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Effects of organophosphorus flame retardant TDCPP on normal human corneal epithelial cells: Implications for human health☆

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ABSTRACT

Tris(1,3-dichloro-2-propyl) phosphate (TDCPP) is one of the most detected organophosphorus flame retardants (OPFRs) in the environment, especially in indoor dust. Continuous daily exposure to TDCPP-containing dust may adversely impact human cornea. However, its detrimental effects on human corneal epithelium are largely unknown. In this study, we investigated the cell apoptosis in normal human corneal epithelial cells (HCECs) after TDCPP exposure and elucidated the underlying molecular mechanisms. Our data indicated a dose-dependent decrease of cell viability after TDCPP exposure with LC_{50} at 202 μg/mL. A concentration-dependent apoptotic sign was observed in HCECs after exposing to ≥2 μg/mL TDCPP. Endoplasmic reticulum stress induction was evidenced by up-regulation of its biomarker genes (*ATF-4*, *CHOP*, *BiP*, and *XBP1*). Furthermore, alternation of Bcl-2/Bax expression, mitochondrial membrane potential loss, cellular ATP content decrease, and caspase-3 and -9 activity increase were observed after exposing to 2 or 20 μg/mL TDCPP. Taken together, the data implicated the involvement of endoplasmic reticulum stress in TDCPP-induced HCEC apoptosis, probably mediated by mitochondrial apoptotic pathway. Our findings showed TDCPP exposure induced toxicity to human cornea. Due to TDCPP's presence at high levels in indoor dust, further study is warranted to evaluate its health risk on human corneas.

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1. Introduction

Since the phase out of polybrominated diphenyl ether (PBDE) flame retardants, organophosphorus flame retardants (OPFRs) have been used as alternatives to meet the demand for fire resistance in many products. Tris (1,3-dichloro-2-propyl) phosphate (TDCPP) as an important OPFR has often been added to polyurethane foams, which is frequently used in many daily products (Stapleton et al., 2011). Its annual production in the United States is at 22,700 tons a year in 2006 and is still increasing in recent years (van der Veen and de Boer, 2012; Betts, 2013).

Like other OPFRs, TDCPP is not chemically bind to the polymeric foam. Therefore, it can be released into indoor environments during daily use and accumulate in indoor dust. The detection frequencies for TDCPP in dust samples from offices, houses and vehicles are >96% with concentrations ranging from <0.03 to 326 μg/g (Carignan et al., 2013). In addition, a recent report showed that elevated concentrations of TDCPP in indoor dust are associated with altered thyroid and prolactin hormone levels and decreased men's sperm quality (Meeker and Stapleton, 2010). A positive trend between TDCPP's urinary metabolite BDCPP and TDCPP concentrations in indoor dust has also been observed in humans (Carignan et al., 2013; Hoffman et al., 2014). Therefore, indoor dust is a primary route for human exposure to TDCPP and its adverse effects via indoor dust exposure warrants investigation.

Human corneal epithelium, which is the outermost cell layer of the eyes and covered by a tear film, acts as the mechanical barrier to exotic substance including dust to minimize interior damage. Continued daily exposure to indoor dust has been associated with increasing risks of corneal injury (Mølhave et al., 2002). Our recent results showed that indoor dust induced significant cytotoxicity to human corneal epithelial cells (HCECs) (Xiang et al., 2016a). Moreover, the organic solvent extract of indoor dust is more potent in inducing HCEC damage than water extract (Xiang et al., 2016b).

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Given the fact that organic solvent extract of indoor dust induces higher toxicity and with the high TDCPP concentrations in indoor dust, it is necessary to evaluate its toxic effects on corneal epithelial cells.

Cell lines have been used to study the toxicity of TDCPP. For example, in primary cultured avian hepatocytes, exposure at ≥ 4.3 μg/mL TDCPP elicited significant cytotoxicity and deregulated the gene expression involved in the thyroid hormone pathway, xenobiotic metabolism, and lipid regulation (Crump et al., 2012). Furthermore, several human carcinoma cell lines have been employed. For example, recent studies demonstrated that exposing to 1–86 μg/mL TDCPP induced cytotoxicity and neurotoxicity in pheochromocytoma neuronal cells and neuroblastoma cells (Li et al., 2017; Liu et al., 2012). However, most of the existing data focus on TDCPP's impact on endocrine disruption and neurotoxicity (Wei et al., 2015). There is limited information available on TDCPP's toxicity on human cornea.

In the present study, the effects of TDCPP on human cornea were investigated by using normal human corneal epithelial cells (HCECs). Changes in cell viability, morphology, apoptosis, mitochondrial membrane potential, cellular ATP level, and caspase-3/9 activity were assessed after 24 h exposure to TDCPP. In addition, mRNA expression levels of endoplasmic reticulum stress, cell apoptosis regulatory genes and Bcl-2/Bax expression were determined to explore the molecular mechanisms associated with the associated effects.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), epidermal growth factor (EGF), penicillin streptomycin solution (PS), and trypsin-EDTA solution were brought from Invitrogen (GIBCO, USA). Cell culture plates and dishes were purchased from Corning Inc. (NY, USA). The CCK-8 cell viability assay kit and SYBR green qPCR master mix were purchased from Yi Fei Xue Biotech. Co., Ltd. (Nanjing, China). The FITC Annexin V apoptosis detection kit, and JC-1 probe and Bradford protein assay kit were obtained from BD Biosciences, USA and Beyotime Institute of Biotechnology (Haimen, China). The TaKaRa MiniBEST universal RNA extraction kit and cDNA synthesis kit were from TaKaRa Biotech. Co., Japan. Tri(1,3-dichloropropyl)- phosphate (TDCPP, CAS No. 13674-87-8) was from J&K Scientific (Shanghai, China) with purity >98%. Dimethyl sulfoxide (DMSO; >99.9%) was obtained from Sigma–Aldrich (MO, USA). In all experiments, final concentrations of DMSO in the exposure media were <0.1% (v/v).

2.2. Cell viability, morphology and apoptosis

Human corneal epithelial cells (HCECs) from the Eye Hospital of Wenzhou Medical University (Wenzhou, China) were cultured in DMEM supplemented with 10% FBS, 10 ng/mL EGF, and 1% penicillin-streptomycin solution in an incubator with 5% CO_2 at 37 °C. After reaching confluence, HCECs were replanted into 6/24/96-well plates or petri dishes with different initial densities and cultured for 24 h to reach ~70% confluence. Then, TDCPP dissolved in n-hexane was solvent-exchanged to DMSO and serially diluted by DMEM to achieve concentrations from 0.034 to 340 μg/mL. For cell exposure, the culture medium was aspirated and treated with TDCPP solutions (DMSO ≤ 0.1%) for 24 h with 0.1% DMSO solution as vehicle control.

To examine the effects of TDCPP on cell viability, HCECs were planted into 96-well plate (100 μL/well) at density of 1×10^5 cells/mL overnight. Then, the medium was changed into fresh medium containing 0.034, 0.34, 3.4, 34, 68, 136, 272, or 340 μg/mL of TDCPP and solvent vehicle (0.1%, v/v) and incubated for 24 h. Cell viability was detected using CCK-8 cell viability assay kit according to the manufacturer's instructions. After exposure, cellular morphology was observed and recorded by an inverted microscopy (TS-100, Nikon, Japan).

Based on the result of LC_{50} calculation, HCECs grew on 24 well plates were exposed to TDCPP at 3 concentrations (i.e., LC_{50} = 200 μg/mL, 1/10 LC_{50} = 20 μg/mL, 1/100 LC_{50} = 2 μg/mL) and incubated at 37 °C for 24 h. After exposure, HCECs were washed twice with PBS and treated with 195 μL/well binding buffer, followed by Annexin V-FITC (5 μL) and propidium iodide (10 μL). Cells were incubated for 20 min at room temperature in the dark. Finally, stained cells were visualized by inverted fluorescent microscopy with NIS-Elements D software (Eclipse Ti-U, Nikon, Japan). Apoptotic cells in early stage were stained with Annexin V-FITC whereas late stage apoptosis or necrotic cells were stained with both Annexin V-FITC and propidium iodide.

2.3. Quantitative real time PCR (q-RT-PCR) analysis

HCECs were seeded in 6-well plates at 1×10^6 cells/well overnight. TDCPP diluted by DMEM was incubated with HCECs at 2, 20, or 200 μg/mL for 24 h, with HCECs treated with DMEM (0.1% DMSO) being served as a control. Then, total RNA from HCECs was extracted by TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, Japan). The purity and concentration of the purified RNA were evaluated by Nanodrop 2000 (Thermo Fisher Scientific, USA). The cDNA was reverse transcribed from 1 μg of total RNA using a PrimeScript RT reagent kit. For amplification, q-RT-PCR reactions were subsequently carried out with SYBR green qPCR master mix using CFX Connect™ Real-Time PCR detection system (Bio-Rad, USA) at the following cycling conditions: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. The specificity value of each primer set was confirmed by comparing the melting curve, which was conducted from 65 to 95 °C with 0.5 °C s^{-1} increments. The specific primers of ER-stress and apoptosis regulatory genes (*CHOP, BiP, ATF-4, XBP-1, Bcl-2, Bax, Caspase-3, and GADD45α*) were synthesized by Nanjing genscript biotechnology co., LTD (Nanjing, China) based on Harvard PrimerBank's data (Spandidos et al., 2010) (Table 1). Each sample was run in triplicate and the threshold cycle values were normalized to housekeeping gene human 18S rRNA (*RN18S1*). The fold changes of target genes were calculated by $2^{-\Delta\Delta CT}$ method.

2.4. Mitochondrial membrane potential and cellular adenosine triphosphate assay

After exposure to TDCPP, the changes in mitochondrial membrane potential (ΔΨm) in HCECs were determined following Xiang et al. (2016a, 2017). Briefly, HCECs were detached with trypsin-EDTA solution and replanted into 24 well-plates at 3×10^5 cells/500 μl/well overnight. The culture medium was changed into fresh DMEM containing TDCPP at 2, 20, or 200 μg/mL and incubated for 24 h. After exposure, 250 μl JC-1working staining solution dissolved in 250 μl DMEM was added into HCECs and incubated in dark for 20 min at 37 °C, followed by washing twice with PBS. The images were observed and recorded using inverted fluorescent microscopy with NIS-Elements D software (Eclipse Ti-U, Nikon, Japan). Additionally, the fluorescence value was detected by an Infinite® 200 PRO fluorescence microplate reader (TECAN, USA). Briefly, for monomeric JC-1, the wavelengths of excitation and emission were 514 nm and 529 nm, while 585 nm and 590 nm were selected to examine aggregate form of JC-1. The fluorescence intensity of increasing green indicates mitochondrial depolarization. Cells