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Original Article

Tris (1,3-dichloro-2-propyl) phosphate treatment induces DNA damage, cell cycle arrest and apoptosis in murine RAW264.7 macrophages

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ABSTRACT — Tris (1,3-dichloro-2-propyl) phosphate (TDCPP) is the most widely used organophosphorus flame retardant, which is now used instead of polybrominated diphenyl ethers (PBDEs). TDCPP has frequently been detected in inorganic environmental matrices, such as soil, water and air as well as biota. *In vitro* effects of TDCPP on cells had not been previously elucidated. Therefore, in the present study, cytotoxicity, DNA damage, cell cycle distribution, apoptosis caused by TDCPP was studied in RAW264.7 macrophage cells. TDCPP reduced viability of RAW264.7 cells in a concentration-dependent manner and caused damage to DNA that was detected by use of the comet assay and caused up-regulation of the level of γ-H2AX. TDCPP increased the intracellular reactive oxygen species (ROS) level in RAW264.7 cells up to 1.44-fold compared to the control group at 12 hr. Percentages of cells in G1 and G2 phases of the cell cycle were dose-dependently greater in cells exposed to TDCPP. TDCPP significantly down-regulated expression of CDK-4, Cyclin D1, Cyclin B1, CDC-2, which are regulators of G1 and G2 phases of the cell cycle. These results demonstrated that TDCPP is cytotoxic and damages DNA in RAW264.7 cells, which resulted in arrest of the cell cycle at G1 and G2 phases and resulted in apoptosis, suggest the necessity to evaluate the effects of TDCPP on the immune system at the cellular level.

Key words: Organophosphorus flame retardant, TDCPP, DNA damage, Cell cycle arrest, RAW264.7 cells

INTRODUCTION

To meet flammability standards set by various jurisdictions, flame retardants (FRs) are added to a wide range of materials, including electronics, textiles, and furniture (Alaee *et al*., 2003; Crump *et al*., 2012; Dishaw *et al*., 2011). After the almost-complete ban in 2009 by the Stockholm convention on persistent organic pollutants (POPs on use of the much used FRs, polybrominated diphenyl ethers (PBDEs)), organophosphorus flame retardants (OPFRs) have been increasingly used in various products (Castro-Jiménez *et al*., 2016). As one of the most commonly used OPFRs, since its first use in 1970s, tris (1,3-dichloro-2-propyl) phosphate (TDCPP), has been widely applied in plastics, foams, electronics equipment and furniture (Dishaw *et al*., 2014; Li *et al*., 2015). Annual production of TDCPP in the United States, in 1998 and 2008 is reported to have been 4500 and 22700 tons, respectively (van der Veen and de Boer, 2012).

TDCPP is an additive FR and thus is not bound to the matrix and can leach from the products to the environment. TDCPP has been detected in a wide range

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of environmental media, such as surface water, drinking water, sediment, air, dust, and tissues of wildlife and humans (Brandsma *et al*., 2013; Cao *et al*., 2012; Giulivo *et al*., 2017; Li *et al*., 2015; Reemtsma *et al*., 2008). In Sweden, TDCPP was detected in an indoor environment (hospital ward) at a concentration of 150 ng/m^3 (Marklund *et al*., 2005). A concentration of 50 ng TDCPP/L was measured in water from the River Ruhr in Germany (Andresen *et al*., 2004). In China, TDCPP was detected in samples of seawater from three coastal cities at concentrations from 24.0 to 377.9 ng/L (Hu *et al*., 2014) and in water from the Songhua River at concentrations of 2.5-40 ng/L (Wang *et al*., 2011) and in sediments from the Pearl River estuary at concentrations up to 4.79 ng/g dry mass (dm) (Hu *et al*., 2017). TDCPP has been detected in a variety of wild biota. For example, concentrations of TDCPP in tissues of fish were as great as 9.56 ng/g lipid mass (lm), glaucous gull eggs (22.54 ± 6.95 ng/g lm), and arctic fox liver with a range of concentrations from < 0.1 to 89 ng/g lm (Hallanger *et al*., 2015). Also, TDCPP was found in food of humans, such as cereals (< 0.5-0.89 ng/g wm), vegetables (< 0.05-1.06 ng/g wm), fruits (< 0.15-0.57 ng/g wm), and meat (< 0.2-0.52 ng/g wm) (Poma *et al*., 2017). TDCPP and its metabolite (bis(1,3-dichloro-2-propyl) phosphate; BDCPP) were detected in human milk and urine of office workers, which indicated that humans are exposed to TDCPP and could thus be at risk of adverse effects (Carignan *et al*., 2013; Sundkvist *et al*., 2010).

Results of previous studies have indicated that TDCPP can cause neurotoxicity, developmental toxicity, endocrine disruption and hepatotoxicity (Li *et al*., 2015; Slotkin *et al*., 2017). Also, TDCPP exposure caused different toxicity in zebrafish, including cell cycle arrest, DNA damage and apoptosis (Chen *et al*., 2018). However, the mechanisms of toxic actions of TDCPP are still limited. TDCPP was toxic to hepatocytes and neuronal cells (Crump *et al*., 2012). TDCPP inhibited synthesis of DNA and altered differentiation in PC12 neuronal cells (Dishaw *et al*., 2011). Concentration-dependent cytotoxicity of TDCPP was observed in HepG2/C3A and A549 cells, with down-regulation of energy metabolism and suppression of pathways relevant to proliferation of cells (e.g. cell cycle, DNA replication) (Zhang *et al*., 2016a).

Structures of OPFRs are similar to organophosphorus pesticides, which are known to cause cytotoxicity and immunotoxicity (Galloway and Handy, 2003; Prabhavathy Das *et al*., 2006), which indicated that OPFRs can exhibit similar modes of toxic actions. TDCPP can perturb expressions of genes involved in immune response, which indicated potential immunotoxic effects (Farhat *et al*., 2014). Macrophages have roles in almost every aspect of an organism's biology, from development, homeostasis and repair, to immune responses to pathogens (Wynn *et al*., 2013). They are innate immune cells with well-established roles in the primary response to pathogens (Martinez *et al*., 2009). Macrophages can ingest and degrade particulate antigens, including bacteria, secrete various cytokines and serve as antigen-presenting cells (Martinez *et al*., 2009). Although the activation/maturation of dendritic cells affected by TDCPP have been reported (Canbaz *et al*., 2017), information on the cytotoxicity and related mechanisms on macrophages induced by TDCPP is still limited. RAW264.7 cells are wildly used as *in vitro* macrophage models in research on inflammation, tumor, immune responses, apoptosis, *et al*. In the present study, the toxic effects of TDCPP on mouse macrophage RAW264.7 cells were studied. The objective of the study was to determine *in vitro* effects of various concentrations of TDCPP on viability of cells, damage to DNA, and alteration of the cell cycle, leading to apoptosis. Also, proteins related to regulation of the cell cycle were analyzed to investigate possible mechanisms for arrest of the cell cycle.

MATERIALS AND METHODS

Materials

TDCPP (CAS no. 13674-87-8, 95.6% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and was dissolved in dimethyl sulfoxide (DMSO) as a stock solution. The Cell Counting Kit-8 was obtained from Dojindo Co. (Kumamoto, Japan). Rabbit anti-Caspase-3, rabbit anti-CDC-2, rabbit anti-Cyclin B1, rabbit anti-Phospho-CDC-2, rabbit anti-Cyclin D1 antibodies were obtained from Cell Signaling Technology (Danvers, CO, USA), mouse anti-γ-H2AX and rabbit anti-CDK-4 antibodies were obtained from Abcam (Cambridge, MA, USA). Secondary horseradish-peroxidase (HRP)-conjugated antibodies and rabbit anti-β-actin antibodies were purchased from Santa Cruz (Dallas, TX, USA). Chemicals for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Amresco (Solon, OH, USA).

Cell culture and treatment

RAW264.7 cells were obtained from the Stem Cell Bank, Chinese Academy of Sciences, and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (Macgene, Beijing, China), and 10% fetal bovine serum (Gibco, Waltham, MA, USA) at 37°C in a humid-

ified atmosphere containing 5% CO_2. Cells were treated with various concentrations of TDCPP for 24 hr. Control cells were incubated with 0.1% DMSO to match the final concentration achieved in culture medium in the experimental exposures.

Cell Viability

Viability of cells was determined by use of the Cell Counting Kit-8 (Dojindo Co.) according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well plate at a cell density of 1×10^4 cells/well and then treated with different concentrations of TDCPP (0, 0.01, 0.1, 1, 10, 50, 100 or 200 μM) for 24 hr. Cells were washed once with phosphate-buffered saline (PBS, pH 7.4). Subsequently, 10 μL of CCK-8 solution was added to each well of the plate, followed by incubation at 37°C for 2 hr. Absorbance was measured at wavelength of 450 nm using a microplate reader (Biorad, Hercules, CA, USA).

Alkaline single-cell gel electrophoresis (comet assay)

The comet assay was performed according to the method described previously by Singh *et al*. (1988) with slight modifications. RAW264.7 cells were harvested after being exposed to various concentrations of TDCPP (0.1, 1, 10, 50, 100 or 200 μM), and 10 μL of cell suspensions were mixed with 70 μL of 0.7% low-melting point agarose, then layered onto microscope slides pre-coated with normal-melting point agarose. Slides were then submerged in an ice-cold lysis solution (10% DMSO, 1% Triton-X in alkaline lysis solution: 2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA; pH 10) and dissolved for 60 min at 4°C in the dark. After washing in PBS (pH 7.4) three times, slides were placed into a horizontal gel electrophoresis chamber with alkaline buffer solution (10 mM NaOH, 200 mM Na_2EDTA, pH > 13) at room temperature for 30 min to facilitate DNA unwinding. Finally, slides were washed in PBS (pH 7.4) and stained with 20 μL of acridine orange (20 μg/L) after electrophoresis at 25 V and 300 mA for 25 min. Slides were then dried in the dark. Images were captured using an Olympus BX-51 fluorescent microscope (Tokyo, Japan). At least 100 cells were randomly selected from each group and analyzed using the Comet Assay Software Project (CASP) 1.2.2.

Determination of intracellular ROS

The level of intracellular ROS was measured using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Jiamay Biotech, Beijing, China). Briefly, cells were seeded at 1×10^5 cells/well in 6-well plates and then treated with different concentrations of TDCPP (0, 0.1, 1, 10, 50, 100 μM) for 6, 12 and 24 hr. Cells were washed once with PBS and incubated for 20 min at 37°C with 10 μM of the probe. Then, the fluorescence intensity was determined using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). At least 1×10^4 cells in the gate were collected for flow cytometry analysis.

Cell cycle analysis

Cell cycle analysis was conducted according to the method described by Yang *et al*. (2014) with slight modifications. In brief, cells were collected after exposure to TDCPP and centrifuged for 5 min at 1000 rpm at 4°C. Supernatants were removed, and the pellets were washed using ice-cold PBS. After centrifugation for 5 min at 1000 rpm at 4°C, the cells were resuspended in 5 mL of ice-cold 70% ethanol, and fixed overnight at 4°C. Followed by centrifugation for 5 min at 1000 rpm at 4°C, the cell pellets were resuspended and stained with 50 μg/mL propidium iodide (PI) containing 0.1% Triton X-100 and EDTA 0.02 mg/mL. DNA content and cell cycle distribution were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using the ModFit software.

Annexin V and propidium iodide (PI) staining assay

Cells exposed for 24 hr to various concentrations of TDCPP were collected and centrifuged for 5 min at 1500 rpm at 4°C. Apoptosis of RAW264.7 cells in each treatment group was then determined by Annexin V/PI apoptosis detection kit (Jiamay Biotech) according to the manufacturer's protocol. Briefly, cell pellets were resuspended in 300 μL of 1 × binding buffer followed by incubation with 5 μL of Annexin V-FITC and 5 μL PI in dark for 15 min. Fluorescence of the cells was then analyzed by flow cytometer (BD Biosciences).

Western Blot analysis

Expression of proteins including γ-H2AX, Caspase-3, Cyclin B1, CDC-2, Phospho-CDC-2, CDK-4 and Cyclin D1 was quantified by use of western blot. Briefly, cells were seeded in 6-well plates for exposure to TDCPP for 24 hr, after which cells were harvested and lysed in the lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (Macgene). Samples were centrifuged at 16900 *g* for 10 min at 4°C. Concentrations of protein were determined using the BCA Protein Assay Kit (Macgene). Each sample (30-40 μg) was loaded and electrophoresed on 10% SDS-PAGE. Proteins were transferred to PVDF mem-

brane after electroblotting at 4°C. Subsequently, membranes were blocked with 5% skim milk and incubated with specific antibodies at 1:1000 dilution at 4°C overnight. Subsequently, membranes were then incubated with the HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG secondary antibodies (1:5000) at room temperature for 1 hr. The protein signals were visualized by enhanced chemiluminescence (ECL) system and the total gray values of each band were analyzed using AlphaEase-FC software V3.1.2. Relative expression of each protein was calculated by normalization to β-actin, and the resulting ratios in the control group were normalized to 1.

Statistical analysis

Experiments were performed at least three times. Values are reported as mean ± standard deviation (S.D.). All data were processed with SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA). The statistical significance of differences was determined by one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

TDCPP inhibited growth of RAW264.7 cells

Effects of various concentrations of TDCPP on growth of RAW264.7 were measured using the CCK-8 kit. Cells were exposed for 24 hr to increasing concentrations of TDCPP (0.01 to 200 μM). Viability of cells displayed a significant decrease ($p < 0.05$) in a dose-dependent manner, and the calculated LC_{50} was 92 μM TDCPP (Fig. 1).

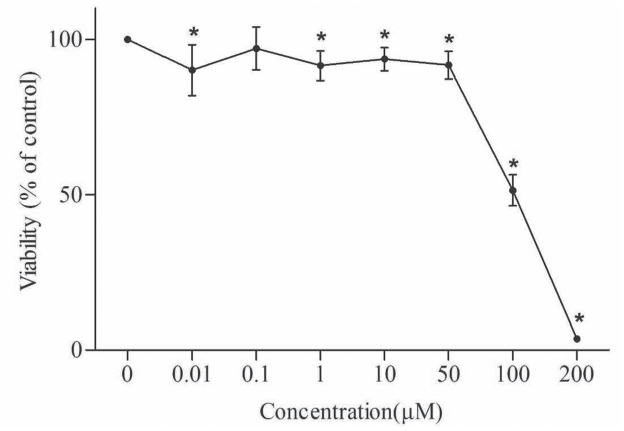

Fig. 1. Effects of TDCPP on viability of RAW264.7 cells as measured by CCK-8 kit after exposure to increasing concentrations of TDCPP (0.01 to 200 μM) for 24 hr. Control group was exposed to solvent (DMSO) only. All data are expressed as mean ± S.D. *p < 0.05, compared with solvent control group.

TDCPP induced DNA damage in RAW264.7 cells

To investigate potential for TDCPP to damage DNA of RAW264.7 cells, the alkaline DNA unwinding assay or comet assay was performed. Distributions of RAW264.7 cells exposed to TDCPP, with respect to the means (± S.D.) of the percentage of tail length, olive tail moment, comet length, and tail DNA are presented (Fig. 2A-D). Concentrations of TDCPP greater than 10 μM caused significant damage to DNA of RAW264.7 cells compared with that in the control group ($p < 0.05$) (Fig. 2). Exposure to TDCPP resulted in DNA comets that exhibited broom-shaped tails and the fluorescence intensity of their heads was weaker than that of controls (Fig. 2E). Expression of the protein γ-H2AX was measured as a marker of DNA double-strand breaks (DSBs). Exposure of RAW264.7 to 0.1, 1, 10, 50 or 100 μM TDCPP resulted in the up-regulation of expression of γ-H2AX in a dose-dependent manner (Fig. 3). Taken together, these results suggested that TDCPP caused DNA damage in RAW264.7 cells.

Production of ROS in RAW264.7 cells exposed to TDCPP

The effect of TDCPP on generation of ROS was measured using the fluorescent dye DCFH-DA. After TDCPP exposure for 6 hr, the intracellular ROS levels in RAW264.7 cells were increased compared with those found in control cells, and the increasing rate peaked at 12 hr (Fig. 4). Treatment with TDCPP higher than 10 μM after 12 hr significantly increased ROS generation up to 1.44-fold of control cells. These results suggested that TDCPP induced cellular oxidative stress in RAW264.7 cells, thus eliciting oxidation damage.

TDCPP induced cell cycle arrest at G1 and G2 phase in RAW264.7 cells

Because damage to DNA usually results in arrest of the cell cycle, the distribution of cells among phases of the cell cycle in RAW264.7 after exposure to TDCPP, was determined by use of flow cytometry. Results of the analysis by flow cytometry indicated that RAW264.7 cells exhibited a statistically significant ($p < 0.05$) arrest in the G1 and G2 phases (Fig. 5A and B). Compared with the control group, treatment with concentrations of TDCPP greater than 10 μM significantly changed the distribution of cells among phases of the cell cycle. The percentage of RAW264.7 cells in G2 phase was greater than the rate of 1% in control cells. Exposure 100 μM TDCPP for 24 hr, resulted in 13% of cells being in G2 phase. Meanwhile, concentrations of TDCPP greater than 10 μM resulted in

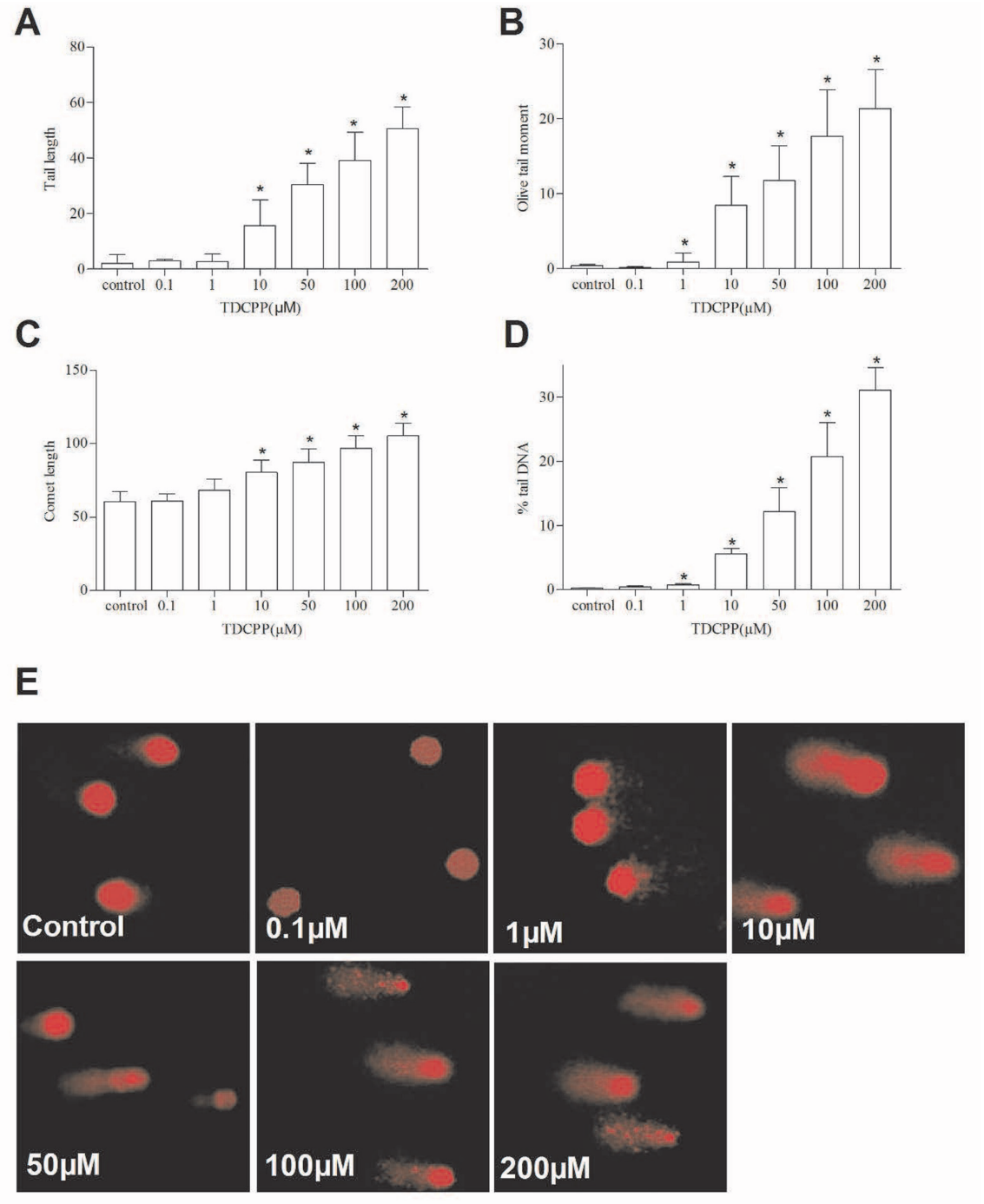

Fig. 2. TDCPP induced DNA damage in RAW264.7 cells, which was calculated by tail length (A), olive tail moment (B), comet length (C), and tail DNA (D) after alkaline comet assay. RAW264.7 cells were treated with various concentrations of TDCPP (0.1, 1, 10, 50, 100 and 200 μM) or DMSO as a control for 24 hr. (E) DNA damage in RAW264.7 cells measured by the alkaline comet assay. The results are representative of three independent experiments. Values are expressed as the mean ± S.D., and at least 100 comets were analyzed in each group. * $p < 0.05$, compared with the solvent control group.

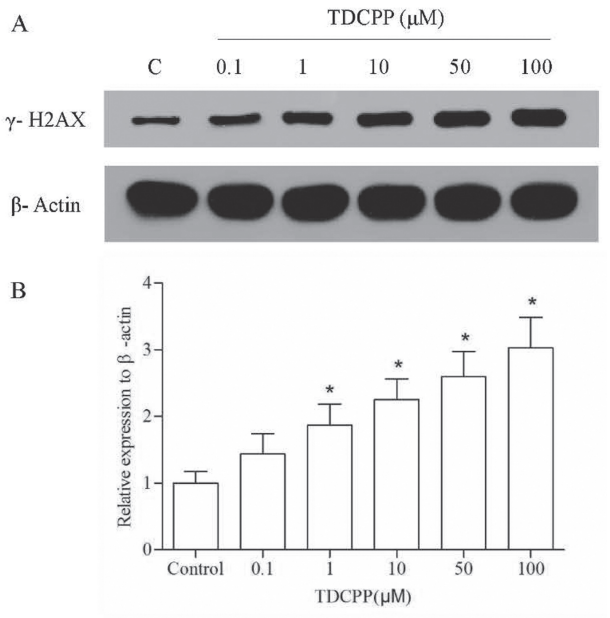

Fig. 3. TDCPP treatment in RAW264.7 up-regulated expression of γ-H2AX. (A) Intracellular concentrations of γ-H2AX were detected by western blot. Cells were exposed for 24 hr to various concentrations of TDCPP and expression of γ-H2AX protein was measured. C, control group. (B) Quantitative results of γ-H2AX level indicated the differences in the intensity after DNA damage. Transcription of protein relative to the control group was normalized to β-actin. Values are expressed as the mean ± S.D., * $p < 0.05$, compared with solvent control group.

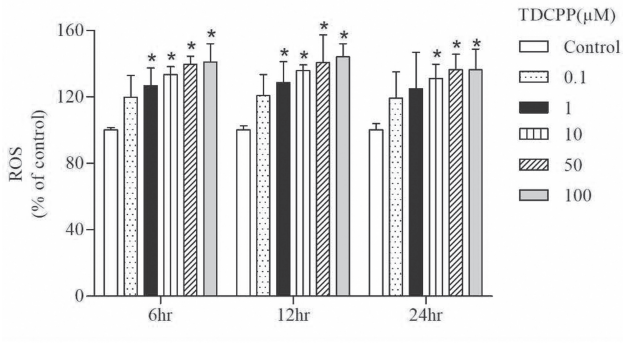

Fig. 4. TDCPP induced production of ROS in RAW264.7 cells. Cells were treated with 0.1, 1, 10, 50 or 100 μM TDCPP or with DMSO as control for 6, 12, and 24 hr. Cells were then stained with DCFH-DA and were subjected to flow cytometry. ROS production was expressed as percentage of control. Values are expressed as the mean ± S.D., * $p < 0.05$, compared with solvent control group.

significantly greater proportions of RAW264.7 cells in the G1 phase.

TDCPP suppressed expression of Cyclin B1, CDC-2, p-CDC-2, CDK-4 and Cyclin D1

In the cell cycle, CDK-4/Cyclin D1 and Cyclin B1/CDC-2 are key regulatory proteins that control transition of cells from G1 to S phase and G2 to M phase, respectively. To investigate the mechanism through which TDCPP causes arrest of the cell cycle at G1 or G2, expression of cell cycle regulators, including Cyclin B1, CDC-2, p-CDC-2, CDK-4 and Cyclin D1, in RAW264.7 cells was measured. Expression of CDK-4, Cyclin D1, Cyclin B1, CDC-2, as well as concentrations of phosphorylated CDC-2 at tyrosine 15 (Tyr15) was significantly ($p < 0.05$) down-regulated after RAW264.7 were exposed to concentrations of TDCPP greater than 10 μM, compared with the control group (Fig. 6). Those results suggest that down-regulation of Cyclin B1, CDC-2, p-CDC-2, CDK-4 and Cyclin D1 might be involved in TDCPP-induced arrest of RAW264.7 cells in phases G1 and G2.

TDCPP induced apoptosis in RAW264.7 cells

Since cells exposed to TDCPP would be likely to undergo apoptosis or death due to arrest of the cell cycle, Annexin V and PI staining was used to detect apoptosis of cells after exposure to various concentrations of TDCPP. That assay has been shown to be able to classify apoptotic cells into two stages, including early apoptotic (Annexin V^+/PI^-) and late apoptotic (Annexin V^+/PI^+) cells (Cui *et al.*, 2010), Percentages of cells occurring as early or late apoptotic cells increased significantly in cells exposed to TDCPP compared with that in the control group (Fig. 7). To further explore the mechanism of apoptosis induced by TDCPP, expression of caspase-3, which is an important regulator of apoptosis was measured. Concentrations of caspase-3 were up-regulated when RAW264.7 cells were treated with TDCPP (Fig. 7B and C). Also, the cleaved form of caspase-3 was observed only in RAW264.7 cells exposed to TDCPP (Fig. 7B and D). These findings confirmed that TDCPP could cause apoptosis in RAW264.7 cells.

DISCUSSION

Several studies have reported toxic effects of TDCPP (Dishaw *et al.*, 2011; Kojima *et al.*, 2013; Xu *et al.*, 2015), but data on mechanisms of these effects are still limited. Effects of TDCPP on the cell cycle of macrophages

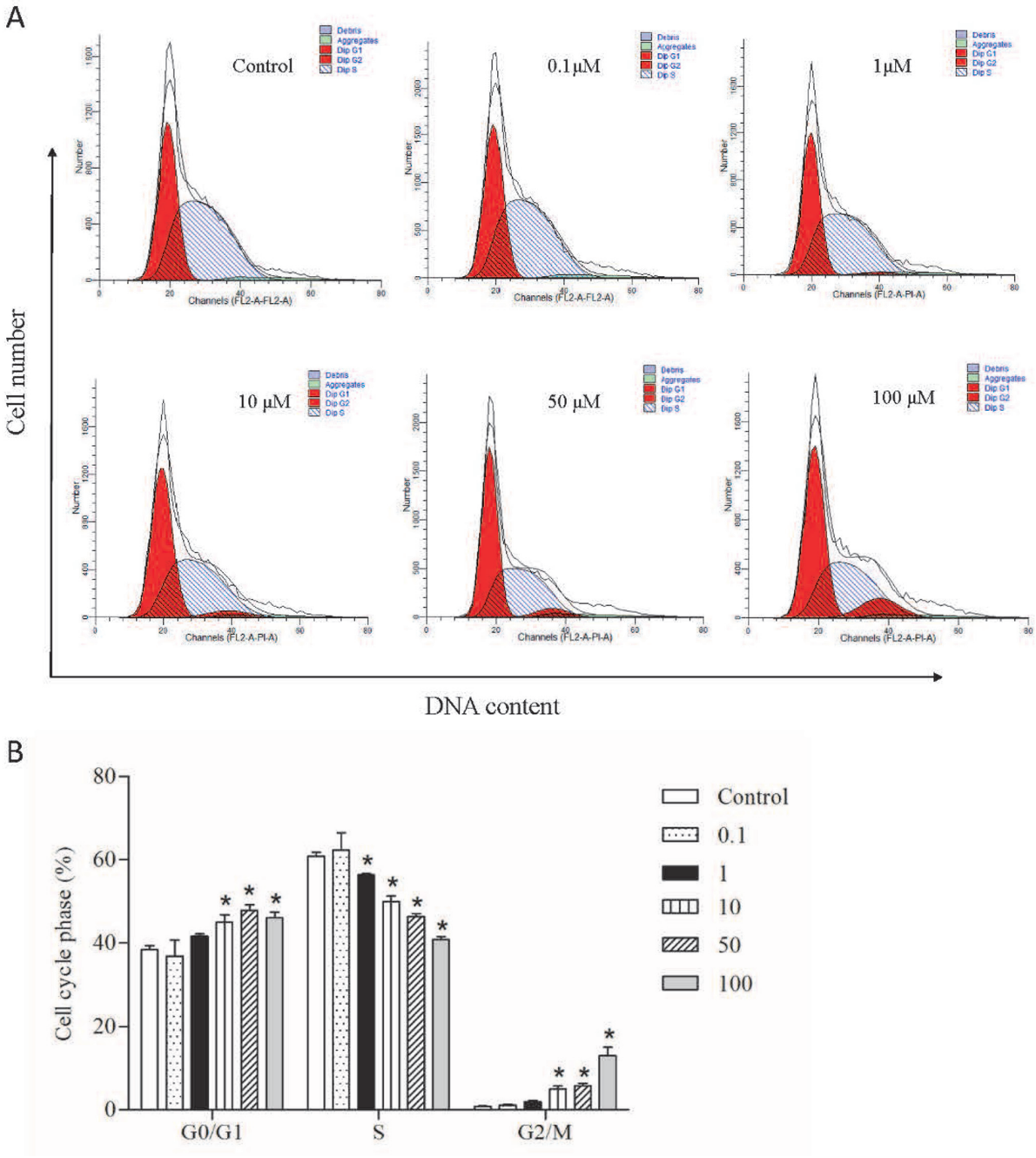

Fig. 5. In RAW264.7 cells, TDCPP caused arrest of the cell cycle at the G1 and G2. (A) Distributions of cells in various phases of the cell cycle when exposed for 24 hr to 0.1, 1, 10, 50 or 100 μM TDCPP or with DMSO as control. Data are shown for one representative experiment. (B) Flow cytometry showed the effect of TDCPP on distribution of RAW264.7 cells in various phases of the cell cycle when exposed to various concentrations. RAW264.7 cells were exposed for 24 hr to 0.1, 1, 10, 50 or 100 μM TDCPP or with DMSO as control. Values are expressed as mean ± S.D., * $p < 0.05$, compared with control group.

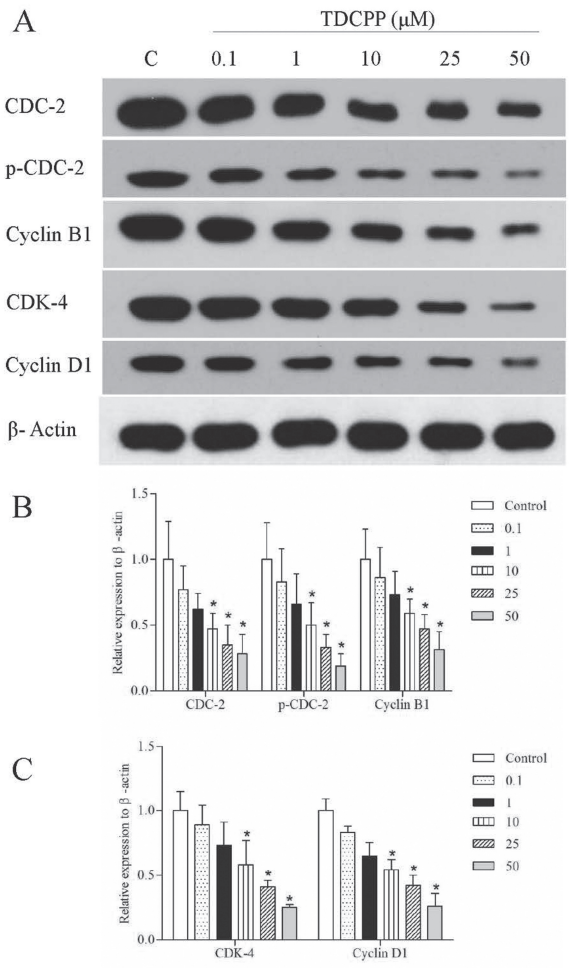

Fig. 6. Expression of cell cycle regulatory protein in RAW264.7 cells in response to TDCPP. RAW264.7 cells were exposed for 24 hr to 0.1, 1, 10, 25 or 50 μM TDCPP or DMSO as control. Western blot analysis results (A) and the relative expression of each protein responsible for G1 (B) and G2 (C) phase arrest are shown. The protein expression relative to the control group was normalized to β-actin. Values are expressed as the mean ± S.D., * $p < 0.05$, compared with solvent control group.

had not been explored. This is the first report to demonstrate TDCPP-induced DNA damage, cell cycle arrest and apoptosis in RAW264.7. The results of the study presented here also provide information for understanding mechanisms of cytotoxicity, carcinogenicity and immunotoxicity caused by exposure to TDCPP.

The observation in the present study, that TDCPP reduced viability of murine RAW264.7 cells in a concentration-dependent manner, was consistent with results of previous studies (Crump *et al*., 2012; Ta *et al*., 2014; Zhang *et al*., 2016a). Other OPFRs, such as tris(2-chloroethyl)phosphate (TCEP) can also cause cytotoxic, genotoxic, or mutagenic effects (Föllmann and Wober, 2006; Ren *et al*., 2008; Zhang *et al*., 2016b), indicated the same toxic potential of TDCPP. The LC_{50} determined from effects observed in this *in vitro* study of RAW264.7 cells, which was 92 μM, was much greater than that determined previously (Crump *et al*., 2012), where the values of LC_{50} were 60.3 ± 45.8 μM in CEH cells and 28.7 ± 19.1 μM in CEN cells. The LC_{50} observed in this study was similar to that observed for human neuroblastoma (SH-SY5Y) cells (Li *et al*., 2017). Based on proliferation of primary cultures, CEH and CEN cells were more sensitive to TDCPP than were RAW264.7 cells.

Here, for the first time, it was reported that TDCPP could cause damage to DNA in RAW264.7 cells. Specifically, TDCPP caused DNA double-strand breaks and up-regulated expression of γ-H2AX. Results of previous studies have demonstrated that some OPFRs, such as TCEP and, tris (2-chloroisopropyl) phosphate (TCPP) also damage DNA (An *et al*., 2016). Alternatively, TCPP did not break DNA of V79 cells or in eight strains of the bacterium *Salmonella*, which indicated the different effect between compounds and cell models used (Föllmann and Wober, 2006). ROS can function as "redox messengers" in intracellular signaling (Yang *et al*., 2014) and inducers of oxidative damage, which are related to various biological processes, such as DNA damage, viability, proliferation and death of cells (An *et al*., 2016). Our results showed that the RAW264.7 cells began to produce ROS when the exposure time reached 6 hr, the increasing rate peaked at 12 hr. TDCPP increased the intracellular ROS level in RAW264.7 cells up to 1.44-fold compared to that of the control group, which indicated that the cells were under high oxidative stress (Liu *et al*., 2018). The results suggested that cytotoxicity and DNA damage induced by TDCPP might result from the high level of ROS caused by TDCPP. ROS also played an important role in cytotoxicity induced by TCPP and tris(2-butoxyexthyl) phosphate (TBEP) on HepG2 and A549 cells, but not on Caco-2 cells (An *et al*., 2016).

Damage to DNA caused by oxidative stress can also result in arrest of the cell cycle and apoptosis. Regulation of the cell cycle and apoptosis is important for maintaining cellular homeostasis between cell division and cell death, and therefore is related to carcinogenesis (Cui *et al*., 2010). Cellular responses to damaged DNA results in arrest of the cell cycle at G1 to S, replication of DNA or G2 to mitosis (Sancar *et al*., 2004). TCEP was recently reported to cause arrest in the cell cycle at the G2/M

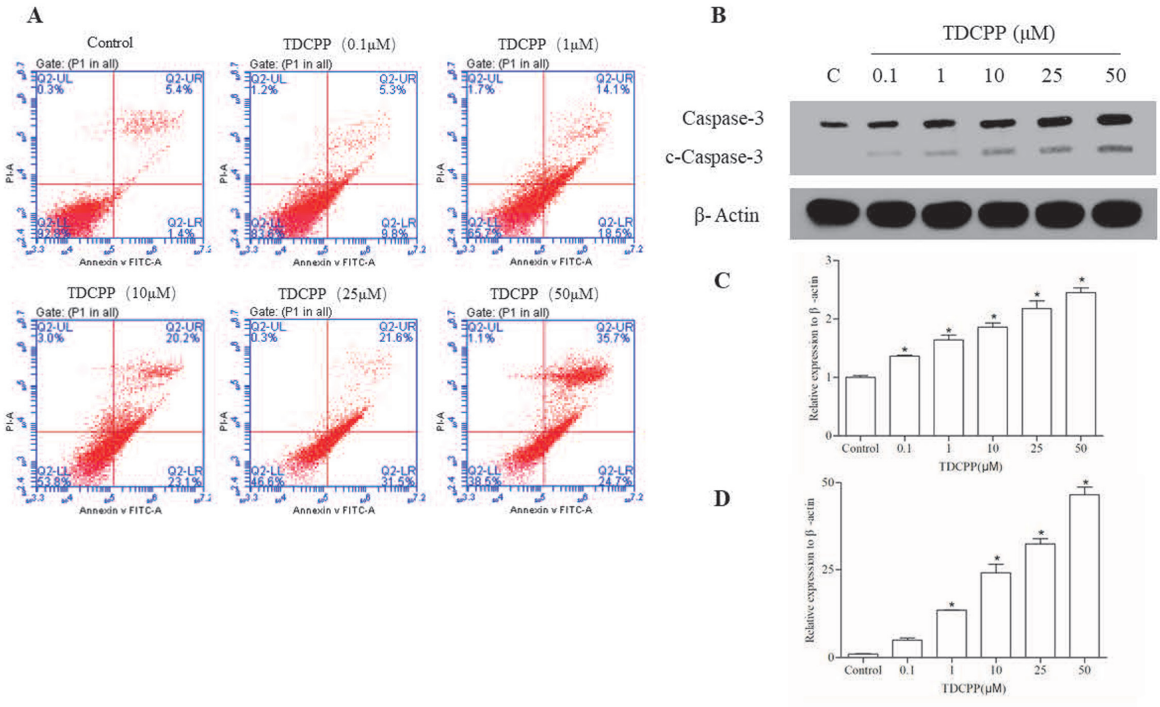

Fig. 7. TDCPP induced apoptosis in RAW264.7 cells. (A) RAW264.7 cells were exposed for 24 hr to various concentrations of TDCPP (0.1, 1, 10, 25 or 50 μM) or with DMSO as control, then stained with Annexin V/PI for flow cytometry analysis. Cell populations shown in the lower left (LL) represented living cells, lower right (LR) represented early apoptotic cells, upper right (UR) represented late apoptotic/necrotic cells and upper left (UL) represented damaged cells. Data were shown for one representative experiment. (B) Cells were treated with TDCPP at different concentrations for 24 hr and the intracellular levels of caspase-3 and cleaved caspase-3 were detected by western blot. C, control group. (C, D) Quantitative results of caspase-3 and cleaved caspase-3 level indicated apoptosis caused by TDCPP in RAW264.7 cells. The protein expression relative to the control group was normalized to β-actin. Values were expressed as the mean ± S.D., * $p < 0.05$, compared with solvent control group.

phase, due to downregulation of SIRT3 in L02 and HepG2 cells (Zhang *et al*., 2016c). In RAW264.7 cells, exposure to concentrations of TDCPP greater than 10 μM resulted in arrest at both G1 and G2 phases. Arrest in G1 phase usually results in cells undergoing repair or in apoptosis (Kavitha *et al*., 2017). In response to cytotoxic effects of TDCPP, RAW264.7 cells exhibited apoptosis. During normal cell growth and function, cell cycle is accurately controlled by checkpoints that permit progress through each phase of the cycle or arrests cells in the G2/M phase. This is often in response to damage or repair of DNA (Visconti *et al*., 2016). Disturbance of cell cycle progress can ultimately lead to apoptosis, especially when damage to DNA is more serious (Visconti *et al*., 2016). DNA damage triggered by exposure to TDCPP might be permanent, and consequently result in cells arrested in the G2 phase to progress to apoptosis. These findings that cells arrested in the G2 phase and subsequent apoptosis in RAW264.7 cells might be associated with inhibition of cell growth and damage to DNA caused by TDCPP are consistent with findings of other investigators (Kavitha *et al*., 2017).

The cell cycle is regulated by cyclins, cyclin-dependent kinases (CDKs), phase-specific protein kinase complexes and other regulatory proteins, and is crucial for maintenance of genomic integrity (Kastan and Bartek, 2004). Cyclin D1/CDK-4 complex is responsible for mitogenic signals in the G1 phase (Clement *et al*., 2001). In RAW264.7 cells, exposure to TDCPP decreased expression of Cyclin D1 and CDK-4, which was considered to contribute to arrest in the G1 phase. Cyclin B1/CDC-2 complex is responsible for progression of cells from G2 to mitosis (Niida and Nakanishi, 2006). Exposure of RWA264.7 cells to TDCPP resulted in arrest in the G2 phase, which was correlated with downregulation of Cyclin B1/CDC-2. Also, phosphorylation of CDC-2 (Tyl-15) in RAW264.7 was less in cells exposed to TDCPP, which

was presumably due to less CDC-2. Data on simultaneous G1 and G2 arrest were limited. Proportions of HT-29 cells in phases G1 and G2/M were dose-dependently increased by exposure to 3,3'-Diindolylmethane, which caused a decrease in activities of CDK-2, CDC-2, Cyclin B1 and Cyclin D1 (Choi *et al*., 2009). Similar results were also reported by Takahashi *et al*. (2014) and Deep *et al*. (2006), but the mechanisms responsible for this simultaneous arrest in phases G1 and G2 remain to be elucidated.

Generation of ROS, in addition to eliciting oxidative stress, is an important proapoptotic signal (Ott *et al*., 2007). In the study results presented here, TDCPP-induced apoptosis in RAW264.7 cells was accompanied with the up-regulated level of caspase-3, together with the overproduction of intracellular ROS. Similarly, TDCPP induced apoptosis via activation of the mitochondrial apoptotic pathway in human neuroblastoma (SH-SY5Y) cells, and generation of excess ROS and increase in $[Ca^{2+}]_i$ were recognized as factors triggering apoptosis (Li *et al*., 2017). The effect of induction of apoptosis observed for TDCPP was similar to that of TCEP, another often used OPFRs, which also induced apoptosis in PC12 cells (Ta *et al*., 2014) and renal proximal tubule cells (Ren *et al*., 2012). The mechanisms by which apoptosis is caused by TDCPP in RAW264.7 still need further elucidation.

Although the present study represents a significant contribution to our understanding of the toxicity and cell cycle arrest pathways induced by TDCPP in cultured macrophage cells *in vitro*, it is likely that general populations are subject to chronic exposure (Li *et al*., 2017), due to the ubiquitous nature of OPFRs, including TDCPP, in both indoor and outdoor environments. Therefore, additional studies are needed to allow evaluation of hazards and risks of TDCPP to animals.

In conclusion, the results of this study suggested that TDCPP could significantly inhibit proliferation of mouse macrophage RAW264.7 cells *in vitro* by arresting cells in the G1 and G2 phases of the cell cycle and then resulting in programmed cell death, apoptosis. The cytotoxic and DNA damage effects induced by TDCPP might result from the overproduction of ROS in RAW264.7 cells. Arrest in the G1 and G2 phase by TDCPP was possibly related to down-regulation of expression of cell cycle checkpoint proteins, such as Cyclin D1, CDK-4, Cyclin B1, CDC-2, and phosphorylation of CDC-2. Arrest of the cell cycle and subsequent apoptosis might play important role in carcinogenesis and immunotoxicity caused by exposure to TDCPP.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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