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 mitochondrial membrane potential loss, cellular ATP content decrease, and caspase-3 and -9 activity increase in involved 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 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 (Melhawe 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 HEC damage than water extract (Xiang et al., 2016b).
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 bought 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 Sigma (MO, USA). The CCK-8 cell viability assay kit and SYBR green qPCR master mix 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.

2.2. 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% CO₂ at 37 °C. After reaching confluence, HCECs were replated 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 × 10⁵ 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 by 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 LC₅₀ calculation, HCECs grew on 24 well plates were exposed to TDCPP at 3 concentrations (i.e., LC₅₀ = 200 μg/mL, 1/10 LC₅₀ = 20 μg/mL, 1/100 LC₅₀ = 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 × 10⁶ 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 with 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 increments. The specific primers of ER-stress and apoptosis regulatory genes (CHOP, BIP, ATF-4, XBP-1, Bcl-2, 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⁻ΔΔCT method.

2.4. Mitochondrial membrane potential and cellular adenosine triphosphate triphosphate assay

After exposure to TDCPP, the changes in mitochondrial membrane potential (ΔΔΨm) in HCECs were determined following Xiang et al. (2016a, 2017). Briefly, HCECs were detached with trypsin-EDTA solution and replant into 24-well plates at 3 × 10⁵ 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-1 working 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
treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10 μmol/L) and DMSO (0.1%) were served as positive and negative control respectively.

The cellular ATP contents were determined in HCECs grown in DMEM containing 0, 2, 20, or 200 μg/mL TDCPP using a luciferase-based ATP assay kit according to the manufacturer’s guideline (Beyotime Institute of Biotechnology, Haimen, China). Briefly, after 24 h exposure, HCECs were lysed with lysis buffer. Lysates were then centrifuged at 12,000 g at 4 °C for 5 min. Then, 100 μL of supernatant was mixed with 100 μL ATP detection working dilution. Luminance was examined by an Infinite® 200 PRO fluorescence microplate reader (TECAN, USA). Standard curves were also prepared and the protein concentration was detected using the Bradford Protein assay. Total ATP levels were normalized to protein content and presented as percent of control.

2.5. Detection of Bcl-2 and Bax protein expression

Expression of apoptotic signaling proteins Bax and Bcl-2 was determined by human Bcl-2 and Bax ELISA kits (Cusabio Biotech, Co. Ltd, Wuhan, China) according to the manufacturer’s instruction. Briefly, HCECs were grown in 24-well plate overnight and then exposed to 2, 20, or 200 μg/mL TDCPP for 24 h. Subsequently, HCECs were washed with cold PBS and lysed with lysis buffer on ice for 30 min. The cell lysates were centrifuged at 12,000 rpm at 4 °C for 5 min. The supernatants were collected and total protein concentrations were measured using Bradford protein assay kit. Then, samples (100 μL) were added to monoclonal antibody pre-coated wells followed by washing and incubating with biotin-antibody working solution for 60 min. HRP-avidin reagent was added into the wells after washing and the microwells were incubated at 37 °C for 60 min. The TMB substrate solution was added and further incubated at 37 °C for 30 min with light-protection and the reaction was terminated by the addition of stop solution. The absorbance was determined using a Multiskan FC microplate reader (Thermo, USA) at 450 nm. The results were presented as folds of DMSO (0.1%)-treated control.

2.6. Caspase-3 and caspase-9 activity assay

Caspase-3 and caspase-9 activity in HCECs after TDCPP exposure were detected using commercial caspase-3 and caspase-9 activity assay kits (Beyotime Institute of Biotechnology, Haimen, China). Briefly, HCECs were seeded into 100 mm petri dishes overnight and then exposed to 2, 20, or 200 μg/mL TDCPP for 24 h. Subsequently, HCECs were washed with cold PBS and lysed with ice-precooling lysis buffer. Lysates were then centrifuged at 12,000 g at 4 °C for 15 min. The supernatant was collected and incubated with the colorimetric substrate Ac-DEVD-pNA for caspase-3 and Ac-LEHD-pNA for caspase-9 at 37 °C for 2 h. After incubation, OD values were measured by using a Multiskan FC microplate reader (Thermo, USA) at 405 nm. The results were presented as percent of control.

2.7. Statistical analysis

The results were presented in terms of mean ± standard error (SE). The statistical difference analysis was performed using one-way ANOVA by Graphpad Prism Version 6 (Graphpad Software, USA). A p-value of <0.05 was considered statistically significant.

3. Results

3.1. TDCPP decreased cell viability and altered cell morphology

Exposure to TDCPP did not significantly affect cell viability until at concentration >68 μg/mL (Fig. 1 A). HCECs showed a 16% cell viability loss after exposing to 136 μg/mL TDCPP. Moreover, TDCPP induced a sharp decrease in viable cells (87%) after exposing to ≥ 272 μg/mL TDCPP. Based on cell viability, the LC50 value for TDCPP was 202 μg/mL using a nonlinear regression (Fig. 1B). The cell morphology changes, a crucial indicator of cytotoxicity, were observed using a microscope. Cell counts and their polygonal appearance were affected at ≤ 68 μg/mL TDCPP (Fig. 2A-E), with visible vacuolization being found at 136 μg/mL (Fig. 2F). When exposure concentrations increased to 272 μg/mL, a majority of HECECs turned round and floated in the medium (Fig. 2G), suggesting cell apoptosis or death. Collectively, cellular morphology changes were consistent with cell viability. To further elucidate the underlying mechanism, 3 concentrations (i.e., LC50 = 200 μg/mL, 10 LC50 = 20 μg/mL, 100 LC50 = 2 μg/mL) of TDCPP were used for exposure in further experiments.

3.2. TDCPP triggered HEC apoptosis

Apoptosis of HCECs following exposure to TDCPP at 2, 20, or 200 μg/mL is shown in Fig. 3. Compared to controls, TDCPP-exposed cells exhibited a concentration-dependent increase in apoptosis. Weak green-signal on HEC cell membrane was observed after exposure to 2 μg/mL TDCPP (Fig. 3J), suggesting TDCPP potentially elicited early apoptosis. At 20 μg/mL TDCPP, the green fluorescence intensity was enhanced (Fig. 3N). In addition, several cell nuclei were positively stained with red propidium iodide (PI; Fig. 3O), indicating TDCPP induced either late apoptosis or necrosis in these cells. Furthermore, after exposure to 200 μg/mL TDCPP, the cell number was significantly declined with HCECs showing Annexin V-FITC positive signal (Fig. 3R). The proportion of PI-positive cells was also elevated, implying more severe damage occurred in HCECs.

3.3. TDCPP upregulated ER-stress and apoptosis regulatory gene expressions

Mounting evidences show that endoplasmic reticulum (ER) plays a key role in toxicant-induced apoptosis. Moreover, ER-stress-mediated apoptosis is also involved in the pathogenesis of several eye diseases (Jing et al., 2012). To figure out whether TDCPP induced ER-stress in HCECs, the ER stress-related genes, consisting of CPEBP

Table 1 Primers for RT-qPCR of ER stress and Cell Apoptosis Regulatory Genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reserve primer (5’-3’)</th>
<th>Accession no.</th>
<th>Production size (bp)</th>
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</thead>
<tbody>
<tr>
<td>ATP4</td>
<td>ATGACCGAAATGCCTTCCTG</td>
<td>GCTGAGAACCCTAGCAGCT</td>
<td>NM_182810</td>
<td>153</td>
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<tr>
<td>BIP</td>
<td>CAGCTCTTGTGAGCCCAAG</td>
<td>CCAATAAAGCTACCAGGGTTT</td>
<td>NM_003474.4</td>
<td>215</td>
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<tr>
<td>CHOP</td>
<td>GGAAAGATCGGTCATCC</td>
<td>CTGCTTCAGGCGCTTCT</td>
<td>NM_00159055</td>
<td>116</td>
</tr>
<tr>
<td>XBP1</td>
<td>TACGCAAGACTACATGGGCC</td>
<td>GGGTTCAAGGGTGACAGTGC</td>
<td>NM_005080.3</td>
<td>283</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>GCTGGCGCTGACGTCG</td>
<td>CGTGCAGGTACCTGCTA</td>
<td>NM_000633.2</td>
<td>89</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>CTTGACGAGCGAATCGACT</td>
<td>CTGTACCGAGGCGATGCA</td>
<td>NM_004546.1</td>
<td>129</td>
</tr>
<tr>
<td>GADD45x</td>
<td>GAGACCAGAGAAGACCAAGGCA</td>
<td>CACTGATGCGTCCGTA</td>
<td>NM_001924</td>
<td>87</td>
</tr>
<tr>
<td>RN1851</td>
<td>GTAACCCCGTGACACCCATT</td>
<td>CCAATCCCTGATAGCAGG</td>
<td>NM_001101.3</td>
<td>151</td>
</tr>
</tbody>
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homologous protein (CHOP), binding immunoglobulin protein (BiP), as well as activating transcriptional factor 4 (ATF-4) and X-box binding protein-1 (XBP-1) were tested by q-RT-PCR. After exposure to 2 mg/mL TDCPP, their mRNA expressions were upregulated by 1.7-3.6 fold (Fig. 4A). The highest increases in BiP (4.5 folds), CHOP (12 folds), and XBP-1 (4.1 folds) were observed after exposing to 200 mg/mL TDCPP. The data indicated that TDCPP caused ER-stress in HCECs.

Both extrinsic and intrinsic apoptosis pathways are involved in ER-stress induced apoptosis (Breckenridge et al., 2003). Bcl-2 and Bax, as important members of Bcl-2 family proteins, have anti-apoptotic and pro-apoptotic activities respectively, both regulating mitochondrial apoptosis pathways. Increased pro-apoptotic Bax mRNA level in HCECs was observed in a dose-dependent manner (Fig. 4B). For example, anti-apoptotic Bcl-2 mRNA expression was not affected at 2 mg/mL, while it increased to 1.4-folds at 20 mg/mL but decreased to 0.6-fold at 200 mg/mL compared to control. Additionally, upstream regulatory gene of Bcl-2/Bax, growth arrest and DNA-damage-inducible protein 45 alpha (GADD45a) and down-stream effector caspase-3 mRNA expressions were also detected in present study, with upregulation of both GADD45a and caspase-3 being observed (Fig. 4B).

3.4. TDCPP altered Bax and Bcl-2 protein expression and induced loss of mitochondrial transmembrane potential

Given that TDCPP perturbed Bax and Bcl-2 mRNA expression (Fig. 4B), herein, we tested their protein expression using ELISA. Consistent with mRNA expression, Bax level was significantly increased in a dose-dependent manner (Fig. 4D). However, anti-apoptotic Bcl-2 protein expression was increased to 1.4 fold after exposing to 2 mg/mL TDCPP, 1.2-folds at 20 mg/mL but dynamically decreased to 0.4-fold at 200 mg/mL compared to control (Fig. 4C), suggesting TDCPP could perturb Bax and Bcl-2 at both mRNA and protein levels and mitochondrial apoptosis pathways might be involved. To further investigate the involvement of mitochondria during TDCPP-induced apoptosis in HCECs, the mitochondrial transmembrane potential (∆Ψm) was measured using inverted fluorescent microscopy combined with JC-1 probe. The JC-1 can aggregate and accumulate in normal cellular mitochondria, showing red fluorescence (Fig. 5A-D). After exposure to TDCPP, red fluorescence intensity was attenuated (Fig. 5JNR), showing an increased green fluorescence signal in a concentration-dependent manner (Fig. 5KQS). For example, the percentage of fluorescence green in the negative control was 4.8%, which increased to 90% in the positive control. More interestingly, the percentage of fluorescence green in HCECs increased from 20 to 53% and to 80% after exposing to 2, 20 or 200 mg/mL TDCPP.

3.5. TDCPP induced loss of cellular ATP content and enhanced caspase-3, 9 activity

Mitochondria are intracellular organelles that generate ATP through the process of oxidative phosphorylation. The loss of mitochondrial membrane potential usually results in decrease of ATP level in mitochondrial apoptotic pathway (Desagher and
As such, we further determined the effects of TDCPP on the cellular ATP content in HCECs. The results showed that TDCPP exposure significantly decreased the cellular ATP levels in a dose-dependent manner (Fig. 6A). The apoptotic process includes the activation of cysteine proteases, which represent both initiators and effectors of cell death. In this study, as one of important apoptotic event initiators, caspase-9 activity was increased to 1.1, 1.5, and 1.9 after exposing to TDCPP at 2, 20, and 200 µg/mL (Fig. 6C). Caspase-3, a critical downstream effector of mitochondrial apoptosis pathways was not activated at 0.034–340 µg/mL TDCPP in this study (Crump et al. 2012; Li et al., 2017). Caspase-3 activity was further increased to 2.1 folds of the control at 200 µg/mL TDCPP, suggesting that TDCPP exposure activated caspase-3 at a dose-dependent manner.

4. Discussion

In this study, we evaluated the adverse effects of TDCPP on human corneal epithelial cells. The parameters evaluated included cell viability and LC50 (Fig. 1), cell morphology (Fig. 2), cell apoptosis (Fig. 3), mRNA expression of biomarkers of ER-stress and cell apoptosis regulatory genes (Fig. 4AB), Bax and Bcl-2 protein levels (Fig. 4CD), mitochondrial membrane potential (Fig. 5), cellular ATP content and caspase-3/9 activity (Fig. 6).

Accumulating evidences demonstrate that indoor dust is a main route for human exposure to TDCPP. Recent study found the association between human health effects (i.e., altered hormone levels and decreased semen quality in men) and TDCPP in indoor dust (Meeker and Stapleton, 2010). Moreover, TDCPP concentrations in indoor dust range from <0.03 to 326 µg/g (Carignan et al. 2013). Previous studies on TDCPP toxicity using mammalian cells used concentrations from 0 to 129 µg/mL compared to 0.034–340 µg/mL TDCPP in this study (Crump et al. 2012; Li et al., 2017). Cell viability is vital to evaluate cellular responses to contaminants, reflecting cell metabolic activities and death (Xiang et al., 2014). Cell viability decrease to <80% was observed in human adrenocarcinoma cells (H295R) after exposing to 10 µg/mL TDCPP (Liu et al., 2012). In this study, TDCPP inhibited HCEC viability at concentration >68 µg/mL, with LC50 being 202 µg/mL, which was higher than that of human neuroblastoma cells (SH-SY5Y) at 43 µg/mL (Li et al., 2017). These difference could be attributed to different cell types (normal HCECs vs. neoplastic H295R and neuroblastoma SH-SY5).

Cell apoptosis is well recognized as a crucial form of cell death (Elmore, 2007). Several studies showed that brominated flame retardants elicit cytotoxicity by inducing cell apoptosis (Al-Mousa and Michelangeli, 2012; Hu et al., 2007). In this study, the effects of TDCPP on apoptosis was investigated using Annexin V-FITC/PI staining combined with inverted fluorescence microscopy. Data showed that TDCPP at 2 µg/mL initiated weak early apoptosis in HCECs while the percentage of early and late apoptotic cells was remarkably increased after exposure to 20 or 200 µg/mL TDCPP (Fig. 3). Similarly, the apoptosis rate of SH-SY5 cells after exposing to 4.6–86 µg/mL TDCPP increased ~18 folds compared with control (Li et al., 2017). Previous result together with our data indicated that apoptosis was involved in TDCPP-induced HCEC cytotoxicity.

To further investigate the associated molecular mechanism of HCECs apoptosis, we hypothesized that endoplasmic reticulum (ER) stress may be involved in TDCPP-induced apoptosis. This was based...
on a recent report that biomarker genes of ER stress are significantly upregulated after TDCPP exposure in zebrafish (Liu et al., 2016). Disruption of the ER protein system by toxicant results in accumulation of unfolded or misfolded proteins in ER lumen, leading to ER stress (Sharma et al., 2008). In ER stress, protein-folding chaperone proteins such as BiP is upregulated after ATF-6 activation. Subsequently, XBP1 expression is also increased followed by PKR-like ER kinase activation. In addition, with ER stress persistence, apoptosis can induce CHOP expression (Oyadomari and Mori, 2004). CHOP is a transcription factor downstream of PERK/ATF4, which is positively controlled by the PERK/ATF-4 (Hetz, 2012) and is expressed when ER homeostasis is disturbed. In a recent study, the expression of GRP78 (BiP) increased 2.1 and 2.4-fold after exposing to 22 and 43 μg/mL TDCPP in human neuroblastoma cells. A dose-dependent elevation at 1.7 to 2.5 folds in CHOP and ATF-4 expression was observed upon exposure to 11–43 μg/mL TDCPP (Li et al., 2017). Similarly, in our study, dose-dependent expression of BiP, XBP-1, CHOP, and ATF-4 were observed after exposing to ≥2 μg/mL TDCPP (Fig. 4), suggesting that HCECs were more susceptible to TDCPP than human neuroblastoma cells in molecular responses and confirming our hypothesis that ER stress was involved in TDCPP-induced HCEC apoptosis.

When the ERs fail to recover from their normal function, an apoptotic pathway is activated (Momoi, 2004). Bcl-2, an inner mitochondrial membrane protein, is a crucial regulator to block apoptotic cell death. The Bax protein is also a member of the Bcl-2 family that promotes apoptosis. CHOP is a sensitive stress-inducible gene that is strongly elevated when ER homeostasis is disrupted. It has been reported that CHOP decreases the ratio of Bcl-2/Bax expression to induce apoptosis (McCullough et al., 2001). Interestingly, in our study, Bcl-2 was initially increased to 1.4-fold after exposing to 20 μg/mL TDCPP but decreased to 0.7-fold at 200 μg/mL, with both CHOP and Bax mRNA expressions being gradually enhanced (Fig. 4B). Moreover, the changes of Bcl-2 and Bax protein expression showed a similar trend with their mRNA expression (Fig. 4CD), implicating that HCECs may defend extracellular stress of TDCPP to alleviate the apoptosis at low concentrations. At higher TDCPP exposure, it exceeded the self-protection capacity and eventually led to more cell apoptosis, which was consistent with our results (Fig. 3). Growth arrest and DNA damage 45 α (GADD45α), another pro-apoptotic factor, is often simultaneously induced with CHOP by DNA damage and apoptotic signals. In this study, upregulation of ER targeting genes (ATF4, BiP, CHOP, and XBP1) (Fig. 4A), GADD45α and pro-apoptotic factors (Bax), and decrease of anti-apoptotic factor (Bcl-2) further demonstrated activation of ER stress during TDCPP exposure in HCECs. In addition, ER stress was involved in the onset of HCECs apoptosis, which was mediated by the mitochondrial apoptosis pathway evidenced by loss of ΔΨm (Fig. 5) and cellular ATP level decrease (Fig. 6A) (Li et al., 2017; Saha et al., 2010). The caspases are a family of cysteine proteases whose activation induces cellular apoptosis. Caspase is produced as inactive zymogens and undergoes proteolytic activation during apoptosis. Caspases-3, a crucial member of caspases family, plays a pivot role in the execution of apoptosis in mitochondrial pathway (Budihardjo et al., 1999). OPFRs such as triphenyl phosphate induce significant dose-dependent increases of caspase 3 activities in human brain blastoma cells, which is an important contributor for triphenyl phosphate-induced cell death.
apoptosis (Belcher et al., 2014). Similarly, our results clearly showed that TDCPP exposure enhanced caspase-3 in a dose-dependent manner (Fig. 6B), which was consistent with cell apoptosis (Fig. 3) and mRNA expression (Fig. 4B). Caspases-9 is a major upstream initiator in mitochondrial-mediated apoptotic pathway, which is responsible for caspase-3 activation (Boatright and Salvesen, 2003). In present study, caspase-9 exhibited similar activation profile as that of caspase-3 after cells were exposed to TDCPP (Fig. 6C). Taken together, these data suggested that TDCPP exposure induced HCEC apoptosis partially by mitochondrial apoptotic pathway through initiating a series of events involving mitochondrial transmembrane potential loss, and caspase-9 and caspase-3 activation.

5. Environmental implications

After PBDE flame retardants being banned, as the main alternative, TDCPP is often added to products including baby merchandises, furniture, carpet padding, and automobiles. Like PBDEs, TDCPP has been widely detected in dust samples from houses, offices, and vehicles with the concentrations ranging from <0.03 to 326 µg/g (Carignan et al. 2013; Van den Eede et al., 2011). More interestingly, recent studies found that people living with higher TDCPP concentrations in indoor dust tend to have higher concentrations of TDCPP metabolite BDCPP in their urine (Carignan et al. 2013; Hoffman et al. 2014; Hoffman et al., 2015). Moreover, TDCPP levels in indoor dust are positively associated with altered
levels of hormones and lower sperm quality (Meeker and Stapleton, 2010). Taken together, the data suggest that indoor dust can be a main exposure pathway to TDCPP and chronic daily exposure to high TDCPP concentrations can occur (Betts, 2013; Wei et al. 2015). The prevalence and high concentrations of TDCPP in indoor dust warrant further studies to evaluate its potential health effects, particularly for human eye surface due to their long-term continuous daily exposure.

Human cornea acts as a protective barrier from exterior dust or particulates. Cornea damage may lead to impaired vision and eventual blindness (Oh et al., 2010). Accumulating evidence shows that indoor dust exposure is an important contributor to human eye surface damages (Mölhave et al., 2002; Pan et al., 2000; Xiang et al., 2016a). Moreover, TDCPP in indoor dust has been linked to adverse health effects. In this study, we found that TDCPP decreased cell viability and triggered cell apoptosis. ER stress induction was evidenced by upregulation of its biomarker genes. More interestingly, ER stress has been associated with many eye diseases via ER stress-induced ocular cell apoptosis (Gould et al., 2007; Jin et al., 2012). Under severe ER stress, apoptosis occurs in stressed cells (Schröder and Kaufman, 2005). Moreover, increased expression of pro-apoptotic genes and decreased expression of anti-apoptotic gene were also confirmed. Besides, loss in mitochondrial membrane potential and ATP contents, and increase in caspase-3 and caspase-9 activity were also observed in HCECs after exposing to TDCPP. These results implicated the involvement of ER stress in TDCPP-induced apoptosis and it may be mediated by mitochondrial apoptotic pathway.

People are exposed to TDCPP-containing dust on a daily basis and such exposure can last for years even decades. Coupled with its increased use in house products and frequent detection in indoor dust, it is important to evaluate its adverse health impacts on human eyes.

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