embryos exposed to BPA (Fig. 2E–H). We measured the angles of expressions of the markers (Fig. 2I,J). Taken together, embryos at the 60–75% epiboly stage exposed to BPA showed reduced expression levels of the ventral markers but increased expression levels of the dorsal markers.

Exposure of embryos at 8–10 somite stage to BPA affects somatic muscle development

To monitor the trend of altered DV patterning, *gata1* and *pax2a* were used as the markers at 8–10 somite stage. *gata1* is ventrally expressed in presumptive hematopoietic cells in 2 lateral stripes (Detrich et al., 1995; Kimmel et al., 1990), while *pax2a* is used for marking the presumptive neural region (Krauss et al., 1991). The *gata1* marker showed widening of the 2 lateral stripes of presumptive hematopoietic cells in BPA-exposed embryos (Fig. 3A,B). Additionally, *pax2a* staining showed a diffused expression pattern in the mid-hindbrain boundary (mhb). The otic vesicles were missing in the embryos exposed to BPA (Fig. 3C,D). On the other hand, the somite muscle widened in BPA-exposed embryos (Fig. 3E,F). The phenotype was further confirmed by using the *myoD* somite marker that is expressed in the dorsal mesoderm and somite muscles (Kimmel et al., 1990; Weinberg et al., 1996). Weak and abnormal *myoD* expression was detected in the BPA-exposed embryos (Fig. 3G,H). In addition to DV patterning, the follow-up *in situ* experiments illustrated the effects of BPA on somatic muscle formation in the segmentation period.

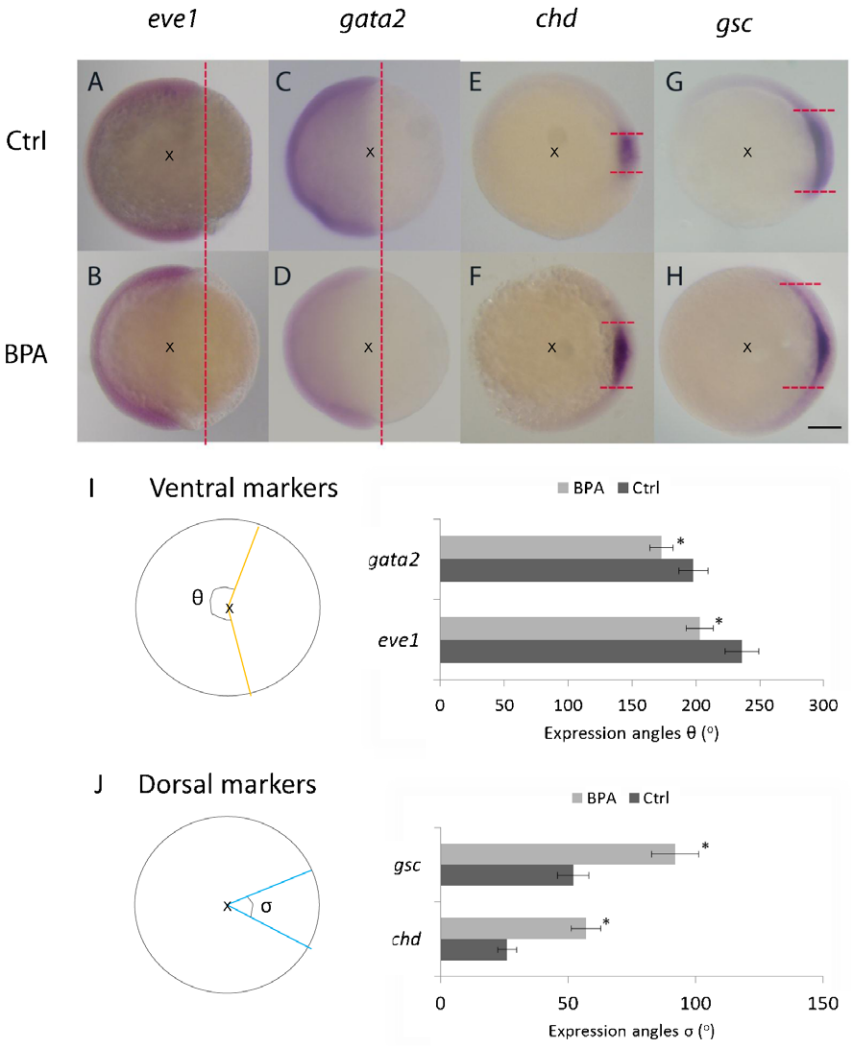

Fig. 2. Bisphenol A affects dorsal–ventral patterning at the 60–75% epiboly stage. Embryos exposed to bisphenol A (BPA) showed narrower expression pattern for the ventral markers *eve1* and *gata2* (**A–D**), but wider expression pattern for the dorsal markers *chd* and *gsc* (**E–H**). Red dotted lines indicate the normal expression margin of the ventral markers (ventricle) or dorsal (horizontal) in both BPA-exposed and control embryos. Images were captured in the lateral view (A–D) and animal pole view (E–H), dorsal towards the right in the 60–75% epiboly stage. Scale bar: 250 µm. Schematic diagrams indicate the expression angles of different markers. x marks the center of the embryos, angle of expression of different *in situ* markers in control and BPA-exposed embryos. θ indicates the angles of the ventral markers (*eve1*/*gata2*) with orange lines (**I**), while σ represents the angles of the dorsal markers (*chd*/*gsc*) with blue lines (**J**). The angles represent the mean of 20 embryos. The expression angles of the ventral markers were smaller (I) but those of the dorsal markers were larger (J), which represents the dorsalization phenotype (*P<0.05).

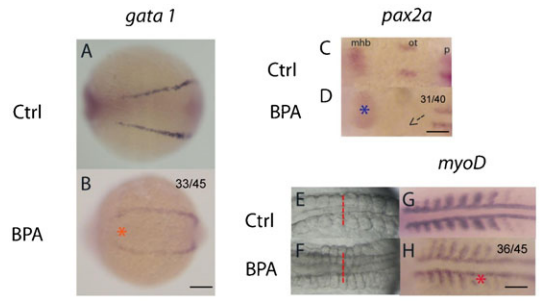

Fig. 3. Bisphenol A alters somite formation at the 8–10 somite stage. Lateral expansion of the presumptive hematopoietic cell marker (*gata 1*) was indicated by an orange asterisk, which indicates the dorsalized phenotype in the bisphenol A (BPA)-exposed embryos (**A**,**B**). *pax2a* expression at the 8–10 somite stage, dorsal view (**C**,**D**). A blue asterisk indicates abnormal developmental pattern in the mid-hindbrain boundary (mhb) in BPA-exposed embryos. Additionally, an arrow marks the missing of the 2 otic vesicles in BPA-exposed embryos (D). Somite morphology of the control (**E**) and the BPA-exposed embryos (**F**) at the 8–10 somite stage. Lateral expansion of somite muscles was observed (red dotted lines). The somite marker, *myoD*, showed widened and diffused expression in the BPA-exposed embryos (red asterisk) as compared to the control (**G**,**H**). mhb, mid-hindbrain boundary; ot, otic vesicle; p, pronephric precursor expression domain. All were head to the left. Scale bars: 75 µm (A,B); 200 µm (C,D); 150 µm (E–H). The number of embryos with the presented phenotype is shown in the top right corner of the panel.

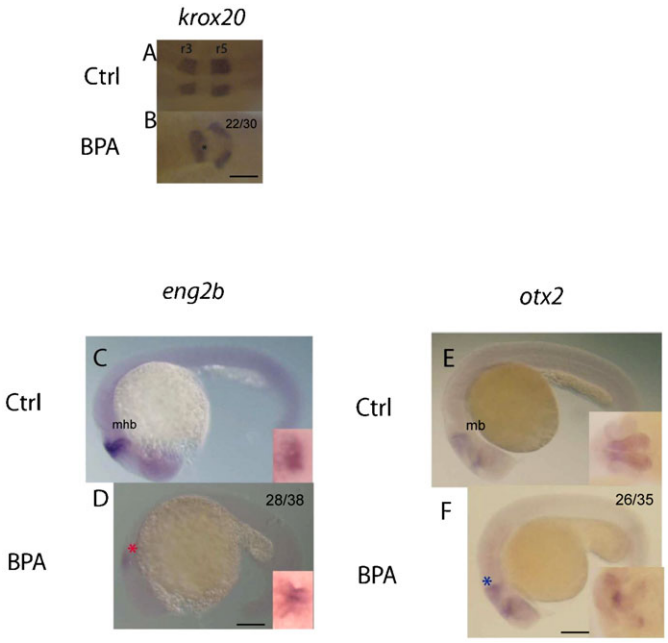

Fig. 4. Bisphenol A influences brain development in the prim-5 stage. The expression patterns of markers in different brain regions (*krox20*, *eng2b*, and *otx2*) at the prim-5 stage are shown. The expression of *krox20*, a marker of rhombomeres 3 and 5, dorsal view (**A**,**B**). Abnormal patterning of rhombomeres 3 and 5 (black asterisk) was found in BPA-exposed embryos (B), which indicates that regionalization was affected. The patterns of *eng2b* expression indicated that the mid-hindbrain boundary (mhb) was minimized in the BPA-exposed embryos (red asterisk). Compared to the controls (**C**), BPA-exposed embryos showed a smaller mhb (**D**), lateral view; magnified views of the dorsal region are shown in the inserts at the right bottom corner. *otx2* expression pattern was restricted in the BPA-exposed embryos (blue asterisk). Compared to the controls (**E**), the BPA-exposed embryos (**F**) showed reduced size of the midbrain. Magnified views of the dorsal section are shown in the corner. mb, midbrain; mhb, mid-hindbrain boundary; r3/r5, rhombomere 3/5. All were head to the left. Scale bars: 100 µm (A,B); 125 µm (C–F). The number of embryos with the presented phenotype is shown in the top right corner of the panel.

BPA exposure of embryos at the prim-5 stage alters brain development

On the basis of the diffused *pax2a* expression in the mhb region at the 8–10 somite stage, we suspected that BPA affected brain development during the developmental process. To prove this assumption, the genetic markers *krox20*, *otx2*, and *eng2b* were used to monitor the brain regionalization process at the prim-5 stage (Finkelstein and Boncinelli, 1994; Joyner and Guillemot, 1994; Stuart et al., 1994). Brain regionalization is one of the fundamental processes in the early stages of vertebrate brain development, including the formation of the mhb and its adjacent brain regions (Joyner and Guillemot, 1994). The hindbrain develops into a series of rhombomeres along the anterior–posterior axis of the neural tube. Rhombomeres are believed to be involved in neuronal organization in brain development (Moens and Prince, 2002), while rhombomere 3 (r3) and 5 (r5) could be identified using the transcription factor, *krox20* (Oxtoby and Jowett, 1993). In this study, BPA exposure resulted in abnormal and unorganized *krox20* expression in the prim-5-stage embryos (Fig. 4A,B). The reduced size of the mhb shown by the *eng2b* mhb structure marker (Ekker et al., 1992; Fjose et al., 1992) was also found in the BPA-exposed embryos (Fig. 4C,D). These data were consistent with the results of decreased *pax2a* expression pattern at the mhb region observed in the 8–10 somite stage embryos exposed to BPA (Fig. 3D). To support this observation, an additional marker *otx2* was used to confirm if BPA affects the development of midbrain structure (Mercier et al., 1995). The expression of *otx2* was decreased in the BPA-exposed embryos, which indicated that the midbrain development was also affected (Fig. 4E,F). The altered percentage was consistent from the early stage to later stages, which suggested the phenotypes might due to the defect in early development. Furthermore, it should be noted that the diffused expression patterns were unlikely to be caused by the developmental delay. Collectively, BPA exposure disturbed the process of brain regionalization, which resulted in the development of abnormal rhombomeres, restricted mhb, and smaller midbrain structure.

Although our study does not provide a detailed mechanism of how BPA affects development, it strengthens our understanding about the developmental defect caused by BPA exposure. The resulting phenotype can be caused by complicated crosstalk between signaling pathways. Further studies should be performed to understand how and why the molecular markers listed above were affected. Zebrafish used in this study can act as a screening model to focus the research on the specific time point, organ, and potential signaling pathway involved in development.

Conclusion

In this study, the standard *in situ* hybridization method was used to examine the effects of EDCs on early embryogenesis. We found that BPA exposure influences DV patterning, somite formation, and brain development in zebrafish embryos. Our study showed the potential use of zebrafish for validating the effects of EDC at a particular developmental stage.

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Competing Interests

The authors have no competing interests to declare.

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