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Toxicological effects of tris(2-chloropropyl) phosphate in human hepatic cells

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HIGHLIGHTS

- TCPP induced hormesis effect in L02 cells.
- TCPP caused ROS overproduction in L02 cells.
- The expressions of *Bax*, *Hrk* and *Bax/Bcl-2* increased significantly in 10^{-4} M group.
- Energy metabolism, signal transduction and cytoskeleton were determined by omics.

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ABSTRACT

Organophosphate flame retardants (OPFRs) are widely used as flame retardants which are ubiquitous in various environment media. As many of OPFRs are toxic and persistent, concerns have been raised in regards to their environmental impact. In this study, the toxicological effects of tris(2-chloropropyl) phosphate (TCPP) in human L02 cells was investigated by cell proliferation and apoptosis, oxidative stress, metabolomic and proteomic responses as well as gene expressions related to apoptosis. Results showed that TCPP did not significantly affect the L02 cell apoptosis, however, a significant increase of ROS production was observed in L02 cells with TCPP treatment compared with that in control group ($p < 0.05$). The expression levels of Bcl-2 family-encoding genes (*Bax, Hrk* and *Bax/Bcl-2*) were up-regulated significantly in 10^{-4} M group ($p < 0.05$). Metabolomic and proteomic responses indicated that TCPP mainly caused disturbance in cell growth/division and gene expression, energy and material metabolism, signal transduction, defense and cytoskeleton, which was further confirmed by the western blot analysis.

1. Introduction

As alternatives to the polybrominated diphenyl ethers (PBDEs) mixtures (i.e., pentaBDE and octaBDE), the use of organophosphate flame retardants (OPFRs) are currently widespread and expected to increase (Dishaw et al., 2011; Moller et al., 2012). The OPFRs have been frequently detected in various different environmental matrices like air (Hartmann et al., 2004), dust (Marklund et al., 2003; Stapleton et al., 2009; Takigami et al., 2009), water (Bacaloni et al., 2008; Regnery and Puttmann, 2010), sediments (Garcia-Lopez et al., 2009), soils (David and Seiber, 1999), landfill leachates (Yasuhara et al., 1999) and even in the aquatic organisms and in human breast milk (Liu et al., 2012).

Trichloroalkyl phosphates, such as tris(2-chloropropyl) phosphate (TCPP), continue to be used as flame retardants and plasticizers in a wide variety of applications resulting in widespread environmental dispersion (Reemtsma et al., 2008; van der Veen and de Boer, 2012). TCPP is mostly used as flame retardants in

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polyurethane foam and epoxy resin (Andresen et al., 2004), which is the most dominant compounds detected in water environment (Martinez-Carballo et al., 2007; Bacaloni et al., 2008; Regnery and Puttmann, 2010). Compared with PBDEs and other brominated flame retardants (e.g., hexabromocyclododecane and tetrabromobisphenol A), however, OPFRs have received less attention with regard to human potential health effects and ecological risk safety.

OPFRs are structurally similar to neurotoxic organophosphate pesticides, raising concerns about exposure and toxicity to humans (Dishaw et al., 2011; Cao et al., 2012). The potential adverse effects of OPFRs to the human health and ecosystem have been determined in several researches (Reemtsma et al., 2008; Ren et al., 2008; Pillai et al., 2014), such as TCPP is suggested to be carcinogenic (Matthews et al., 1990). Exposure to OPFRs has been associated with adverse reproductive, neurologic and other systemic effects (Liu et al., 2012). Meeker and Stapleton (2010) suggested that tris-(1,3-dichloro-2-propyl) phosphate (TDCPP) might disrupt the hormone levels and decrease the semen quality among adult human males. Fu et al. (2013) reported that TDCPP caused developmental toxicity in vertebrates and the studies on the potential health risks of TDCPP were necessary, because of its frequent presence and the potential exposure for human. However, there are limited data on the toxicological and molecular effects of OPFRs exposure. Therefore, the molecular toxicological mechanism of OPFRs remains to be further investigated.

The "-omic" approaches are capable to discover broader ranges of biomarkers at molecular levels, such as genomics, transcriptomics, proteomics and metabolomics (Santos et al., 2010; Weckwerth, 2011; He et al., 2012). The determination of toxicological biomarkers at a certain level (e.g., gene, protein and metabolite) can illustrate the toxicological effects caused by the environmental contaminants. Proteomics is defined as a large-scale study on proteins expressed by the genome in a given organism (Anderson and Anderson, 1998). A comparison of protein profiles between control conditions and contaminant-stressed can detect the alterations in the proteome, and can interpret the contaminants-caused toxicological effects and interaction mechanisms (Gomiero et al., 2006). Metabolomics usually focuses on the end products of metabolism in a biological sample (Liu et al., 2011). Proteomics and metabolomics can directly characterize the perturbations of metabolic pathways, link enzymes and stress-responsive proteins. Hence, the integrated two-omic technique may present a combined insightful view and better understanding of the toxicological effects of contaminants (D'Alessandro et al., 2011). In this study, the possible molecular mechanism of toxicity effects caused by OPFRs was investigated. Considering the liver is a major target organ in exposure, the human hepatic cell (L02) was used in this study as an *in vitro* cell model (Tian et al., 2012). The toxicological effects of TCPP exposure in L02 cells through analysis of ROS production and cell apoptosis were evaluated by the flow cytometry. An integrated metabolomics and proteomic approach was used to elucidate the toxicological effects of TCPP. Moreover, the expression levels of related proteins were measured to explore the potential mechanism.

2. Materials and methods

2.1. Chemicals and reagents

TCPP (CAS No. 13,674-84-5) was purchased from J&K Chemical Co., Ltd.. Roswell park memorial institute (RPMI 1640) medium, fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin-streptomycin solution and trypsin were obtained from Hyclone (Logan, Utah, USA). 2′,7′-dichlorofluorescein diacetate (DCFH-DA), dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Cell counting kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). Annexin V-FITC/PI detection kit was purchased from Promega, USA. SYBR Green Real-time PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). All antibodies were purchased commercially from Abcam, USA, including anti-APE1, anti-PGK1 (Ser473) and anti-Rabbit IgG(H + L)/HRP. All other reagents used were of analytical grade. All experiments were carried out in at least triplicate.

2.2. Cell culture and OPFRs treatment

Human L02 cells are an immortalized non-tumor cell line derived from normal liver tissue, which are considered to be an *in vitro* model of nonmalignant liver (Tian et al., 2012). L02 cells were cultured in 1640 medium (Hyclone, USA) supplemented with 100 unit/mL penicillin, 100 unit/mL streptomycin and 10% (v/v) fetal bovine serum (Hyclone, USA). The cells were grown at 37 °C with 5% CO_2 in a humidified incubator (MEMMERT, Germany). When cells grew to 60–80% confluence, the media were discarded and the fresh medium containing various concentrations of TCPP were supplemented. Dimethyl sulfoxide (DMSO) was used as solvent for L02 cell assay. The final concentrations of solvent in the exposure media were less than 0.1% (v/v).

2.3. Cell proliferation assay

In the study, cell growth was evaluated following exposure to TCPP for 24 h. Cells were seeded in 96-well plates at a concentration of 10^4 cells per well and left to attach for 24 h. Then the cells were exposed to various concentrations (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M) of TCPP in 100 μL of medium, respectively. After exposure for 24 h, 10 μL of CCK-8 reagent was added to each well, and then incubated at 37 °C for 1 h. After that, the absorbance in each well was measured with a spectrophotometric plate reader at 450 nm.

2.4. Analysis of cell apoptosis

After the experiment of cell proliferation, the dose responsive curve of L02 cells to TCPP exposures were determined (Fig. 1). Then two concentrations (10^{-6} and 10^{-4} M) that could describe the low-

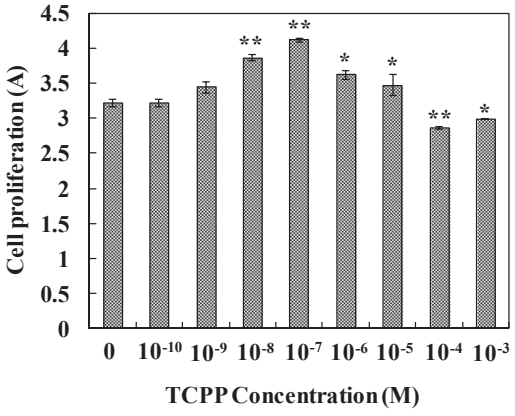

Fig. 1. The hormesis effect of TCPP on cell proliferation in L02 cells. Data represent the mean ± S.D. of three separate determinations in triplicate (*$p < 0.05$, **$p < 0.01$, compared with the control group).