SDRP Journal of Earth Sciences & Environmental Studies (ISSN: 2472-6397)

Triphenyl phosphate (TPP) and tris (2-chloroisopropyl) phosphate (TCPP) induced apoptosis and cell cycle arrest in HepG2 cells

DOI: 10.25177/JESES.4.1.RA.409

Research

Received Date: 02nd Nov 2018
Accepted Date: 21st Nov 2018
Published Date:28th Nov 2018

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CITATION

Jing An, Triphenyl phosphate (TPP) and tris (2-chloroisopropyl) phosphate (TCPP) induced apoptosis and cell cycle arrest in HepG2 cells(2018)SDRP Journal of Earth Sciences & Environmental Studies 3 (4)

Abstract

The purpose of this study was to investigate the effects of organophosphate flame retardants (OPFRs) exposure in human liver carcinoma cell line HepG2 in terms of cell viability, apoptosis induction and cell cycle changes. The results data showed that triphenyl phosphate (TPP) and tris (2-chloroisopropyl) phosphate (TCPP) could significantly inhibit the cell proliferation and enhance the level of reactive oxygen species (ROS) and mitochondrial membrane potential (MMP). Moreover, TPP reduced the activities of c-Jun N-terminal kinase (JNK), extracellular regulated protein kinases (ERK1/2) and p38 pathways, while TCPP activated the p38 pathway in HepG2 cells. In addition, TPP mainly induced apoptosis by activating p53-regulated apoptotic pathway, whereas TCPP activated both mitochondrial and p53-mediated apoptosis pathway in HepG2 cells. Furthermore, by regulating the expression of cell cycle proteins, TPP induced cell cycle arrest at G1 and S phase; while TCPP mainly lead to cell cycle arrest at G1-phase. Taken together, the toxicity of TPP and TCPP in the HepG2 cells is associated with ROS generation and MMP alterations, which could induce apoptosis and cell cycle arrest through different molecular pathways. The more severe cytotoxicity of TPP than TCPP might relate to the benzene rings structure of TPP.

Keywords: TPP; TCPP; oxidative stress; apoptosis; cell cycle arrest

INTRODUCTION

Recent years, due to the forbidden of polybrominated diphenyl ethers (PBDEs), organophosphate flame retardants (OPFRs) have been used as an excellent substitute in many aspects of production such as

chemical, electronics, textile, home and building materials industries [1, 2] OPFRs have a good resistance to heat, water and oil and other properties. According to the report of European Flame Retardant Association (EFRA), North America, Western Europe and China had become the largest areas in consuming flame retardants (FRs) in 2010, accounting for about 60% of the world's FRs consumption. In 2011, the sales of phosphorous FRs were far exceeded that of brominated FRs in Europe. [3] As a new kind of organic pollutants, OPFRs have attracted considerable attention from researchers of environmental monitoring and health risk assessment.

Since OPFRs are added to the material in an additive manner, and most of the OPFRs are semi-volatile, they could easily enter into various environmental media by means of volatilization, abrasion and leakage. [4] In reality, a wide range of OPFRs have been detected in water, [5, 6] atmospheric, [7] sediments [8] and biological samples. [9, 10] The concentrations of OPFRs are even higher than PBDEs measured simultaneously at the same areas. Especially, the exposure concentration of OPFRs in indoor dust particles is 2-3 orders of magnitude higher than that of PBDEs. [11, 12]

Triphenyl phosphate (TPP) containing the formula OP $(OC_6H_5)_3$ has been used as a plasticizer in lacquers, varnishes, and hydraulic fluids, and as a fire retardant in a wide variety of settings and products since 1970s. [13, 14] TPP was reported to be one of the most frequently detected organophosphate compounds in fishes and mussels from Swedish lakes and coastal areas with concentrations ranging from 21 to 180 ng/g lipid weight. [15] Tris (2-chloroisopropyl) phosphate (TCPP) containing the formula $CHC_{l0}P$ is a chemical compound resistant to ultraviolet light. As a flame retardant, TCPP is widely used in flexible and rigid polyurethane foam, polyvinyl chloride, polyvinyl acetate, phenolic resins, epoxy resins, and other materials. TCPP has become one of the most dominant compounds detected in water, which is a preferred distributing medium for OPFRs. [16, 17] Until now, the highest concentrations of TCPP in water as reported in United Kingdom Rivers reached 26 μg/L. [18]

Considerable documents have shown that TPP and TCPP exhibit various activities including teratogenicity and potential carcinogenicity, neurodevelopmental toxicity and endocrine disruption. It was reported that TPP could significantly reduce the activity of acetylcholinesterase (AChE) in minnows with the 96 h LC_{50} 2 mg/L. [19] Kim et al. [20] found that TPP elevated the concentration of thyroid hormones T3 and T4 in zebrafish embryos, interfering with the normal development process. In mammal, TPP could inhibit the carboxylesterase (Ces) enzymes in vivo in mouse liver, altering hepatic lipid metabolism, and causing serum hypertriglyceridemia. [21] Compared with tetrabromodiphenyl ether (BDE-47), TCPP has comparable or greater neurodevelopmental toxicity in zebrafish embryos and larvae, resulting in weight loss, hatchability reduction, survival and heart rate, etc. [22-24] Dishaw et al. reported that TCPP exposure impaired the larval swimming activity in early life stage of zebrafish. [23] It has been found that TCPP could alter multiple hormone concentrations and mRNA transcription levels at 9240 ng/g and 51600 ng/g (egg) exposed doses, with significant delayed effects on chicken's incubation. [25, 26]

The latest research of health risk assessment has focused on the environmental impact of OPFRs on the human body. Liu et al. [27] reports that OPFRs can disrupt the balance of sex hormones in humans by influencing steroid production or estrogen metabolism. Individually, TPP has a strong inhibitory effect on human carboxylesterase, altering liver lipid metabolism, leading to serum hypertriglyceridemia. [28] TPP in indoor dust can inhibit human hormone levels and severely reduce male semen quality. [29] In vitro reporter gene assays showed that TPP and TCPP have estrogen receptor alpha (ERα) and receptor beta (ERβ) biological activity. In addition, TPP and TCPP also showed glucocorticoid receptor (GR) antagonistic activity and progesterone X receptor (PXR) biological activity. [30] However, the toxicity and health risk data available for OPFRs is still limited, and the mechanisms under the toxicity are less well understood. In the present study, we used the human hepatoma cells (HepG2) as an in vitro model system for cytotoxicity investigation. This study examined the effects of TPP and TCPP on cell proliferation, reactive oxygen species (ROS), and mitochondrial mem-

brane potential (MMP), apoptosis and cell cycle distribution in HepG2 cells. Then, the expression of different signal pathways such as mitogen activated protein kinases (MAPKs), mitochondrial apoptotic pathways and cell cycle related proteins were measured to explore the potential mechanism. These results will provide additional supplement to the health risk assessment of exposure to OPFRs.

2. Materials & Methods

2.1. Chemicals and reagents

Triphenyl phosphate (TPP) and Tris (2-chloroisopropyl) phosphate (TCPP) were purchased from Lab of Dr. Ehrenstorfer (Augsburg, Germany). Dulbecco's modified eagle's medium (DMEM) with high glucose concentration, D-Hank's and fetal bovine serum (FBS) were obtained from Invitrogen (Paisley, UK). Cell counting kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). 2,7-Dichlorodi -hydrofluorescein diacetate (DCFH-DA), Rhodamine 123 (Rh123) and dimethyl sulfoxide (DMSO) were purchased from Sigma (Saint Louis, MO, USA). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was purchased from BD Biosciences (Sparks, MD, USA). Mammalian protein extraction reagent (M-PER) and bicinchoninic acid (BCA) protein assay were purchased from Thermo Fisher Scientific Inc (Waltham, MA, USA). Polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Darmstadt, Germany). All other reagents were from Sigma (Saint Louis, MO, USA) and were analytical grade chemicals, if not stated otherwise.

2.2. Cell culture and treatments

Human hepatoma cells (HepG2) were purchased from ATCC (American Type Culture Collection) and cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were maintained in an atmosphere of 5% CO_2 and 100% relative humidity at 37°C. For all experiments, cells were passaged every two days and used for experiments at exponential growth phase. Before exposure to OPFRs, cells were seeded into 35-mm culture dishes (5×10^4 cells per well), six-well plates (5×10^4 cells per well) or 96-well plates (3×10^3 cells per well) and cultured for 24 hours. Then fresh culture medium containing different concentrations of TPP or TCPP (50, 100, 200 μM) were supplemented and co-incubated for specified time. TPP and TCPP (200 mM) were dissolved in DMSO as storage solution and the working solutions were freshly diluted by the DMEM with 10% FBS. The final concentration of DMSO in experiments was 0.1% (v/v). Controls cells were treated with corresponding vehicle alone. All experiments were carried out in triplicate with at least 3 replicates.

2.3. Cell viability

CCK-8 assay, a sensitive colorimetric assay to determine the number of viable cells, was used to measure the cell proliferation. In brief, HepG2 cells were seeded in 96-well plates with 6 replicates for 24 h to allow cell adhesion at a cell density of 3,000 cells per well. Then cells were exposed to various concentrations of TPP or TCPP (50, 100, 200 μM) for 24 h or 48 h. After OPFRs treatment, the culture medium was removed, and 10 μL of CCK-8 reagent in 90 μL of fresh serum-free DMEM was added to cells per well, and then incubated at 37°C for 30-60 min. Finally, absorbance at 450 nm of each well was measured with Multiscan Mk3 plate reader (Thermo Electron Corporation, Waltham, MA, USA) to ascertain the number of viable cells indirectly.

2.4. Detection of reactive oxygen species (ROS)

DCFH-DA assay was used to detect the presence of ROS in this study. Briefly, HepG2 cells were seeded in 35-mm culture dishes at a density of 2×10^4 cells/well and treated with TPP or TCPP (50, 100, 200 μM) for 24 h. Then cells were washed twice with warm D-Hank's solution and incubated with DCFH-DA (10 μM in fresh serum-free medium) at 37 °C for 30 min in the dark. After incubation, the medium was removed, and cells were washed with D-Hank's for three times. The cells were observed under a fluorescence microscope (Olympus BX-51, Japan). The intensity of fluorescence was analyzed by Image-pro plus 6.0 software, and the fluorescence intensity of

the control group was normalized to 100%.

2.5. Measurement of mitochondrial membrane potential (MMP)

Rh123 is a positive charged cationic dye, which is predominantly combine with negatively charged mitochondrial matrix enriched into the cells, so it is proportional to the level of the mitochondrial membrane potential (MMP). HepG2 cells at a density of 2×10^4 cells/well cultured in 35-mm culture dishes were treated with different concentrations of TPP or TCPP (50, 100, 200 μM) for 24 h, then washed with warm D-Hank's solution, and incubation at 37°C for 30 min in the dark. Then the medium was removed, and cells were washed with D-Hank's for three times. Finally, the fluorescence of HepG2 cells was observed under a fluorescence microscope (Olympus BX-51, Japan). The fluorescence intensity was analyzed by Image-pro plus 6.0 software with the fluorescence intensity of control group normalized to 100%.

2.6. Cell apoptosis assay

Annexin V is used to detect early apoptotic cells by binding to the extracellular phosphatidylserine. PI is a nucleic acid dye that can not pass through the normal cell or early apoptotic cells of the integral cells membrane but can penetrate the late apoptotic and necrotic cells of the incomplete cell membrane. The Annexin V- FITC/PI detection kit was used to identify and quantify the apoptotic cells according to the instructions of supplier. After treatment with TPP or TCPP (50, 100, 200 μM) for 24 h, cells were rinsed twice with warm D-Hank's buffer, then harvested with 0.25% trypsin and washed twice with cold D-Hank's buffer. Approximately 4×10^5 cells from each sample were suspended in 400 μL binding buffer. The cell suspensions were incubated with Annexin V -FITC at 37°C for 15 min, and then with 10 μL of PI for 5 min. Notably, the staining procedure was conducted in the dark. The stained cells were immediately analyzed by flow cytometry using the FL2 detector with excitation wavelength at 488 nm. The data were analyzed with Summit 5.2 Software. The apoptosis rates referred the percentage of apoptotic cells in total cells.

2.7. Cell cycle assay

Cell cycle analysis in HepG2 was assessed using flow cytometry. In brief, cells were plated in six-well plates at 10^5 cells per well for 24 h to allow cell adhesion, and then treated with TPP or TCPP (50, 100, 200 μM) for 24. Cells were harvested with 0.25% trypsin and fixed with 70% ethanol at -20°C for 24 h. The cell pellets were washed with D-Hank's and resuspended in 1 mL of D-Hank's containing 0.1 mg/mL RNase A at 37°C for 30 min. Before analysis, PI was added to the cell samples to a final concentration of 50 μg/mL. At last, the cell samples were filtered with 300 mesh nylon filter before the test.

2.8. Western blotting

Western blotting was performed according to our previous study. [31] After treatment with TPP or TCPP (50, 100, 200 μM) for 24 h, HepG2 cells were washed with warm D-Hank's buffer and lysed using M-PER to collect the total protein. Cell suspension was made with ultrasonic cell disruptor (VC 130PB-1, Sonic, USA). Protein concentrations were determined by BCA protein assay using Multiscan Mk3 plate reader (Thermo Electron Corporation, Waltham, MA, USA) at 590 nm. After denaturing by boiling for 3 min, proteins were mixed with 6 × Laemmli buffer. Equal amounts of protein (60-80 μg) were subjected to 8-12% sodiumdodecylsulfate- polyacrylamide gel electrophoresis (SDS-PAGE) at 70 V for 30 min and then at 120 V until the bromophenol blue dye reached the bottom and transferred from gel to a PVDF membrane. The PVDF membrane was then blocked with 5% skim milk powder in 3% tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The membrane was incubated with primary antibodies overnight at 4°C, washed with TBST, and incubated with secondary antibodies for 1 h, and washed with TBST for three times. The blots were visualized using chemiluminescence, and the optical densities of individual bands were quantitated using the Chemi-Imager digital imaging system (Alpha Innotech, San Leandro, CA, USA).

All antibodies were purchased commercially as follows: anti-phosph-p38, anti-p38, anti-phosph-p44/42 extracellular regulated protein kinases 1/2 (ERK1/2), anti-p44/42 ERK1/2, anti-B cell lymphoma 2 (Bcl-2), anti-Bcl-2 associated X protein (Bax) , anti-Caspase 3, anti-Caspase 9, anti-p21, anti-p27, and anti-Cyclin D1 (Cell Signaling, Beverly, MA, USA), anti-phosph-c-Jun N-terminal kinase 1/2/3 (JNK1/2/3) (T183+T183+T221), anti-JNK1/2/3 and anti-heme oxygenase-1 (HO-1) (Abcam, Cambridgeshire, UK), anti-p53 (Proteintech, Chicago, IL, USA) anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Multisciences Biotechnology, Hangzhou, China), anti-Rabbit IgG (H + L)/HRP and anti-Mouse IgG (H + L)/HRP (Dingguo, China).

2.9. Statistical analyses

Data were analyzed with SPSS software. All experiments were performed in triplicate and data were expressed as mean ± standard error of the mean (SEM) and were compared using one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison test. In all cases, $p < 0.05$ was considered statistically significant.

3. Results & Discussion

3.1. Structures of TPP and TCPP may influence the toxic effect on cell proliferation.

The results of CCK8 displayed that both TPP and TCPP could inhibit the proliferation of HepG2 cells in a time-dependent and dose-dependent manner, and the cytotoxicity of TPP was greater than that of TCPP (Fig. 1C and D). After treated with TPP and TCPP for 24 h, the rate of cell survival reduced to 97.6±2.8%, 92.3±7.1%, 85.5±10.3%, and 93.7±5.6%, 90.3±6.0%, 87.3±7.9%, respectively, as compared with the control groups, and which were continually reduced to 75.0±4.2%, 57.1±7.1%, 51.1±8.0%, and 88.1±8.6%, 84.9±13.1%, 75.4±10.3%, respectively, after another 24 h treatment. The LC50 of TPP and TCPP is 100 and 360 μM for 48 h treatment, respectively. Previous studies have reported the consistent results of higher toxicity of TPP than TCPP. TPP could induce the AChE and BChE activities in Chinese rare minnow, and the 96 h-LC50 values of TPP was 0.5-2 mg/L (1.5-6 μM). [19] Dishaw et al. [23] found that TCPP could impair the swimming activity during the early life stage of Zebrafish at levels of above 100 μM concentration. The molecular structures of TPP and TCPP were showed in Figure 1 A and B. Comparing with TCPP, TPP has three benzene rings rather than chlorine substituents in TCPP. Our previous study has shown that the different chemical structure of xenobiotics could affect the cytotoxicity and metabolic pattern. [31] In this study, our results suggested that the different structure of TPP and TCPP might be one of the reasons for different toxic effects.

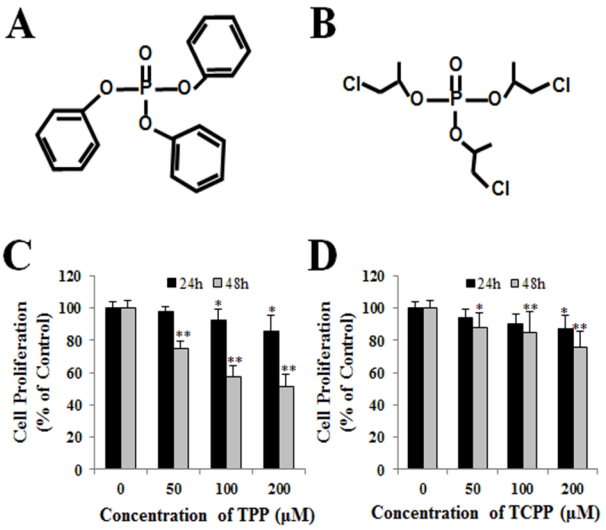

Figure 1. The chemical structure and the toxic effect of TPP and TCPP on cell proliferation in HepG2 cells

Molecular structure of (A) TPP and (B) TCPP. HepG2 cells were treated with different concentrations of (0, 50, 100 and 200 μM) (C) TPP and (D) TCPP for 24 h or 48 h, and the cell proliferation activity was detected by CCK-8 assay. * $p<0.05$, ** $p<0.01$ vs the control (0 μM).

3.2. TPP and TCPP could induce oxidative stress and activate the heme oxygenase (HO) antioxidant protection mechanism in HepG2 cells.

The organism is prone to produce ROS during aerobic metabolism, and the most important ROS is $O_2\bullet^-$ and H_2O_2, which mainly derived from the mitochondrial respiratory chain and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. [32] In this

study, cells were treated with 50, 100 or 200 μM of OPFRs for 24 h, and then the level of intracellular ROS was assessed by DCFH-DA assay. As shown in Figure 2A, both TPP and TCPP significantly increased the ROS levels in a dose-dependent manner. Under the concentration of 200 μM of TPP or TCPP treatment, the level of ROS increased more than 50% (p<0.01) as compared with control groups. The heme oxygenase (HO) plays an important role in resisting against oxidative stress and maintaining the stability of intracellular environment. Among the three kinds isoenzymes of HO (HO-1, HO-2 and HO-3), HO-1 is easily activated by oxidative stress inducer, playing a protective role against oxidative stress injury. [33] As shown in Figure 2B, the outcomes of immunoblot revealed that exposure of TPP and TCPP resulted in a significant accumulation of HO-1 expression, which further indicated that the induction of oxidative stress with OPFRs exposure activated antioxidant protection mechanism in HepG2 cells.

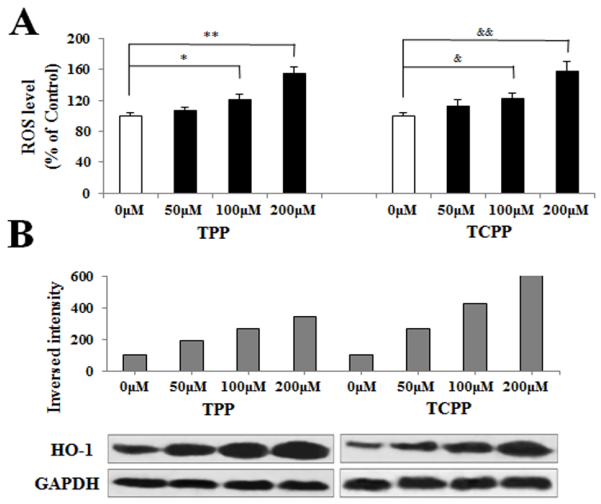

Figure 2. TPP and TCPP induced oxidative stress and heme oxygenase (HO) expression in HepG2 cells

HepG2 cells were treated with TPP and TCPP (0, 50, 100 and 200 μM) for 24 h. (A) The quantification analysis of intracellular ROS generation using the DCFH-DA assay. (B) Protein expression of HO-1 was measured with a western blot assay and quantified by Chemi-Imager digital imaging system. GAPDH protein expression was used as internal reference. * p<0.05, ** p<0.01 vs the control (0 μM), & p<0.05, && p<0.01 vs the control (0 μM).

3.3. TPP and TCPP could influence the MAPKs pathway in HepG2 cells.

The MAPKs signaling pathway was involved in various fundamental cellular processes, such as growth, proliferation, differentiation, migration, metabolism, and apoptosis. The C-Jun amino terminal kinase (JNK) can phosphorylate the NH2 terminus of the transcription factor c-Jun, which is associated with regulation of cell proliferation and apoptosis. [34] The extracellular signal-regulated protein kinase (ERK) 1/2 pathway is commonly activated by various mitogens to modulate multiple processes such as proliferation, differentiation, and survival signals. [34] P38 MAPK is strongly activated by various stress conditions and inflammatory cytokines, including osmotic and oxidative stress. It has been found that ROS activates MAPKs pathway in many cell systems. Thus, we inspected three major families of MAPKs: ERK, JNK and p38 in HepG2 cells after exposure to OPFRs (Fig. 3A). The results showed that there was no difference in total-p38 and ERK1/2, while the expression of P-ERK1/2 and P-JNK1/2/3 were all decreased in cells treated with TPP and TCPP. However, the P-p38 showed exactly opposite outcomes: it was decreased in cells treated with TPP but was increased in TCPP-treated cells (Fig. 3). The role of MAPK families in cellular responses varies depending on the cell type and stimulus. Different excitation of MAPKs to organic compound has also been reported in series of previous studies. Abella et al. [35] reported that non-dioxin-like (NDL)-PCBs 101, 153 and 180 could activate p38 MAPK in murine chondrogenic cell line ATDC-5. TCDD treatment could induce a transient upshift in ERK activity, followed by a decline, but a concomitant dramatic activation of p38 in RAW 264.7 cells. [36] These results suggested that MAPKs families have different response to TPP and TCPP exposure, which might also be one of the consequences of different structure impact.

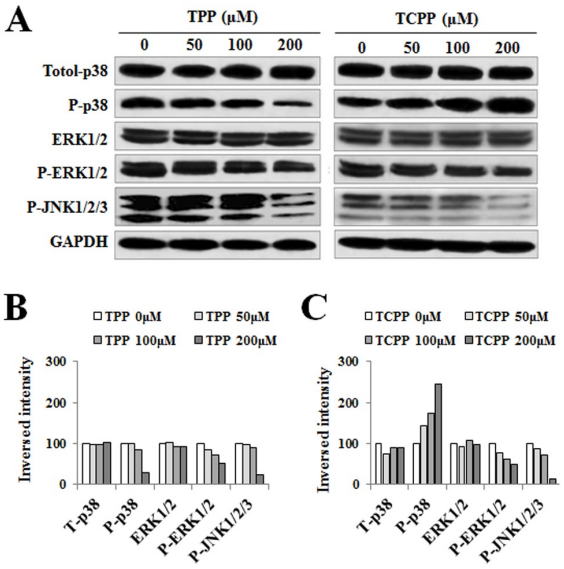

Figure 3. TPP and TCPP could affect the MAPKs pathway in HepG2 cells

HepG2 cells were treated with TPP or TCPP (0, 50, 100 and 200 μM) for 24 h. (A) Protein expression of Total-p38, P-p38, ERK1/2, P-ERK1/2, JNK1/2/3 and GAPDH were measured with the western blot assay. Ipwin 32 software was used for quantitative analysis for protein expression in HepG2 cells treated with (B) TPP and (C) TCPP.

3.4. TPP and TCPP induced apoptosis in HepG2 cells.

Mitochondria are not only the productive sites for ROS generation, but also the important organelles that regulate apoptosis. The increased ROS level can directly or indirectly destroy the integrity of the mitochondrial membrane and change the mitochondrial membrane potential (MMP), resulting in release of the apoptotic factor and induction of irreversible apoptosis. [37] In this study, the apoptosis rate of HepG2 cells treated with TPP or TCPP was investigated by flow cytometry using the Annexin V-FITC/PI staining method. The results displayed that the early apoptosis rate and the necrosis rate were both elevated in a dose-dependent manner in cells treated with TPP, while there was only the necrosis rate showed an obviously increase in TCPP treated cells (Fig. 4A). Then MMP in HepG2 cells was assessed by the Rh123 assay. As shown in Figure 4 B, the hyperpolarization of the MMP in HepG2 cells after treated with TPP or TCPP indicated the potential mitochondria damage. The MMP increased 17.9%, 51% and 15.5%, 25.3% after 100 and 200 μM of TPP and TCPP treatment for 24 h. Furthermore, the greater ability of TPP than TCPP in inducing apoptosis and mitochondria damage was consistent with the more inhibiting of TPP than TCPP on cell proliferation.

Bcl-2 family is closely associated with cell death (apoptosis) process, with Bcl-2 acting as anti-apoptotic factor and the Bcl-2-asslciated protein X (Bax) acting as pro-apoptotic factor. [38] In the cysteinyl aspartate specific proteinase (Caspase) family, Caspase-9 is an apoptosis initiator and Caspase-3 is an executioner to induce apoptosis. [39] For the mechanism study, the expressions of mitochondrial apoptosis-related proteins such as Bax, Bcl-2, Caspase-3 and Caspase-9 in HepG2 cells after TPP or TCPP exposure were measured. As shown in Figure 4C, Bax, Caspase-3 and Caspase-9 were increased in HepG2 cells after TCPP exposure and the Bcl-2 was correspondingly decreased. However, in cells treated with TPP, expression of Bax, Caspase-3 and Caspase -9 were reduced and Bcl-2 expression was elevated after treatment. These results suggested that TCPP might cause cell death through the mitochondrial apoptotic pathways in HepG2 cells, whereas TPP does not.

Besides the mitochondrial-pathway apoptosis, activation of p53 gene by DNA damage signal also leads to apoptosis. Mirella et al. has reported that ROS can directly damage DNA, which would then activate the p53-p21 pathway to cause cell cycle arrest and apoptosis. [40] To explore the potential mechanism of TPP induced cell proliferation inhibition and apoptosis, the expression of p53 was detected with western blot assay. As shown in Figure 4C, p53 expression was up-regulated after TPP and TCPP treatment, which reminded us that the p53 pathway might also play an important regulating role on the apoptosis induced by OPFRs, especially for TPP treatment.

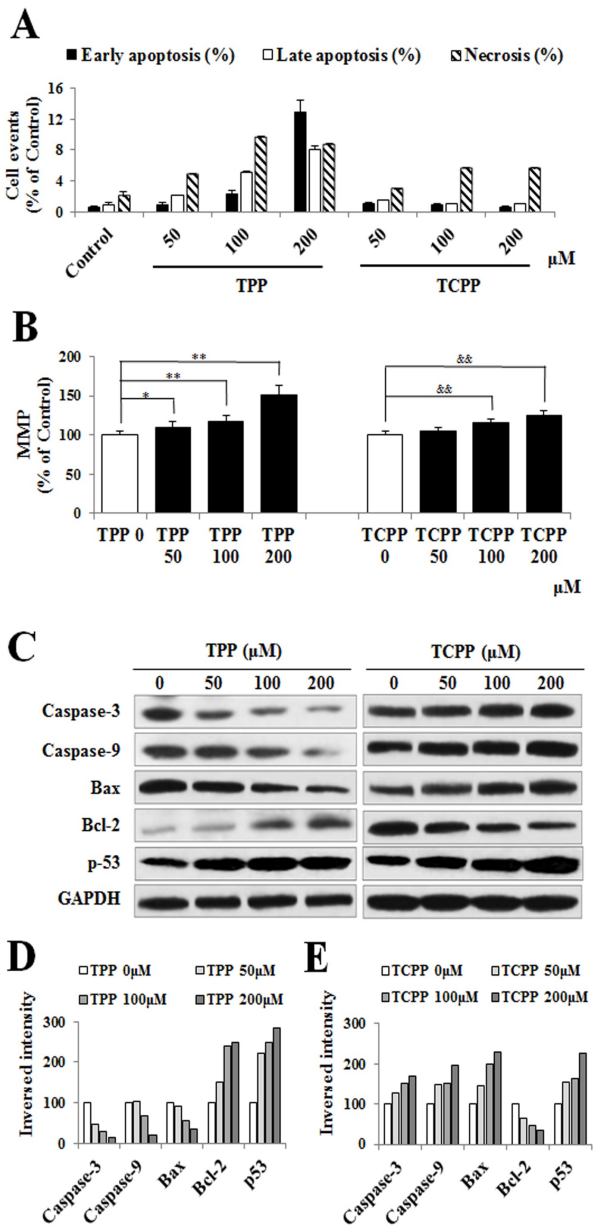

Figure 4. TPP and TCPP could induce cell apoptosis in HepG2 cells

HepG2 cells were treated with TPP or TCPP (0, 50, 100 and 200 μM) for 24 h. (A) The apoptosis was detected by flow cytometry. (B) The fluorescence intensity of mitochondrial membrane potential (MMP) was quantified by Ipwin 32 software. (C) Protein expressions of Caspase-3, Caspase-9, Bax, Bcl-2 and GAPDH were measured using the western blot assay. Ipwin 32 software was used for quantitative analysis in cells treated with (D) TPP and (E) TCPP. * p<0.05, ** p<0.01 vs the control (0 μM), & p<0.05, && p<0.01 vs the control (0 μM).

3.5. TPP and TCPP could induce cell cycle arrest in HepG2 cells.

Except cell death, the rate of cell growth is another rate limiting process to influence cell proliferation, and the speed of cell division determines the rate of cell growth. In this study, the cell cycle after TPP and TCPP exposure was assayed and the distribution of cell cycle phase were arranged in Table 1. Compared with control groups, the proportion of cells in G1 phase increased 6.3-9.2% after treatment with 100 and 200 μM of TPP, respectively (p<0.05). And the proportion of cells in S phase increased 11.2% and 49.5% after treatment with 200 μM TPP for 24 and 48 h, respectively (p<0.05). While TCPP treatment induced only G1-phase arrest, with a 6.5% and 10.5% increase of G1-phase proportion after 100 and 200 μM of TCPP treatment, respectively.

The endogenous regulation of cell cycle in each dividing cycle (including G1, S, and G2/M phase) is mainly mediated by the network of specific cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs). [41] Cyclin D1 is a key protein to regulate cell cycle during G1 phase by combining with CDK4/6 to modulate the cell cycle process. Cyclin A2 is mainly expressed in the proliferation of somatic cells, which can bind with CDK2 during S phase and activate CDK1 during the transition from G2 to M phase. [42] The broad spectrum of CDK inhibitors p21 and p27 mainly block G1 to S-phase conversion during cell cycle by inhibiting the CDK activity. [43]

In order to explore the underlying mechanism of OPFRs-induced cell arrest, the expressions of cell cycle protein Cyclin D1, Cyclin A2, CDK2, p21, and p27 were measured by western blot assay (Fig. 5). TPP treatment induced increase of CDK inhibitor p21 and p27 and reduction of cyclin A2 and cyclin D1, which is expected to inhibit cell proliferation through cell cycle arrest at G0/G1 and S phase. TCPP induced a concentration-dependent decrease on the expression of cyclin D1 and an increase in the expression of p21 and p27. In addition, TCPP had an opposite effect on cyclin A2 and CDK2 expression, which is consistent with the results of the cell cycle distribution that TCPP treatment only induced G1-phase arrest. This finding was consistent with previ-

ous work on cell cycle changes induced by organic compounds. PCB29-pQ could increase the S-phase cell population by down-regulating cyclins A/D1/E and CDK 2/4/6, and up-regulating p21/p27 protein expression.[44] PAHs could reduce expression of cyclin D1 and increased p21 level to mediate G1-phase cell cycle arrest. [45]

Table 1 The distribution of cell cycle phase after OPFRs treatment.

Groups	Time / Dose	24h			48h		
		G1	S	G2/M	G1	S	G2/M
Control	0μM	61.5±0.1	24.9±0.2	13.6±0.3	67.8±1.2	19.4±0.3	12.8±0.4
TPP	50μM	63.9±0.8	23.2±0.7	12.9±0.4	**71.4±0.6***	19.7±0.7	8.9±0.1
	100μM	**65.4±1.2***	23.9±0.7	10.7±0.4	**72.5±1.8***	**23.5±1.2***	7.0±0.6
	200μM	**67.2±0.2****	**27.7±0.2***	5.1±0.5	**70.4±0.4***	**29.0±0.46****	0.6±0.1
TCPP	50μM	63.4±0.6	22.3±0.1	14.3±0.6	**73.3±0.9***	17.0±1.1	9.7±2.0
	100μM	**65.5±0.3****	22.6±0.1	11.9±0.5	**74.9±0.7****	16.9±2.1	8.2±1.4
	200μM	**66.5±0.6****	22.8±0.8	10.7±0.3	**74.5±0.4****	16.5±0.1	9.0±0.5

HepG2 cells were treated with TBP (50, 100, and 200 μM) and TBEP (50, 100, and 200 μM) for 24 or 48 h. The proportion of cell cycle phase was analyzed by flow cytometry. * $p<0.05$, ** $p<0.01$ compared with the control groups.

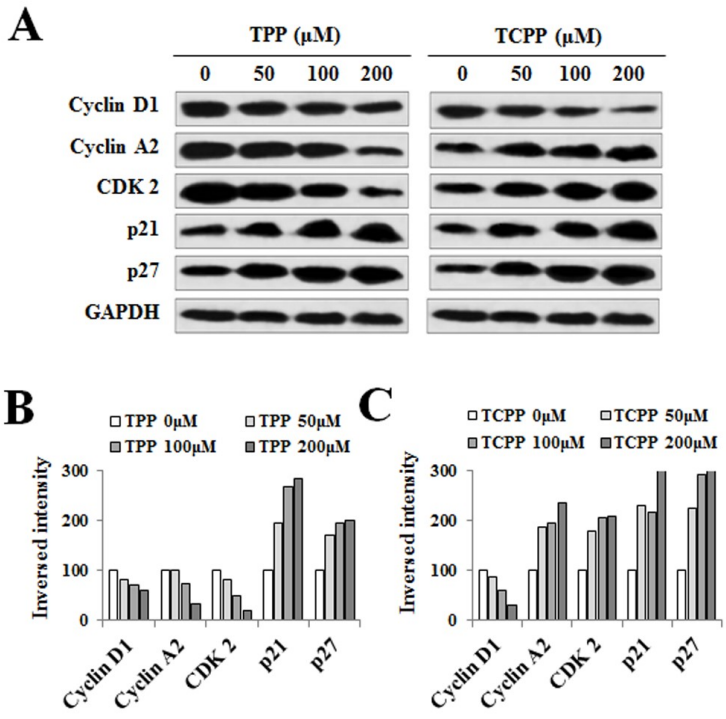

Figure 5. TPP and TCPP induced cell cycle arrest in HepG2 cells

HepG2 cells were treated with TPP (0, 50, 100 and 200 μM) and TCPP (0, 50, 100 and 200 μM) for 24 h. (A) The expressions of cell cycle related protein Cyclin D1, Cyclin A2, CDK2, P21, P27, P53 and GAPDH were measured with western blot assay. Ipwin 32 software was used to quantify protein expression in cells treated with (B) TPP and (C) TCPP.

4. CONCLUSIONS

TPP and TCPP have significant toxic effects in HepG2 cells as evidenced by proliferation inhibition, intracellular ROS generation, MMP changes, apoptosis induction and cell cycle arrest. ROS generation induced by TPP and TCPP may be a key factor of cytotoxicity, which can cause abnormal proteins expression in signal pathways, and finally lead to apoptosis and cell cycle arrest. TPP and TCPP induced apoptosis and cell cycle arrest through different molecular mechanism via diverse signal transduction pathways. TPP reduced all the three MAPK families (p38, ERK1/2, and JNK) activities, while TCPP activated the p38 MAPK pathway. TPP dominantly activate the p53 mediated apoptosis, while TCPP could activate both mitochondrial and p53 mediated apoptosis. Furthermore, TPP and TCPP could cause a concentration-dependent decrease of cyclin D1 expression and an increase in the expression of CDK inhibitor proteins p21 and p27, resulting in significant cell cycle arrest at the G0/G1 phase. In addition, TPP could significantly inhibit the expression of cyclin A2 and CDK2, leading to cell cycle arrest at S phase. In chemical structure, TPP has three benzene rings rather than chlorine substituents in TCPP, which might partly explain the difference of toxic effect and regulation mechanism between TPP and TCPP.

Conflict of interest

The authors declare that there are no conflicts of interest.

ACKNOWLEDGMENTS

The study was supported by grants from the National Natural Science Funds for Distinguished Young Scholars (41225013), the State Key Program of National Natural Science Foundation of China (41130752), the National Natural Science Foundation of China (No. 81072335; 41561144007); Innovative Research Team in University (No. IRT13078).

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