Full Length Research Paper

Combined exposure of di (2-ethylhexyl) phthalate, dibutyl phthalate and acetyl tributylcitrate: Toxic effects on the growth and reproductive system of zebrafish (Danio rerio)

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Received 16 October, 2016; Revised 11 November, 2016; Accepted 03 January, 2017 and Published 21 January, 2017

This study examined the growth and reproductive toxicity of combined plasticizer mixture of two phthalate esters of di (2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP) and one phthalate substitute of acetyl tributyl citrate (ATBC) to adult zebrafish after three-month exposure at an environmentally relevant concentration (50 µg/L). Obtained data demonstrated significant adverse effects on the growth and reproductive system of adult zebrafish, as evidenced by significant decrease in body lengths, weight and gonado-somatic index, impairment of fecundity, disruption of oogenesis and spermatogenesis, etc. Male zebrafish were more sensitive to the combined exposure with respect to relatively lower fecundity and testis in male were damaged more than the female ovaries observed. The maturation of oocytes was inhibited as evidenced by more underdeveloped perinucleolar and early cortical alveolar oocytes as well as atretic oocytes in the exposed ovary. In contrast, most of the spermatocysts were broken and almost all of the spermatogenetic cells of spermatogonia, spermatocytes and spermatids were lost in the exposed testis, inferring that Sertoli cells might be the main target cells of the combined plasticizer mixture and subsequent infertility of the exposed male fish. Thus great concerns should be raised on the reproductive safety of aquatic organisms when exposed to combined plasticizer mixture.

Keywords: Combined exposure, plasticizer, reproductive toxicity and zebrafish.

INTRODUCTION

Plasticizers of di (2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP) are two most commonly used phthalate esters (PAEs) with similar molecular structure but different side ester chains, representative of high and low molecular weight, respectively. DEHP and DBP, as synthetic industrial chemicals, are widely used in plastic polymers primarily in polyvinylchloride to enhance their flexibility and workability (Stales et al. 1997). As imparted plasticizers, they constitute up to 40 percent of the total weight of some plastics, but do not covalently bind to the polymers, hence easily leaching out over time into the environment (Bauer and Herrmann 1997). Today, DEHP
and DBP have been ubiquitously detected in the worldwide water, soil and air, and even the human urines due to the extensive consumption of PAEs-containing goods such as food packaging, cosmetics, pharmaceuticals, medical devices, toys, building materials, etc. (Latini 2005; Lin et al. 2009; Xu et al. 2013; Zeng et al. 2008). Thus, great concerns have been raised on the safety of PAEs exposure to environment and organisms (Jobling et al. 1995).

Accumulated evidences have shown that DEHP and DBP have weak endocrine disruption activity, imposing developmental and reproductive toxicity on both male and female mammals after extremely high dose exposure (Agarwal et al. 1989; Anas et al. 2003; Andrade et al. 2006; Barlow and Foster 2003; Guerra et al. 2010; Herreros et al. 2013; Kim et al. 2004; Li et al. 2000; Liu et al. 2009; Tyl et al. 1988; Xi et al. 2012; Zhang et al. 2013). There were also sufficient evidences suggesting that DEHP and DBP could cause liver cancer in rodents through the activation of peroxisome proliferator-activated receptor (Ryu et al. 2007). Among phthalate esters, DEHP and DBP are the most widely used plasticizer, where reports on the concentrations of phthalate esters (PAEs) in the marine environment discovered in surface sea water, surface sea sediment and marine organisms were 0–300 µg/L, 3µg/g and 4.07ng/g (Liu et al. 2009).

The aquatic environment is the major repository of pollutants. The sperms, eggs and juveniles of aquatic organisms are usually more sensitive to toxicants than adults. Therefore, in the PAEs exposure, aquatic organisms are under higher risks than terrestrial organisms with respect to their feature of spawning, fertilization and development in vitro. (Ohtani et al. 2000)reported the disruption of gonadal sex differentiation of male tadpoles of Ranarugosa by DBP exposure. (Yang et al. 2009)examined the toxic effects on the embryogenesis and larvae development of abalone by DEHP and DBP. In addition the urban lakes of Guangzhou, China, such compounds are unremarkably discovered in the aquatic environment, the detected concentrations were 0.94 to 3.60 µg/L DBP and 0.015 to 0.32 µg/L DEP, whereas the concentrations of DBP were detected between 0.35 µg/L and 40.68 µg/L in Haiwe river respectively (Wang and Chi 2012).

In contrast, fish have been more extensively studied to assess the endocrine disruptor activity of DEHP or DBP to zebrafish (Carnevali et al. 2010; Chen et al. 2015; Corradetti et al. 2013; Ortiz-Zarragoitia and Cajaraville 2005; Uren-Webster et al. 2010; Ye et al. 2014)and Chinese rare minnow(Wang et al. 2013). For example, three-week exposure to environmentally relevant concentration of DEHP (ranging from 0.02 to 40 µg/L) was found to be associated with impaired spermatogenesis and decreased fertilization capacity of male zebrafish (Corradetti et al. 2013), and a significant decrease in ovulation and embryo production in female zebrafish (Carnevali et al. 2010). Similar results were also observed in the male zebrafish after short-term and higher-dose exposure to DBP (Ortiz-Zarragoitia and Cajaraville 2005). Up to date, all the published toxic data in fish were obtained from individual exposure of DEHP or DBP, or combined exposure of DBP and strong estrogen of 17α-ethynylestradiol (EE2). However, no work has been done on the combined exposure of DEHP and DBP in fish species (Chen et al. 2015; Oehlmann et al. 2009).

The acetyl tributyl citrate (ATBC) is a newly produced plasticizer as a phthalate substitute to be used in children's articles and pharmaceuticals. This high-production-volume chemical has been approved by the U.S. Food and Drug Administration (FDA) for use as a food additive and food contact substance due to its extremely low oral toxicity in mammals (Finkenstei and Gold 1959; Oehlmann et al. 2009; Toxicology-Regulatory Services 2003). But it is reported to have a higher leaching rate from plastic polymers than DEH (Commission 2007). Thus, the widespread distribution of ATBC can be anticipated in the future and concerns are also raised on the safety of ATBC on environment and organisms. Although the environmentally concentration of ATBC was still unknown to our knowledge, the environmentally concentration ranges of DEHP and DBP have been widely reported in the marine surface water (0-300µg/L) (Liu et al. 2009), Chinese reservoir (0.18-11µg/L) and rivers (0.35-101.1 µg/L) (Chi 2009; Liu et al. 2013), German surface waters (0.33-97.8µg/L) (Fromme et al. 2002), and Japanese freshwater (up to 58 µg/L) (Naito et al. 2006). Therefore, the combined concentration of 50µg/L tested in the present study was at an environmentally relevant exposure level. However, no work has been done on the toxic effects of ATBC on aquatic organisms including fish yet.

The main purpose of the present study was to investigate the reproductive toxicity of combined exposure of environmentally relevant concentration (50 µg/L = 50 µg/L) of DEHP, DBP and ATBC on adult zebrafish (Danio rerio).

**MATERIALS AND METHODS**

**Chemicals**

Three plasticizers of technical grade di-(2-ethylhexyl) phthalate (DEHP, PubChem CID: 8343), dibutyl phthalate (DBP, PubChem CID 3026) and acetyl tributyl citrate (ATBC, PubChem CID 6505), with a purity ≥ 99% was obtained from Fuyu Chemicals Co. Ltd. (Shanghai, China). and DMSO purity ≥ 99% was obtained from...
Preparation of stock solution

Three chemicals (DEHP, DBP and ATBC) each 0.1 ml, i.e., total 0.3 ml altogether was mixed with 1.5 ml of DMSO and then the dilution was carried out. To obtain desired concentration of each plasticizer in working solution, the stock solution of each plasticizer was diluted to 0.05 µg/L using double-distilled water, combine concentration of all three plasticizers in working solution was 0.15 µg/L, which contained the individual 0.05 µg/L concentration of each plasticizer.

Zebrafish and exposure design

Three-month old zebrafish (D. rerio) of WT (wild-type) strains were purchased from a local fish dealer (Qingdao, China) and acclimatized in glass aquaria of 10 liter each filled with double distilled water for one week before used in this study. The photoperiod was set at 12:12 hours dark: light and the temperature was maintained at 28 ± 1 °C. Zebrafish were fed twice a day with freshly hatched Artemia nauplii and tropical flake food (Westerfield 2000). A total of 18 male and 18 female zebrafish were randomly selected and divided into two groups: control group and experimental group. Each group consisted of 9 males and 9 females which were maintained in two 6-L glass tanks separately to avoid spontaneous mating. Individuals in each tank were exposed to 5 liters of double distilled water with (experimental group) or without (control group) 50 µg/L of combined mixture of DEHP, DBP and ATBC for three months. The water in each tank was aerated and continuously filtered by top filter water pump, and replaced with fresh water having 50 µg/L of combined plasticizer mixture once a week. After three months of exposure, the individuals were taken for reproductive toxicity or histopathological analysis.

Reproductive toxicity analysis

The toxic effects of combined plasticizer mixture on the reproductive system of adult zebrafish were examined by fecundity and gonado-somatic index (GSI) analysis. For fecundity analysis, four kinds of combinations were set up as followed: Group 1st, exposed females were mated with exposed males; Group 2nd, exposed males were mated with control females; Group 3rd, exposed females were mated with control males; Group 4th, control males were mated with control females. For mating, one male and one female fish were placed in a spawning box in the evening. The spawning was triggered once the light was turned on the next morning and the fertilized eggs were collected and examined under a stereomicroscope. The number of spawned eggs and fertilization rate for each mating were calculated. Three matings for each combination group were carried out in parallel. The GSI analysis was carried out just before histological analysis. The body weight (g) and lengths (mm) of fish samples which were stored in 70% ethanol were first measured and then the abdomen of each fish was incised, and the ovary or testis were excised and weighed again. The GSI of each individual was calculated according to the following formula (Panter et al. 1999).

\[ GSI(\%) = \frac{\text{ovary + testis weight} \times \text{total body weight}}{100} \]

Histopathological analysis

After three-month exposure period, fish were killed by allowing it in ice water and removed when the fish was not responding to external stimuli. Anaesthetized specimens were fixed in Davidson’s fixative for 24 hours and then transferred into 70% ethanol, stored at 4 °C. After weighing for GSI analysis, the dissected testis or ovary tissues were serially dehydrated and embedded in paraffin wax, sectioned transversely in 5–6 µm thickness, and stained with hematoxylin-eosin (H & E) and evaluated under microscope (Olympus BH2, Japan) (Johnson et al. 2009). In specific, the dehydration of specimens were carried out on automated vacuum tissue processor (Leica ASP300 S), the embedding of specimens were done on Paraffin Embedding Station (Leica EG1150 H), the chilling and blocking out of histological tissue samples were done on Cold Plate (Leica EG1150 C), the section of tissue samples were taken on Rotary Microtome (Leica RM2235), the staining of specimen slides were done on Leica Auto Stainer (XL Automated Slide Stainer).

Statistical analysis

All the experiments were conducted in triplicate. The statistical analysis was performed with SPSS software (version 21.0, IBM SPSS Statistic). One-way analysis of variance (ANOVA) was used to detect the significant difference (Tukey's Honestly Significant Difference (HSD)\(^2\), \(p < 0.05\)) between treatment and control. All data are given as mean ± SD (standard deviation).

RESULTS

Toxic effects on body lengths, body weight and GSI of exposed zebrafish

The body lengths and weights as well as GSI index of the male and female adult zebrafish in both the exposed and
control groups were examined. As shown in Table 1, three-month combined exposure of plasticizers DEHP, DBP and ATBC significantly decreased the body lengths, body weights and GSI index of the exposed male and female fish in comparison with the corresponding control male and female fish, but there was a sex-related difference. In detail, the mean body length and weight of exposed male fish was about 63% and 60% of that of the control male fish, respectively, however, the mean body length and weight of exposed female fish was about 90% and 47% of that of the control female fish, respectively. In contrast, the GSI index of exposed male and female fish were about 61% and 67% of that of the control male and female fish, respectively.

Toxic effects on the fecundity of exposed zebrafish

The breeding results of the four groups in zebrafish were obtained at the age of six months. As shown in Figure 1, significant toxicological effect was observed in the first group of exposed female to exposed male, which produced 338 eggs in total, of which, only 80 were fertilized (~76.3% non-fertilization rate). In the second group, exposed female to control male, a total number of 202 eggs were accounted showing 56 fertilized eggs (~72.2% non-fertilization rate). And the third group of exposed male to control female produced 253 eggs in total, from which 82 fertilized eggs were obtained (~67.6% non-fertilization rate). In contrast, only 12.5% non-fertilization rate was observed for the normal breeding in the group of control male to control female (488 eggs in total). The above results revealed that combined exposure of the three plasticizers mentioned-above imposed a significant toxic effect on the fecundity of the adult zebrafish after three-month exposure.

Histopathological changes in the ovary and testis of exposed zebrafish

The ovary of six-month old zebrafish was well developed and the prevailing late cortical alveolar and vitellogenic oocytes occupied the whole ovary tissues. And atretic oocytes were scarcely found (Figure 2, a). In contrast, the ovary of exposed zebrafish was far to be well developed as evidenced by the presence of much more perinucleolar oocytes with varied sizes and dark blue staining and early cortical alveolar oocytes in the exposed ovary. And atretic oocytes were frequently found in the exposed ovaries, characteristic of disruption of yolk vesicles, hyalinization, disintegration of zona radiata, and hypertrophy of perifollicular cells (Figure 2, b).

In contrast to the exposed ovaries, the exposed testis was more heavily damaged. In the exposed testis, most of the spermatocysts were broken and consecutive spermatogenesis was disrupted (Figure 3, C and D). In the control testis, unbroken spermatocysts, encircled by the cytoplasmic arms of Sertoli cells, enclosed varied stages of spermatogenic cells (spermatogonium, spermatocyte, spermatid) instead (Figure 3, A and B). The spermatogonia were the largest spermatogenic cells generally with light red staining and prominent nucleoli. The spermatocytes had comparatively dense nuclei (blue color) and intermediate cell size in comparison with spermatogonia and spermatids. The spermatocyte nuclei were usually evident in one of three meiosis phases: pachytenite, leptotene, or zygotene. The spermatids had dense nuclei and narrow rims of eosinophilic cytoplasm. They were the smallest cells within the germinal epithelium. The spermatocytes had dark, round nuclei and minimal or no apparent cytoplasm. Tails were generally not apparent in histological sections. Spermatozoa were the smallest spermatogenic cells, and they existed as scattered individual cells within tubular lumen. The Sertoli cells tended to have sharply-defined elongated or triangular nuclei. Sertoli cells are usually present in low numbers, usually as single cells located adjacent to lobular septa.

DISCUSSION

In this study, the obtained results for the combined exposure of DBP, DEHP and ATBC at an environmentally relevant concentration for three months showed significant toxic effects on the growth and reproductive system of adult zebrafish. In comparison with the control fish, the body lengths, weight and GSI of both exposed male and female zebrafish were significantly inhibited by 37%, 40% and 39% for males, 10%, 53% and 33% for females, respectively, indicating an interruption of both body growth and gonad development by the combined exposure of the three plasticizers tested. Similar decrease of body weight (~35.2%) was reported in male rats treated with 500 mg/kg/day DEHP for 90 days (Dorostghoul et al. 2010), and significant decrease of body weight (~1.4%) in female rats exposed with 300 mg/kg/day DEHP for 28 days (Liu et al. 2014). However, no significant impact on the body lengths and weight was found in the fathead minnows and three-spined sticklebacks after long term exposure of di-n-butyl phthalate (DnBP) at environmentally relevant concentrations (6~200 µg/L) (Strohker et al. 2005). It was uncertain for us to attribute this inconsistency to the difference of plasticizer or fish species.

Whereas, it was also found in the present study that combined exposure of these three plasticizers tested significantly affected the fecundity of the adult zebrafish after three-month exposure. Both the total number of spawned eggs and fertilization rate decreased in the three exposed mating combinations compared with the control mating combination. From the view of non-
Table 1. Comparison of the average of body lengths, body weights and GSI of exposed and control male and female zebrafish after three-month exposure.

<table>
<thead>
<tr>
<th>Group</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (mm)</td>
<td>Weight (g)</td>
</tr>
<tr>
<td>Control</td>
<td>33.33 ± 1.52</td>
<td>0.46 ± 0.00</td>
</tr>
<tr>
<td>Exposed</td>
<td>21 ± 1.73*</td>
<td>0.28 ± 0.01*</td>
</tr>
</tbody>
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*Note: Significant difference, \( p < 0.05 \); data are expressed as mean ± SD (n=3).

Figure 1. Toxic effects on the fecundity of adult zebrafish after three-month combined exposure of DEHP, DBP and ATBC (0.05 µg mL\(^{-1}\)). Four mating groups were tested as indicated. Exp is the abbreviation of Exposed. Asterisk (*) indicates values that are significantly different from the control (Tukey HSD\(^a\), \( p < 0.05 \)). All data were expressed as mean ± SD (n=3).

Fertilization rate, the fecundity of exposed male fish was more seriously impaired than the exposed female fish. This is in agreement with the histopathological results. The maturation of oocytes was inhibited and the number of atretic follicles obviously increased in the ovaries of exposed female fish. In contrast, the histological structure of the testis of exposed male fish was heavily damaged, evidenced by the breakage of most of the spermatocysts and lack of varied spermatogenic cells of spermatogonium, spermatocyte and spermatid. The absence of spermatogenic cells inferred a source loss of spermatozoa production and declared an infertility of the exposed male fish.

In addition, spermatogenesis in zebrafish occurs in cysts, spermatocysts, formed by Sertoli cells which encircle clonal spermatogenic cells within one cyst by their cytoplasmic arms (Hess and França 2005; Johnson et al. 2009; Pudney 1993). The widespread disintegration of spermatocysts in the exposed testis suggested that Sertoli cells might be the main target cells...
Figure 2. Representative histopathological micrographs of the ovaries of control (a) and (b) exposed female zebrafish at the age of 6 months by hematoxylin and eosin staining (H & E). The combined concentration of DEHP, DBP and ATBC was 0.05 µg mL\(^{-1}\). The exposure period was 90 days. The exposed ovary showed ovary atresia and a proportional change of varied stages of oocytes as evidenced by an increase in the number of the smaller, non-granulated perinucleolar oocytes. Pe, Perinucleolar oocyte. Ec, Early cortical alveolar oocyte. Lc, Late cortical alveolar oocyte. Vo, Vitellogenic oocyte. Ao, Atretic oocyte.

Figure 3. Representative histopathological micrographs of the testis of control (A and B) and exposed (C and D) male zebrafish at the age of 6 months by hematoxylin and eosin staining (H & E). The combined exposure concentration of DEHP, DBP and ATBC was 0.05 µg mL\(^{-1}\). The exposure period was 90 days. The exposed testis was heavily damaged as evidenced by the breakage of spermatocyst and lack of varied spermatogenic cells. Sg, spermatogonium. Sc, spermatocyte. St, Spermatid. Sz, spermatozoa. Se, Sertoli cells.

of the combined mixture of DEHP, DBP and ATBC and were the causative of the breakage of spermatocysts. And also, many studies have indicated that Sertoli cells were involved in the progression of spermatogenesis through a variety of paracrine signals regulating gene expression and metabolism of germ cells. Sertoli cells regulated sur-
vival of germ cells via paracrine secretion of trophic factors such as insulin growth factor, nerve growth factor, growth factor derived from glia and stem cell factor(Skinner 2005). Thus, it can be concluded that the interruption of the normal secretion of paracrine signals of Sertoli cells by the combined plasticizer mixture might be responsible for the loss of spermatogenic cells in the exposed testis. On the other hand, the obtained data also suggested that adult zebrafish were perfect animal models to evaluate the reproductive toxicity of environmentally endocrine disruptors for their special physiological structure of gonads and capacity of continuous spawning. All stages and process of oogenesis and spermatogenesis can be clearly observed in the ovary and testis of three-month old and above zebrafish. Evaluation of the inhibitive effects on the maturation of oocytes and sperms by toxicants becomes possible and easy. And also, the mating experiments can be set up immediately after exposure (Johnson et al. 2009; Maack and Segner 2003).

However, the developmental and reproductive toxicity of DEHP and DBP have been widely reported in animals, toxic data on ATBC were quite limited to date (Finkelstein and Gold 1959; Mochida et al. 1996). Thus, the contribution of ATBC to the combined toxic effects was uncertain until further data for individual exposure of DEHP, DBP and ATBC at the same exposure concentration (50 µg/L) were obtained and compared.

CONCLUSION

This study demonstrated that combined exposure of plasticizers DEHP, DBP and ATBC at environmentally relevant concentration (50 µg/L) imposed significant adverse effects on the reproductive system of adult zebrafish after three-month exposure, as evidenced by significant decrease of body lengths, weight and GSI, impairment of fecundity, interruption of oogenesis and spermatogenesis, etc. Male zebrafish were more sensitive to the combined plasticizer exposure with respect to relatively lower fecundity and more heavily damage of the testis than female zebrafish. The maturation of oocytes of the exposed ovaries was inhibited as evidenced by the occurrence of more underdeveloped perinucleolar oocytes and early cortical alveolar oocytes as well as atretic oocytes in the exposed ovary. However, most of the spermatocysts were broken and almost all of the spermatogenic cells were lost in the exposed testis, inferring that Sertolii cells might be the main target cells of the combined plasticizer mixture and subsequent infertility of the exposed male fish. Hence great concerns should be raised on the reproductive toxicity of combined plasticizer exposure to the aquatic environment and organisms.

ACKNOWLEDGEMENTS

The author thanks Dr. Manish Raj Pandey (National Trust for Nature Conservation, Nepal) for providing guidance in English proofreading and statistical analysis.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

FUNDING

This work was supported by National High-tech R&D Program of China (863 Program) [Grant Number 2012AA100402] and National Natural Science Foundation of China [Grant Number 31172391 and 31472274].

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