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Research paper

Developmental toxicity and cardiac effects of butyl benzyl phthalate in zebrafish embryos

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ABSTRACT

Phthalic acid esters (PAEs), commonly called phthalates, have become ubiquitous environment pollutants. Studies have focused on reproductive toxicity, neurotoxicity, teratogenicity, tumourigenesis, and mutagenesis of phthalates. However, relatively little is known about the phthalates effects on the heart. Butyl benzyl phthalate (BBP), a member of PAEs, is classified by the US Environmental Protection Agency as a priority environmental pollutant. We studied the developmental toxicity of BBP, especially its effects on the heart development, in zebrafish (*Danio rerio*) embryos. Embryos at 4 hr post-fertilization (hpf) were exposed to 0, 0.1, 0.6 and 1.2 mg/L BBP until 72 hpf. BBP caused abnormalities in embryo morphology, including yolk-sac edema, spinal curvature, tail deformity, uninflated swim bladder and cardiac defects. Exposure to 0.6 mg/L BBP significantly increased the malformation rate, caused growth inhibition, increased the cardiac malformation rate as well as the distance between the sinus venosus (SV) and bulbus arteriosus (BA), and reduced the heart rate of embryos. Exposure to 1.2 mg/L BBP significantly affected all endpoints, except survival rate at 24 hpf. To preliminarily elucidate the potential mechanism of heart developmental toxicity caused by BBP, we examined the expression of two genes related to heart development, Nkx2.5 and T-box transcription factor 5, by real-time quantitative PCR. The expression of the two genes was dose-dependently downregulated with BBP. BBP could induce developmental toxicity, with adverse effects on the heart development in zebrafish embryos, and alter the expression of genes related to heart development.

1. Introduction

Among hazardous pollutants, phthalic acid esters (PAEs), commonly called phthalates, are used extensively as plasticizers for flexible polyvinyl chloride (Chai et al., 2014), and as non-plasticizers in consumer products, including medical devices, building materials, paints, pesticides, fertilizers, and food packaging (Wan et al., 2013; Zhang et al., 2014). Millions of tons of phthalates are produced annually (Liang et al., 2008). Because phthalates are not covalently bound to the plastic matrix or other chemicals in formulations (Bang du et al., 2011), they can leach, migrate or evaporate into the indoor air and atmosphere, foodstuff, and other materials. Phthalates have been found in water, air, sediment, soil, food and aquatic organisms (Bang du et al., 2011); they have become ubiquitous environmental pollutants. Dimethyl phthalate, dibutyl phthalate, butyl benzyl phthalate (BBP), diethyl phthalate, diethylhexyl phthalate and di-*n*-octyl phthalate are classified by the US Environmental Protection Agency as priority environmental pollutants. Studies have investigated the phthalate uptake by diet, dust ingestion, inhalation, and direct skin contact with phthalate-containing products (Heudorf et al., 2007). Phthalates is not easily degradable and can accumulate in the tissues of various organisms. Therefore, they can accumulate in the food chain by biomagnification as one organism consumes food lower in the chain and is then consumed by an organism higher in the chain (Chatterjee and Karlovsky, 2010). Humans are generally at the top of the chain, which increases their exposure (Chatterjee and Karlovsky, 2010). Phthalates and/or their metabolites have been detected in the saliva and urine of children and adults, amniotic fluid of second-trimester foetuses, cord blood of newborns, human breast milk, and urine of neonates in intensive care units (Howdeshell et al., 2008).

The environmental pollution and food safety problems caused by phthalates have attracted extensive attention worldwide. Studies have focused on the reproductive toxicity, neurotoxicity, teratogenicity, tumourigenesis, and mutagenesis of phthalates (Bang du et al., 2011; Wang et al., 2012; Abdul-Ghani et al., 2012; Holahan and Smith, 2015). However, relatively little is known about the effects of phthalates on the heart.

Congenital heart defects (CHDs) are defects in the structure of the

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heart and great vessels that occur at birth, and are the most frequent major malformations seen at birth worldwide. CHDs account for nearly one third of infants with major congenital anomalies diagnosed prenatally or during infancy in Europe (Dolk et al., 2011), occurs in 7–8/1000 live births in China (Wang et al., 2013), and an estimated 32,000 infants with congenital heart disease (CHD) are born each year in the United States (Roger et al., 2012). After years of study, the aetiology of CHD remains unknown, but is commonly believed to involve the interaction of multiple environmental and genetic factors (Pierpont et al., 2007; Jenkins et al., 2007). Environmental chemicals have been shown to have critical roles in the etiology of many human diseases. Therefore, whether the worldwide prevalence of CHD is associated with the worldwide use of phthalates is of interest. Singh and Li analysed the curated interactions between 16 phthalates and genes/proteins by using the Comparative Toxicogenomics Database. Analysis showed that the top three phthalate toxicity categories were cardiotoxicity, hepatotoxicity and nephrotoxicity, and the greatest risk for human disease was cardiovascular diseases, followed by liver diseases, urologic diseases, endocrine diseases, and genital diseases (Singh and Li, 2011). A case–control study showed parental occupational exposure to phthalates associated with increased risk of some CHD phenotypes (Wang et al., 2015). Data support that CHD is associated with phthalates (Singh and Li, 2011; Wang et al., 2015). Further research is needed to elucidate a possible experimental relation between CHD and phthalates.

BBP is widely used in plastics, the automotive industry, lubricants, cosmetics, clothing, pesticides and other industries (Martín et al., 2008; Zhang et al., 2014). Because of its slow hydrolysis, photolysis, volatility, high production volume and widespread use, BBP has been considered a major global environmental pollutant, representing the “second global PCB (polychlorinated biphenyls) pollution” (Zhang et al., 2014). Kohn et al. (2000) estimated that 95% of people in contact with BBP are exposed to approximately 4 μg/kg body weight/day, and BBP is efficiently absorbed, metabolized, and excreted (Martín et al., 2008). Therefore, we need to investigate the toxic effects of BBP on organisms and the environment, to provide a scientific basis for establishing an ecological risk assessment and food safety standards.

Zebrafish (*Danio rerio*) has been a prominent model vertebrate, and it is useful for developmental and toxicological studies, with many advantages over other species, such as high fecundity, transparency of embryos, rapid development, advanced genomic resources as well as similar organ systems and gene functions as humans (Bakkers, 2011; Bailey et al., 2013; Dai et al., 2014). Zebrafish is especially suitable for studying cardiovascular development and cardiotoxicity (Heideman et al., 2005; Bakkers, 2011) because cardiac defects do not cause immediate lethality as in many vertebrate models and zebrafish embryos can survive the first 7 days of their development without cardiovascular function (Stainier, 2001). This situation allows for readily observing the progressive development of cardiac abnormalities.

Here, we studied the developmental toxicity of BBP, focusing on its effects on the heart development, by using zebrafish embryos as a model. Endpoints were survival, hatching success, growth, malformation, and cardiac structure and function. We also examined the expression of two genes associated with heart development to preliminarily elucidate the potential mechanisms of the cardiac toxicity caused by BBP.

2. Materials and methods

2.1. Chemicals

BBP (> 99% purity) and 1-phenyl-2-thiourea (PTU) were from Sigma-Aldrich (St. Louis, MO, USA). Tricaine (MS-222), used for anaesthesia, was from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was used as the vehicle to solvate the chemicals from Sinopharm (Shanghai). All other chemicals were of analytical grade.

2.2. Embryo collection

Wild-type zebrafish (AB) were maintained and embryos were collected as described (Westerfield, 2000). Briefly, embryos were collected during the first hour of the light period in the 14:10 h light-dark cycle, then incubated in fresh egg water (60 mg/L Instant Ocean Salts) (Westerfield, 2000). At 4 h post-fertilization (hpf), embryos were examined under a stereo-microscope (SZX-16, Olympus, Tokyo, Japan) and embryos developing normally at blastula stage (30% epiboly) were used for subsequent experiments.

2.3. Embryo exposure

Zebrafish embryos at 4 hpf were exposed to a series of BBP doses, 0, 0.1, 0.6 and 1.2 mg/L (about 0, 0.3, 1.9 and 3.8 μmol/L), and the exposure time was up to 72 hpf. The exposure was carried out using glass petri dishes with 35 embryos in 7 mL of the control or BBP solutions. 35 embryos were exposed to every dose and six replicates were run for each concentration. The pH and dissolved oxygen (DO) of egg water were 7.2 ± 0.2 and 6.5 ± 0.2 mg/L, and were monitor daily. DMSO was used as a carrier solvent for BBP in fresh egg water (60 mg/L Instant Ocean Salts) (Westerfield, 2000); the final concentration of DMSO used was 0.01% (v/v). The control group received 0.01% DMSO. Each group contained 0.003% PTU (w/v) to inhibit the formation of pigment cells. Embryos were kept with 28 °C and 14:10 (light: dark) condition. The solutions were changed completely once daily, and dead embryos and detritus were removed daily.

2.4. Embryo development assessment

The development of zebrafish embryos was monitored by using a SZX16 stereo microscope equipped with a DP72 camera and DP72 software (Olympus, Tokyo). The survival of zebrafish embryos was examined at 24, 48 and 72 hpf, and hatching success, malformation and body length were examined at 72 hpf. Mortality was identified by missing heartbeat, coagulation of the embryos, failure to develop somites, and a non-detached tail. The length of each embryo along the body axis from the anterior-most part of the head to the tip of the tail was measured by using digital images produced with Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA). 8 embryos randomly selected from each concentration were used for taking the morphological image and measuring the body length. Four replicates were run for each concentration.

2.5. Assessment of the effect of BBP on the heart

BBP-treated zebrafish embryos at 72 hpf were anesthetized by using 0.0168% Tricaine and mounted on 3% methylcellulose. The looping of the heart tube was quantified by measuring the distance between the sinus venosus (SV) and bulbus arteriosus (BA) as described previously (Antkiewicz et al., 2005). Heart rates of zebrafish embryos were counted by visual observation in 20-s intervals under the stereo microscope. 8 embryos used for the assessment of the effect of BBP on the heart were the same as those used for the image capture and body length measurement. Four replicates were performed for each concentration.

2.6. Real-time quantitative PCR assay

Total RNA was extracted from 12 embryos randomly selected from each concentration at 24 and 72 hpf using TRIzol reagent (Invitrogen). Approximately 2 μg of total RNA was used for reverse transcription for first-strand cDNA with Superscript II reverse transcriptase (Invitrogen, USA). Real-time quantitative PCR, with the first-strand cDNA used as a template, involved use of the primer sequences for β-actin (5′-TGGCTTCTGCTCTGTATGGC-3′ and 5′-CCCTGTTAGACAACT