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Effects of low-level hexabromocyclododecane (HBCD) exposure on cardiac development in zebrafish embryos

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Abstract Hexabromocyclododecane (HBCD) is one of the most widely used brominated flame retardants. In the present study, zebrafish embryos were exposed to HBCD at the low concentrations of 0, 2, 20 and 200 nM. The results showed HBCD exposure resulted in an increase in heart rate and cardiac arrhythmia after exposure for 72 h, though the survival rate and the whole malformation rate were not significantly affected. These results demonstrated that the heart might be a target of HBCD. Low-level HBCD exposure may not share the same mechanisms as exposure to high concentrations, since no obvious increase of apoptotic cells around the heart was observed in the HBCD-treated groups. It was observed that the expression of *Tbx5* and *Nkx2.5* was significantly elevated by HBCD treatment in a dose-dependent manner using real-time quantitative PCR, which may be mainly responsible for the alteration of heart rate, given that Tbx5 and Nkx2.5 are two factors regulating ventricle conduction. The mRNA expression of *RyR2* and *Atp2a2b* (SERCA2a) was up-regulated in the exposure group, which may be one of reasons to affect the normal heart rate, since SERCA2a and RyR2 play an important role in calcium ion transport of cadiomyocytes. However, HBCD exposure did not significantly change the expression of Actc1l, Tnnt2, and Myh6, which are mainly muscle contractile genes that play key roles in the formation of cardiac structure. These results were consistent with the lack of effect seen on the other measurements of cardiac function, end diastolic volume, end-systolic volume, stroke volume, and cardiac output.

Keywords HBCD · Developmental toxicity · Heart rate · Mechanism · Zebrafish

Introduction

Tetrabromobisphenol A, polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) are most widely used as brominated flame retardants in the world. HBCD is added as an additive or reactive component in a variety of polymers, such as polystyrene foams, high-impact polystyrene, and epoxy resins. These polymers are then used in a medley of consumer products, including electronics, textiles, foam furniture, and other building materials (Birnbaum and Staskal 2004). The commercial HBCD product usually contains three diastereomers α-, β-, and γ-HBCD, whereof γ-HBCD is the main component (>70 %) (Peled et al. 1995). The demand of HBCD has dramatically increased over the past three decades. According to data released by Bromine Science and Environmental Forum (BSEF 2001), the global production is about 16,700 t (56.9 % in Europe, 23.4 % in Asia). In 2007, the production capacity of HBCD in China was 7,500 t (Luo et al. 2010). Many studies have shown that HBCD can easily bioaccumulate and biomagnify through various food chains (Drottar et al. 2001; deBoer et al. 2002; Lindberg et al. 2004). Now, HBCD has been found in all

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environmental media such as atmosphere, sediments, aquatic system (Morris et al. 2004), and even in animal and human samples (Per et al. 2011), indicating its high bioavailable and bioaccumulative potential.

In China, monitoring of HBCD has been performed mainly in eastern and southern coastal areas. In two species of marine fish from nine Chinese coastal cities, the average total HBCD concentration is 3.7 ng/g lipid weight (lw.), the levels in Fuzhou and Xiamen are 0.85–4.9 and 0.78–5.9 ng/g lw, respectively (Xia et al. 2011). The concentrations of HBCD range from 12 to 330 ng/g lw in muscle of freshwater fish from the Yangtze River (Xian et al. 2008), and range from 27 to 44 ng/g lw in pooled muscle of skipjack tuna (*Katsuwonus pelamis*) collected from east china sea and offshore area of Taiwan (Ueno et al. 2006). The HBCD levels in fish mentioned above are lower than that from other regions of the world, especially in Europe. The highest reported HBCD levels in fish are 139 and 1,110 ng/g lw from pooled muscle of sole (*Solea solea*) in the Dutch Western Scheldt estuary (Janák et al. 2005), and individual values exceeding 10,000 μg/kg wet weight (Law et al. 2006).

HBCD has a very low acute toxicity, does not cause eye and skin irritation. The median lethal concentrations (LC50) in bluegill (*Lepomis macrochirus*) and golden orfe (*Leuciscus idus*) are above 100 mg/L and 10,000 mg/L, respectively, and the half maximal effective concentration (EC50) values in various fish are all above the water solubility of HBCD (Hardy 1999). A few studies have suggested that liver weight can be increased and that thyroid hormone system can be affected by HBCD (Darnerud 2003; Birnbaum and Staskal 2004; van der Ven et al. 2006). HBCD inhibits the plasma membrane uptake of neurotransmitters dopamine, glutamate and γ-amino-*n*-butyric acid into synaptosomes (Mariussen and Fonnum 2003). HBCD can impair spontaneous behavior, concerning learning and memory capability in mice (Eriksson et al. 2006). In rats, oral exposure to HBCD induces drug-metabolising enzymes probably via the pregnane X receptor and constitutive androstane receptor signalling pathways (Germer et al. 2006).

Although studies have reported HBCD can cause developmental toxicity, reproductive toxicity, neurotoxicity and endocrine disrupting effects on animals or human (Eriksson et al. 2006; Ema et al. 2008; Saegusa et al. 2009), limited information can be found about its cardiac toxicity. The heart rates are significantly reduced in zebrafish embryos exposed to HBCD (0.05, 0.1, 0.5, and 1.0 mg/L) for 96 h (Deng et al. 2009), but the exposure concentrations tested are greater than those measured in surface waters (Deng et al. 2009). In the present study, we investigated the effects of HBCD at 0, 2, 20 and 200 nM (0, 1.28, 12.83 and 128.3 μg/L) on zebrafish (*Danio rerio*) embryos. Our objectives were to validate: (1) whether low-level HBCD exposure causes cardiac abnormalities; (2) what is the mechanism of HBCD, at a low concentration, on cardiac defects.

Materials and methods

Chemicals

1,2,5,6,9,10-HBCD (>95 % purity) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Dimethylsulfoxide (DMSO, >99 % purity) was purchased from Sinopharm (Shanghai, China). HBCD was dissolved in DMSO to reach stock concentrations of 0.04, 0.4 and 4 mM.

Zebrafish exposure

Wild-type TU zebrafish were maintained according to routine procedures (Westerfield 2000). Thirty embryos within 1 h post fertilization (hpf) were exposed to 15 ml HBCD solution (nominal concentration: 0, 2, 20 and 200 nM), with the final DMSO concentrations of 50 μL/L. The control group received an equal volume of the solvent DMSO (50 μL/L) instead of HBCD. There were six replicates for each treatment solution in Petri dishes. The embryos were maintained at 28 ± 0.5 °C with a 14:10 h light/dark cycle, and the solutions were completely changed twice a day. Water parameters were: pH, 7.0; NH_3, <0.005 mg/L; nitrites, <0.03 mg/L; and dissolved oxygen, 7.5 mg/L.

In order to measure the developmental toxicity of HBCD, embryos were observed under a Nikon TE300 (Tokyo, Japan) microscope at 24, 48 and 72 hpf. The endpoints included survival rate, whole malformation rate and hatching rate. Morphological deformities included cardiac abnormalities, spinal deformity, altered axial curvature and tail malformation (Deng et al. 2009; Tyor et al. 2012).

Cardiac function analysis

Cardiac functions were determined from 20-s video segments for individual embryos (n = 6 for each treatment), which were selected randomly from each concentration after exposure to HBCD for 72 h. The segments were imaged with a digital video camera (Nikon, Tokyo, Japan). Followed by the method of Incardona et al. (2009), cardiac arrhythmia was obtained by determining the interbeat variability. The number of frames between cardiac contraction initiations was calculated using NIS-Elements Imaging Software (Nikon, Tokyo, Japan). The mean and standard deviations (SD) were analyzed for each embryo.

These deviations for individual embryos were then averaged to obtain a mean interbeat variability for each exposure group.

The end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), and cardiac output (CO) were measured and calculated using PureCodec Build as described by Chen et al. (2008). The equations used were as follows: SV = EDV − ESV, and CO = SV × HR (heart rate).

Acridine orange (AO) staining and caspase-3 activity measurement

We used acridine orange (Sigma, St. Louis, MO, USA) staining and caspase-3 activity measurement to identify embryo cell apoptosis after exposure to HBCD for 72 h. The staining procedure was performed as described by Chan and Cheng (2003). Briefly, embryos were stained with 5 μg/mL of AO at 28 °C for 20 min and washed three times with PBS, each for 5 min. Before examination, embryos were anaesthetised with 0.016 M tricaine (Sigma, St. Louis, MO, USA). Fluorescence was measured at excitation wavelength 490 nm and emission wavelength 520 nm by a fluorescence microscope (Nikon, Tokyo, Japan).

The enzyme activity was determined using caspase-3 colorimetric assay kit (Keygene, Nanjing, China) according to the manufacturer's instructions. Briefly, 500 μg of protein were diluted to 50 μl with lysis buffer, then added 50 μl reaction buffer. After that, the solution was incubated with 5 μl substrate at 37 °C for 4 h, and the absorbance value was read at 405 nm in a microtiter plate reader (Thermo, FL, USA).

RNA extraction and reverse transcription

The method of RNA extraction and reverse transcription has been described previously (Huang et al. 2012). Total RNA was extracted from the whole embryos using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA using a Revert Aid Mu-MLV cDNA synthesis kit according to the manufacturer's protocol.

Real-time Quantitative PCR

We performed QPCR analysis on an Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA, USA) using the Brilliant SYBR Green QPCR reagent kit (Stratagene) according to the manufacturer's protocol. Gene expression levels were normalized to zebrafish *Gapdh*. Standard curves and primer efficiencies were determined for all genes analyzed by QPCR. mRNA expression was calculated using the Relative Expression Software Tool (version 2) by the Pair Wise Fixed Reallocation Randomization Test© (Pfaffl et al. 2002). Primer sequences designed with software Primer Premier 5.0 are listed in Supplementary Table 1.

Data processing

Results are reported as mean ± SD. The data were checked for homogeneity of variance using the Levene statistic in SPSS. Then, data analysis was performed using SPSS statistics Version 17.0 (SPSS Inc., Chicago, IL, USA). Significant difference between control and treated groups was analyzed with one-way ANOVA followed by post hoc analysis according to Duncan. P values <0.05 were considered significant.

Results

Developmental toxicity

After exposure to HBCD for 72 h, the survival rate was not affected by any concentration of treatment (Fig. 1a). Pericardial and yolk sac edema was observed in the HBCD-treated groups [Supplementary material (SM), Fig. SM-1], but the rates of heart malformation and whole-embryonic malformation did not significantly change (Fig. 1b). The hatching rates in the HBCD-treated groups were higher than the control at 48 hpf, and a significant increase (by 1.8-fold) was observed in the 2 nM group compared with the control (Fig. 1c).

Cardiac dysfunction

It was apparent that the heart rate was affected by HBCD at different exposure times (Fig. 2). The heart rate in the group of 20 nM at 36 hpf and in the group of 200 nM at 48 hpf were significantly higher than that of the controls. There was a same tendency after exposure for 60 h and 72 h, the heart rates in all the treatment groups were significant increased compared with the control.

In the method of measuring cardiac rhythm, a regular rhythm would have a low SD. In the present study, the control embryos had established a regular rhythm, with a very low interbeat variability (±1.90 ms), while HBCD-treated embryos displayed a significant increase in irregularity of the rhythm, with a mean interbeat variability of ±2.77 and ±2.67 ms in the 20 and 200 nM groups (Fig. 3).

EDV, SV and CO did not show significant difference after exposed to HBCD for 72 h (Fig. SM-2), while there was a significant reduction of ESV in the 20 nM group compared with the control.

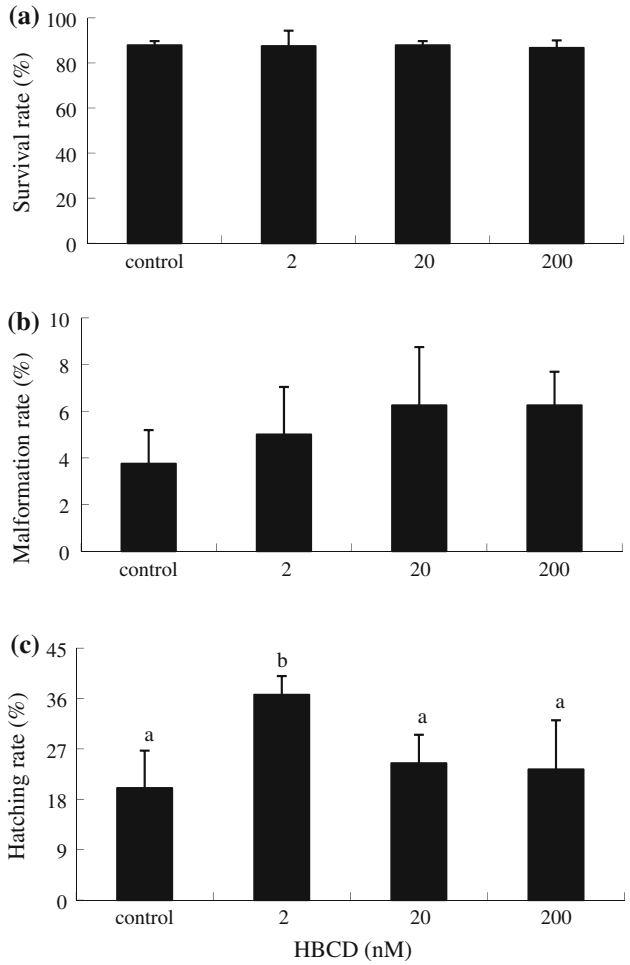

Fig. 1 Developmental effects of zebrafish embryos exposed to HBCD (0, 2, 20 and 200 nM). **a** Survival rate at 72 hpf; **b** whole malformation rate at 72 hpf; **c** hatching rate at 48 hpf. Results are reported as mean ± SD (n = 6). Means of exposures not sharing a common letter are significantly different at $P < 0.05$ as assessed by one-way ANOVA followed by the Duncan test

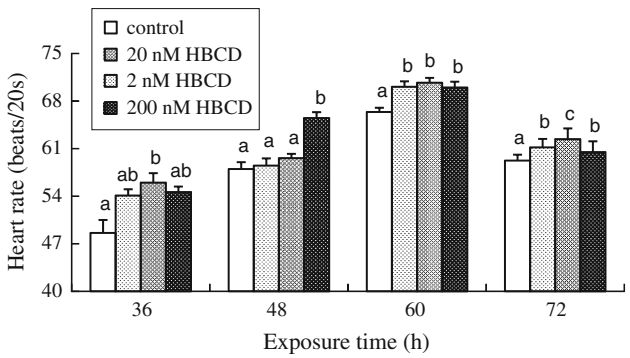

Fig. 2 Heart rate in the zebrafish embryos exposed to 0, 2, 20 and 200 nM HBCD for 36, 48, 60 and 72 h. Results are reported as mean ± SD (n = 10). Means of exposures not sharing a common letter are significantly different at $P < 0.05$ as assessed by one-way ANOVA followed by the Duncan test

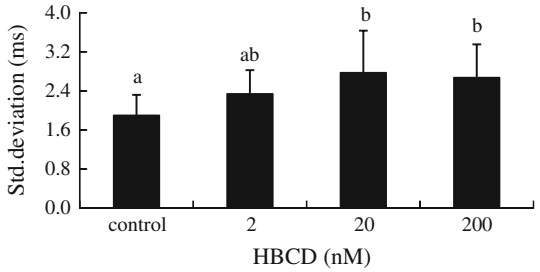

Fig. 3 Cardiac arrhythmia in zebrafish embryos exposed to 0, 2, 20 and 200 nM HBCD for 72 h. Results were reported as mean ± SD. (n = 6). Means of exposures not sharing a common letter are significantly different at $P < 0.05$ as assessed by one-way ANOVA followed by the Duncan test

Acridine orange staining and caspase-3 activity

The number of apoptotic cells and caspase-3 *activity* in the HBCD-treated groups showed no significant change compared with the control group (Figs. SM-3, SM-4).

Quantitative analysis of transcript levels of important genes

Five cardiac developmental related genes were analyzed by real-time quantitative PCR. A concentration-dependent elevation has be observed in the expression of *Tbx5* (1.78-, 1.91- and 2.03-fold) and *Nkx2.5* (1.47-, 1.46- and 1.54-fold) in the 2, 20 and 200 nM groups compared to the control. The mRNA expression of *RyR2* and *Atp2a2b* in the HBCD exposure fish was up-regulated and reached a significant change in the 200 nM group compared with the control. However, HBCD exposure did not cause significant alteration in the mRNA levels of *Atp2a2a, Actc1l*, *Tnnt2* and *Myh6* (Fig. 4).

Discussion

The hatched zebrafish by 72 hpf has completed most of its morphogenesis (Kimmel et al. 1995). So we chose the period of 72 hpf to investigate the majority of endpoints. In fish and aquatic mammals, HBCD has been reported to accumulate in livers, eggs, gonads, muscle, and adipose tissue (Janák et al. 2005; Xian et al. 2008). The highest concentration of HBCD occurs in zebrafish eggs after parental exposure (Nyholm et al. 2008). Fish embryos or larvae are more sensitive to HBCD than adult fish (Deng et al. 2009). HBCD (0.1, 0.5 and 1 mg/L) could increase malformation rate and reduce survival in zebrafish embryos, the mechanism of this developmental toxicity appears to be the generation of reactive oxygen species (ROS) and the consequent triggering of apoptosis genes

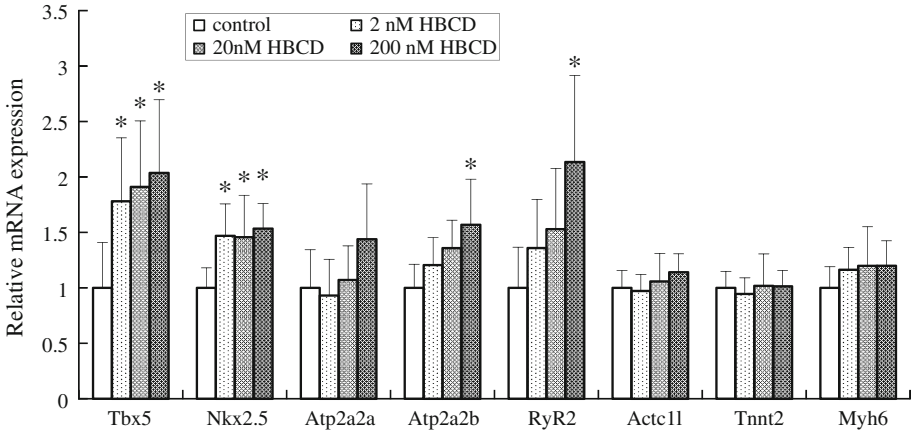

Fig. 4 Gene expression profiles in zebrafish embryos exposed to 0, 2, 20 and 200 nM HBCD for 72 h. Values were normalized against *Gapdh*. *Bars* represent the gene expression changes (mean fold change ± SD) compared with the corresponding control (n = 6, $*P < 0.05$)

(Deng et al. 2009). The generation of ROS is an important apoptotic signal (Livingstone 2001). However, exposure to 0.002–10 mg/L of HBCD for 96 h does not change the survival rate in the zebrafish embryos. Malondialdehyde contents (an indicator of lipid peroxidation) in 0.5, 2.5, and 10 mg/L group are significantly higher compared to the control, but have no significant alteration in 0.002, 0.01 and 0.1 mg/L group (Hu et al. 2009). These results suggest that low concentrations HBCD may not cause oxidative damage to zebrafish embryos. ROS-induced oxidative stress is one of the reasons causing abnormal development during embryogenesis (Yamashita 2003). In the present study, no effects were observed in survival, malformation in the fish embryos exposed to HBCD, which might be due to no oxidative damage and apoptosis caused by the low concentrations of HBCD. However, the hatching rate in the group of 2 nM was significantly affected compared with the control, which may be related to the mechanism of hormesis. The phenomenon has also been reported in exposure to bis(2-ethylhexyl) phthalate and benzo[a]pyrene (Chikae et al. 2004), methoxychlor (Versonnen et al. 2004) and perfluorooctane sulfonate (Wu et al. 2012).

The heart rates of the zebrafish embryos after exposure HBCD for 96 h are significantly reduced in all of the exposure groups (0.05, 0.1, 0.5, and 1.0 mg/L), and considerable numbers of apoptotic cells mainly appeared around the heart area, and the expression patterns of caspase-9 and caspase-3 are up-regulated, suggesting that this effect is possibly due to the high percentage of apoptotic cells in the heart (Deng et al. 2009). In contrast, the heart rates in the HBCD-treated group increased and abnormal heart rhythm appeared in the present study. The number of apoptotic cells and caspase-3 activity showed no significant change in the fish treated with the low concentrations of HBCD, which indicated that the alteration of heart rate was not caused by apoptosis.

In the present study, HBCD could induce an abnormal heart rhythm, which has not been previously reported. Zebrafish larvae (96 hpf) exposed to PBDE 47 had significant tachycardia, which progressed into atrioventricular block arrhythmias (Lema et al. 2007). Cardiac arrhythmia suggests the disturbance of the conduction system, which is a special structure to maintain the normal electrophysiological response and cardiac work (Hatcher and Basson 2009). Cardiac arrhythmia is closely related to hypertrophic cardiomyopathy, coronary heart disease, heart failure and rheumatic heart disease (Ghuran and Camm 2001; Lo and Hsia 2008; O'Mahony et al. 2012).

The expression analysis of genes was performed to investigate into the mechanisms of the alteration of heat rate and cardiac arrhythmia by real-time qPCR. Tbx5 and Nkx2.5 are two essential transcription factors, and they affect conduction system development and cardiac morphogenesis (Takeuchi and Bruneau 2009; Balci and Akdemir 2011). The cardiac-specific expression of *Tbx5* in mice can first be detected at embryonic day 7.5 in the cardiac crescent, and later in the posterior heart tube, ventricle and atria (Bruneau et al. 1999). According to Herrmann et al. (2011), overexpressed *Tbx5* induced cell differentiation and cause an earlier and increased appearance of contracting cardiomyocytes, which led to a higher frequent beat than control cells. Elevated *Nkx2.5* transcript levels have been detected in the developing cardiac conduction system, particularly in the atrio-ventricular bundle, bundle branches and Purkinje fibers (Thomas et al. 2001). They directly bound to the promoters of target genes to affect their transcription. Tbx5 and Nkx2.5 have been identified in patients with congenital heart diseases (CHD) and conduction system abnormalities, presenting atrial septal defects and atrioventricular conduction blocks (Schott et al. 1998; Moskowitz et al. 2004; Pashmforoush et al. 2004). Two different types of cardiac transcription factors are cooperatively required for specification of the ventricular conduction system and synergistically induce cardiac development (Hiroi et al. 2001; Moskowitz et al. 2007). Site-directed mutagenesis of the potential binding

sites revealed that Nkx2.5 was functionally positive for the expression of Tbx5 (Sun et al. 2004). In the present study, the up-regulation of *Nkx2.5* and *Tbx5* might be one of the main reasons causing the increase of heart rate.

Each contractile cycle in the heart, each heartbeat, requires an efflux of Ca^{2+} from the intracellular sarcoplasmic reticulum (SR) Ca^{2+} store through cardiac ryanodine receptor (RyR2) Ca^{2+} release channels, which is followed by the reuptake of Ca^{2+} into the SR by the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) Ca^{2+} pump (Dulhunty et al. 2012). Mice with genetically reduced RyR2 exhibit a lower basal heart rate and fatal arrhythmias (Bround et al. 2012). There are five distinct SERCA isoforms encoded by three separate genes (Lytton et al. 1992). SERCA2a, which is encoded by *Atp2a2a* and *Atp2a2b* in zebrafish, is expressed at high levels in cardiac myocytes (Ebert et al. 2005; Lai et al. 2011). Both decrease and increase of SERCA activity in the heart result in heart failure and arrhythmia (Nilüfer 2007; Schillinger et al. 2002; Yao et al. 1998). In the present study, the mRNA expression of *RyR2* and *Atp2a2b* (SERCA2a) showed an increased tendency after treatment with HBCD, which might be responsible for the arrhythmia.

Actc1l and *Tnnt2* are two major muscle contractile genes and the primary efficiency determinant of muscle contraction in heart, they play key roles in the formation of cardiac structure (Laing 2007; Qi et al. 2007). Mutations or abnormal expression of *Actc1l* and *Tnnt2* can cause hypertrophic cardiomyopathy (Maron 2002; Laing 2007). Reduced *Actc1* expression might contribute to the onset of CHD through induction of cardiomyocyte apoptosis (Jiang et al. 2010). Down-regulation of *Actc1* also leads to a dramatic decrease in cardiomyocyte proliferation and severe morphological defects including thin compact layer, disorganized trabeculae and ventricular septal defect (Qi et al. 2007). Nkx2.5 binds weakly to the serum response element of the cardiac a-actin promoter, resulting in modest activation of endogenous cardiac α-actin gene transcription (Chen et al. 1996). Tbx5-overexpression results in up-regulated levels of *Troponin T*, endogenous *Tbx5*, and *Nkx2.5* (Herrmann et al. 2011). Myh6 is also known to control muscle contraction in the heart. Myh6 is a fast ATPase, which expressed at relatively low-levels in the prenatal heart and is up-regulated during the early postnatal period (Morkin 2000). The cardiac transcription factor Tbx5 strongly regulates expression of *Myh6*, but mutant forms of Tbx5 do not (Ching et al. 2005). In this study, no significant changes of *Actc1l*, *Tnnt2*, and *Myh6* expression were consistent with the stabilization of cardiac function such as EDV, ESV, SV and CO.

In summary, this study showed that exposure to low concentrations of HBCD elevated the heart rate of zebrafish embryos, which is different with that in zebrafish exposed to high concentrations of HBCD. The results demonstrated that the heart might be a main target of HBCD. The irregular heartbeat and arrhythmia were mainly due to the up-regulation of *Tbx5*, *Nkx2.5*, *Atp2a2b and RyR2* mRNA expression that plays a central role in cardiac development, since no apoptosis was observed in the embryos exposed to low concentrations of HBCD.

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