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Developmental toxicity of dibutyl phthalate and citrate ester plasticizers in *Xenopus laevis* embryos

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HIGHLIGHTS

- ATEC has the highest 96-h-LC_{50} and EC_{50} values in *Xenopus* embryos.
- The 96 h-LC_{50} value of ATBC is lower than those of ATEC and ATHC in *Xenopus* embryos.
- DBP and ATHC increased apoptosis and necrosis in *Xenopus* embryos, respectively, but ATBC and ATEC did not.
- DBP and ATHC increased oxidative stress in *Xenopus* embryos.
- DBP and ATHC increased head malformation with decreased the *FoxN3* and *Col2a1* mRNA in *Xenopus* embryos.

GRAPHICAL ABSTRACT

Chemicals	Dibutyl phthalate (DBP)	Citrate esters		
		Tributyl O-acetyl citrate (ATBC)	Trihexyl O-acetyl citrate (ATHC)	Triethyl 2-acetyl citrate (ATEC)
LC_{50} (mg/l)	13.3	13.3	98.0	409.6
EC_{50} (mg/L)	7.5	12.2	-	413.8
Teratogenic indices	1.79	1.09	-	0.99
Oxidative stress	+++	-	+++	-
Pro-apoptotic gene expression / DNA fragmentation	+++	-	Necrosis	-

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ABSTRACT

Citrate esters have been considered as alternatives to phthalate plasticizers. Being considered to have low toxicity in mammals, their toxicological information for aquatic animals remains poorly understood. We examined the developmental toxicity of citrate esters including tributyl O-acetylcitrate (ATBC), triethyl 2-acetylcitrate (ATEC), and trihexyl O-acetylcitrate (ATHC) together with dibutyl phthalate (DBP) based on the frog embryo teratogenesis assay-*Xenopus* (FETAX). ATBC has the lowest 96 h LC_{50} and 96 h EC_{50} values. In RT-qPCR, the ratio of *bax* and *bcl-2* mRNA was significantly increased by DBP, but not by ATBC, ATEC and ATHC. DNA fragmentation was obvious in DBP-treated tadpoles, but not in those treated with ATBC and ATEC, whereas ATHC caused necrotic DNA degradation. Lipid hydroperoxide levels in tadpoles were significantly increased by DBP and ATHC, but not by ATBC and ATEC, suggesting that induction of oxidative stress by DBP and ATHC in embryos. In tadpoles with head abnormalities, basihyal bone, ceratohyal bone and Meckel's cartilage were frequently missed together with reduction in branchial gill bones. *Col2a1* mRNA in the head of tadpoles was significantly decreased by low concentration of DBP, ATHC, and high concentration of ATEC. In stage 25 embryos *FoxN3* mRNA, a master regulator for differentiation of neural crest cells to chondrocytes in head, was significantly decreased by DBP and ATHC, but not by ATBC and ATEC. In conclusion, ATEC was recommended as the alternative to phthalate plasticizer having the lowest developmental toxicity in amphibian embryos.

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1. Introduction

Approximately 50 types of plasticizers have been commercially

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used to increase the plasticity or viscosity of materials. In 2014, the total global market for plasticizers was 8.4 million tons (Malveda, 2015). The most commonly used plasticizers are phthalate esters, such as dibutyl phthalate (DBP) and bis (2-ethylhexyl) phthalate (DEHP), well known endocrine disruptors (Alexander, 2015; Zarean et al., 2016; Giribabu and Reddy, 2017). Recently, citrate esters, such as tributyl O-acetylcitrate (ATBC), triethyl 2-acetylcitrate (ATEC) and trihexyl O-acetylcitrate (ATHC), have been considered as alternative plasticizers. As people have become more concerned about the safety of life supplies, the use of citrate ester plasticizers has also increased dramatically (Takeshita et al., 2011). Although the *in vivo* toxicity of citrate esters in rats was explored decades ago, toxicological information including endocrine activity and developmental toxicity remains poorly available (Finklestein and Gold, 1959; Gold et al., 1998; Meyers et al., 1964). Considering various plasticizers contaminated the freshwater system together with surfactants that would have increased the solubility of citrate esters (Shi et al., 2015), citrate ester plasticizers at environmentally relevant concentrations may affect the development of amphibian embryos. The frog embryo teratogenesis assay-*Xenopus* (FETAX; ASTM, 2004) has been widely used to determine the toxicity of common environmental contaminants, such as metals (Bosisio et al., 2009; Fort et al., 2006), organic compounds (Bacchetta et al., 2008; Chae et al., 2014; Gutleb et al., 2007), and nanomaterials (Nations et al., 2011). Recently, FETAX has been efficiently applied to the examination of safety for chemicals including pesticides, surfactants, plasticizers, and synthetic drugs (Park et al., 2010, 2016). In addition, FETAX produces toxicological information important for management of chemical pollutants in aquatic environments to protect the health of aquatic vertebrates, such as amphibians. To elucidate the developmental toxicity of citrate ester plasticizers, commonly used citrate esters including tributyl O-acetylcitrate (ATBC), triethyl 2-acetylcitrate (ATEC), and trihexyl O-acetylcitrate (ATHC) were subjected to FETAX. As a positive control for developmental toxicity assessment of citrate esters, dibutyl phthalate (DBP) was examined. To elucidate the developmental toxicity of ATBC, ATEC and ATHC, we examined the mortality, developmental abnormality, and growth of *Xenopus* embryos following 96 h of exposure. To reveal the toxic mechanisms of citrate esters, the expression of mRNA of genes related with apoptosis and head development, cleaved caspase-3, genomic DNA fragmentation, and lipid peroxidation were analyzed in *Xenopus* embryos.

2. Materials and methods

2.1. Animal husbandry and collection of embryos

Xenopus laevis were bred at Hanyang University Aquarium and maintained on a diurnal cycle (14 h light and 10 h dark) at 20–22 °C. Experiments on amphibian embryos were conducted following the Guidelines for the Use of Live Amphibians and Reptiles in Field and Laboratory Research (ASIH, 2004). Adult males and females, bred in the laboratory, were given a single injection of 450 IU and 750 IU hCG, respectively, in the dorsal lymph sac. Fertilized eggs were obtained. Unfertilized and necrotic eggs were removed, and healthy fertilized eggs with first cleavage furrows were selected 2 h post-fertilization (p.f.). When the fertilized eggs developed to the blastula stage, the embryos were de-jellied with a 2% L-cysteine solution (pH of 8.1) for 2 min. Normal embryos were selected according to FETAX (ASTM, 2004).

2.2. Chemicals and test solution preparation

DBP (Cat. No. 524980-100ML; purity >99%), ATBC (Cat. No. 388378-500ML; purity >98%), ATEC (Cat. No. 388351-500ML; purity >99%), butylated hydroxytoluene, Bouin's solution, HPLC grade methanol, sodium bicarbonate, sulfuric acid, ferrous sulfate, trypsin, magnesium sulfate, calcium sulfate, calcium chloride, potassium hydroxide, L-cysteine, dimethyl sulfoxide (DMSO), sodium tetraborate, hematoxylin, and eosin were purchased from Sigma-Aldrich Korea (Seoul, Korea). ATHC (Cat. No. sc-229580; purity >95%) was purchased from Santa Cruz (Santa Cruz, CA, USA). Sodium chloride was purchased from Samchun Co. Ltd (Pyeongtaek, Korea). Potassium chloride was purchased from Showa Co. Ltd. (Ikoma, Japan). Human chorionic gonadotropin (hCG) was purchased from DaeSung Microbiological Labs Co. Ltd (Seoul, Korea). Taking into account the lower solubility of these chemicals, DMSO was used to make the stock solutions of chemicals. Stock solutions of DBP, ATBC, ATEC and ATHC were made and serial dilutions from the stock solutions were used to obtain the final test solution concentrations. Then, the DMSO stock was diluted with FETAX solution to create the appropriate stock concentration. The final highest concentration of DMSO in test solutions dishes was 0.5%. Solutions with increasing chemical concentrations were prepared every day for each assay.

2.3. FETAX for developmental toxicity

Four wells containing only FETAX solution were used as negative controls in each experiment. For ATBC, ATEC, ATHC and DBP with low solubility, DMSO was used as a solubilizer, since it was shown not to produce detrimental effects to developing *X. laevis* embryos up to concentrations of 1% v/v (ASTM, 2004). Four wells of 40 embryos were used for each concentration. After embryo placement, developing embryos were kept in an incubator (MIR550, Sanyo, Osaka, Japan) at 23 ± 1 °C as directed by the ASTM guide (ASTM, 2004). At 24 h intervals, dishes containing developing embryos were removed from the incubator to determine mortalities, and to perform static renewal of solutions. Preliminary range-finder assays were conducted for ATBC, ATEC and ATHC to assess the range of concentrations over which systemic toxicity might occur. The three following experiments were then performed with established concentrations obtained from the range finding assays. ATBC: 0, 5.3, 10.5, 15.8, 21.0, 26.3, and 31.5 mg/L (0, 5, 10, 15, 20, 25, and 30 ppm); ATEC: 0, 90.9, 181.6, 272.6, 363.5, 454.4 and 681.6 mg/L (0, 80, 160, 240, 320, 400, and 600 ppm); ATHC: 0, 14.9, 22.3, 33.5, 50.3 and 75.4 mg/L (0, 14.8, 22.2, 33.3, 50, and 75 ppm). For DBP, the established concentrations obtained were 0, 6.3, 9.4, 14.1, 21.0 and 31.5 mg/L (0, 6, 8.9, 13.4, 20 and 30 ppm). At the end of the culture, surviving embryos were counted and fixed with Bouin's solution, and then observed under the stereomicroscope (MZ8; Leica, Heerbrugg, Switzerland) to define the morphological abnormality. Staging and patterning of abnormal development were conducted as described by Nieuwkoop and Faber (1967) and Rugh (1962). Dead embryos were not counted as malformed (ASTM, 2004). When the embryonic survival rate of the control group at 96 h p. f. was higher than 90%, then the developmental toxicity data were accepted. Otherwise, batches were discarded. We conducted another FETAX with logarithmic concentrations for real-time PCR, DNA fragmentation, and lipid hydroperoxide determination analysis. At the end of the culture, surviving embryos were frozen using liquid nitrogen and stored in a −70 °C deep freezer.

2.4. Histological analysis

For histological examination, tadpoles were fixed in Bouin's solution and processed for paraffin sections. The serial paraffin sections were 5 μm thick and were processed for hematoxylin-eosin (H-E) staining. Observation and photography were

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