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Flame retardant tris(1,3-dichloro-2-propyl)phosphate (TDCPP) toxicity is attenuated by *N*-acetylcysteine in human kidney cells

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ABSTRACT

Prolonged exposure to the flame retardants found in many household products and building materials is associated with adverse developmental, reproductive, and carcinogenic consequences. While these compounds have been studied in numerous epidemiological and animal models, less is known about the effects of flame retardant exposure on cell function. This study evaluated the toxicity of the commonly used fire retardant tris(1,3-dichloro-2-propyl)phosphate (TDCPP) in cell line derived from the kidney, a major tissue target of organohalogen toxicity. TDCPP inhibited cell growth at lower concentrations (IC_{50} 27 μM), while cell viability and toxicity were affected at higher concentrations (IC_{50} 171 μM and 168 μM, respectively). TDCPP inhibited protein synthesis and caused cell cycle arrest, but only at higher concentrations. Additionally, the antioxidant *N*-acetylcysteine (NAC) reduced cell toxicity in cells treated with TDCPP, suggesting that exposure to TDCPP increased oxidative stress in the cells. In summary, these data show that low concentrations of TDCPP result in cytostasis in a kidney cell line, whereas higher concentrations induce cell toxicity. Furthermore, TDCPP toxicity can be attenuated by NAC, suggesting that antioxidants may be effective countermeasures to some organohalogen exposures.

1. INTRODUCTION

Flame retardants are a diverse group of chemicals that are designed to slow or prevent the spread of fire. These compounds are added into many household products, but can be gradually released into the environment as the products age, or more rapidly if the products are damaged by flood or fire. The halogenated phosphate triester tris(1,3-dichloro propyl) phosphate (TDCPP) is a high volume additive flame retardant with primary application in polyurethane foams, resins, plastics, textile coatings, and rubber [1]. In fact, TDCPP and other flame retardants can comprise as much as 5% of the total weight in foam products [1]. A recent study found TDCPP in dust from 96% of US households at > 2 ppm, with some as high as > 50 ppm [2]. Furthermore, studies monitoring TDCPP levels in people have found detectable to alarming levels of TDCPP in breast milk, adipose tissue, semen and urine [3–8]. Therefore, more studies are needed to understand the risks and consequences of exposure to TDCPP and other organohalogens.

Although the environmental penetrance of TDCPP is widespread, the compound generally is considered to have low toxicity by regulatory agencies, including the Environmental Protection Agency, International Agency for Research on Cancer, or National Toxicology Program [9]. California's Proposition 65 does list TDCPP as a potential carcinogen, but the No Significant Risk Level (NSRL) is listed as 5.4 μg/day as a "safe harbor value" for industry [10]. In contrast, reports in animal models have shown that TDCPP can disrupt development, reproduction, and endocrine functions, along with increasing risk for some types of cancers [11,12]. The mechanisms of TDCPP actions are mammalian and some mammalian systems [13–16]. To study TDCPP toxicity, the use of a cell culture model is powerful because of the degree of control for dose, duration, and target types. Several studies of TDCPP toxicity have been reported from culture model systems, but only a few of these used human cells [17–21]. Of the human cell studies, only Ren and colleagues utilized cells that were derived from the kidney, although they were used mainly as a transfection system to test organohalogens on thyroid hormone receptor (TR) signaling. The lack of reports with kidney cells is surprising since the kidney is a critical target for organohalogen toxicity due to the accumulation of chemicals prior to excretion. Furthermore, studies in animal have specifically

Abbreviations: ATSDR, Agency for Toxic Substances and Disease Registry; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetraacetic acid; FBS, fetal bovine serum; NAC, *N*-acetylcysteine; SFFCPF, San Francisco Firefighters Cancer Prevention Foundation; TDCPP, tris(1,3-dichloro-2-propyl)phosphate; Tris, tris(2,3-dibromopropyl)phosphate; TR, thyroid hormone receptor

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shown the development of tumors in the kidneys after TDCPP exposure [11].

Therefore the effects of TDCPP were tested in a cell line derived from the human kidney. The study objective was to quantify the changes in cell morphology, growth, viability, and toxicity after exposure to TDCPP over a range of doses and times. Additionally, specific antioxidants were tested in hopes of attenuating the detrimental effects of TDCPP in these cell types. There are many options for antioxidants with demonstrated activity in endothelial cells both in culture and *in vivo*, including single compounds and botanical extracts [22–25]. *N*-acetylcysteine (NAC) was chosen for this study because it is a readily available and cost-effective compound that is listed in the World Health Organization's List of Essential Medicines as being safe and effective [26].

2. MATERIALS AND METHODS

2.1. Chemicals & supplies

TDCPP, NAC, CellLytic M, and other chemicals not for cell culture were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated. Dulbecco's Modified Eagle Medium (DMEM), trypsin-ethylenediamine tetraacetic acid (EDTA), and other cell culture reagents were purchased from InVitrogen (ThermoFisher Scientific, Waltham, MA, USA), except for fetal bovine serum (FBS; Hyclone, Logan, UT, USA).

2.2. Cell culture

HK-2 (human papillomavirus 16-transformed kidney proximal tubule) cell lines were a kind gift from Dr. Pankaj Kapahi, Buck Institute for Research on Aging. The cell line was authenticated by the University of California Berkeley Cell Culture Facility (http://bds.berkeley.edu/facilities/cell-culture) at the beginning of the study; short tandem repeat analysis indicated 100% match for HK-2. Cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin and maintained at 37 °C in 100% humidity. Cell density was maintained below 100% confluency with cultures split with trypsin-EDTA typically once per week. Population doubling time was approximately 3.6 days. Cell counts were measured with a Z2 Coulter Counter equipped with multisizing capabilities (Beckman Coulter, Fullerton, CA, USA). Cell morphology was monitored using a conventional inverted light microscope. For TDCPP treatment, stock TDCPP was first diluted into dimethyl sulfoxide (DMSO), 0.22 µm filter-sterilized, and frozen at −20 °C in small aliquots. DMSO concentration was kept constant at 0.1% v/v in media. All control cells received equivalent volume of DMSO vehicle. For NAC treatment, NAC was dissolved into phosphate-buffered saline (PBS), 0.22 µm filter-sterilized, and frozen at −20 °C in small aliquots. After each aliquot was thawed, residual drug volume was discarded.

2.3. Cell viability

Cell viability was measured using the vital dye exclusion method [27]. Briefly, adherent cells were enzymatically released and incubated in 0.2% trypan blue for 5 minutes at room temperature. Cells with and without dye exclusion were then scored on a hemocytometer by trained technicians.

2.4. Cell toxicity

Cell toxicity was measured using a commercial tetrazolium reduction assay (CellTiter-Blue Cell; Promega Corporation, Madison, WI) according to manufacturer's instructions. Once dye was added to each well, the microplates were incubated for 1 hour at 37 °C. Fluorescence yield was monitored at an excitation of 560 nm and emission at 590 nm.

2.5. Cell protein levels

Protein levels in cell cultures were measured using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Briefly, cell pellets from exposure cultures were lysed in CellLytic M and analyzed using microplate version of assay procedure. Absorbance was monitored at a wavelength of 562 nm.

2.6. Cell cycle analysis

Randomly cycling cell populations were analyzed for cell cycle distribution by propidium iodide staining [28,29]. Cultures were routinely 60–80% confluent prior to drug exposure. Briefly, adherent cells were enzymatically released, washed in PBS, and fixed using ice-cold 100% ethanol. When ready for processing, cells were suspended in staining solution (50 µg/ml propidium iodide, 0.1 mg/ml sodium citrate, 2 lg/ml ribonuclease A, and 0.03% Triton X-100) for 5 minutes prior to analysis using an LSR-Fortessa flow cytometer (BD, Franklin Lakes, NJ, USA). Data were collected using 100,000 events per sample and mean fluorescence was determined using native DIVA software. Cell cycle model analysis was performed using FlowJo software, version 10 (Ashland, OR, USA).

2.7. Statistics

Graphing, regression, and statistical analysis was conducted using Prism software, version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Significance was defined as $p < 0.05$.

3. RESULTS

3.1. Effects of TDCPP on HK-2 morphology

HK-2 cell cultures were exposed to a range of TDCPP concentrations over a range of times. After 24 hours of exposure, 10 µM TDCPP caused a noticeable decrease in cell numbers although the morphology of the cells was similar to controls, whereas cultures with > 100 µM TDCPP showed evidence of cell death (Fig. 1A). Longer exposures to TDCPP resulted in lower concentrations needed to produce the cytostatic and cell death, as expected (data not shown). These observations were quantified using measures of cell growth, viability, and toxicity.

3.2. Effects of TDCPP on HK-2 cell growth

HK-2 cell cultures were exposed to increasing TDCPP concentrations for up to 96 hours, with cell numbers measured at every 24 hours (Fig. 1B). Cellular growth rate began to decline at 10 µM TDCPP, relative to control. At 10–100 µM TDCPP, cell growth rate was inversely proportional to TDCPP concentration, with an IC_{50} of 27 µM (20–36 µM, 95% confidence interval) determined by comparison of the slopes of the growth curves (Supplemental Fig. 1A). At 100 µM TDCPP, cell growth rate was minimal and the slope of the linear function was not significantly different from zero. At TDCPP concentrations above 100 µM, there was no measurable cell growth; cell growth curves yielded negative slopes due to substantial levels of cell death (data not shown).

3.3. Effects of TDCPP on HK-2 cell viability

To determine the cause of TDCPP-induced cell growth inhibition, HK-2 cell cultures were exposed to increasing TDCPP levels to measure the effect on cell viability (Fig. 1C). Unlike cell growth, cell viability was not significantly affected by 24-hour TDCPP exposure until over

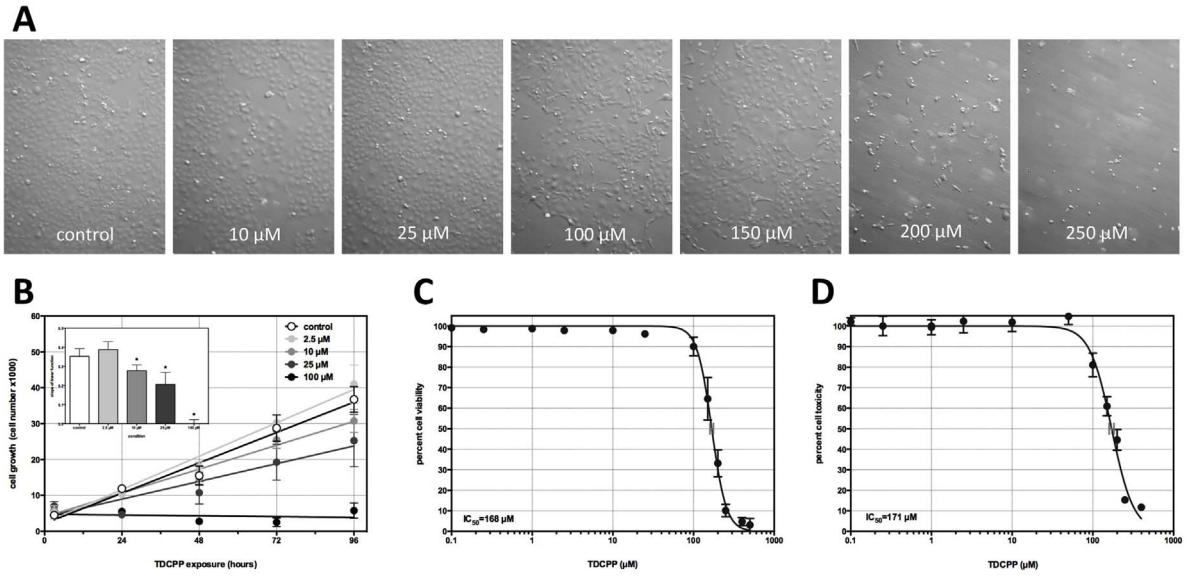

Fig. 1. TDCPP inhibits the growth and viability of HK-2 cells in a dose-dependent manner. (A) Representative light micrographs of cultures exposed to increasing concentrations of TDCPP for 24 hours (100× magnification). A reduction in cell number was evident at 100 μM TDCPP, whereas cell death was evident at 200 μM TDCPP. (B) Changes in cell growth were measured in cultures with continuous exposure to increasing concentrations of TDCPP for up to 96 hours. The mean ± SEM from 3 independent experiments is shown and fit to a linear function. Inset shows the slope for each linear function; asterisks indicate significant difference from slope of control ($p < 0.05$). (C) Changes in cell viability were measured in cultures with continuous exposure to increasing concentrations of TDCPP for 24 hours. The mean ± SEM from 18 independent experiments is shown and fit to a sigmoidal dose-response function. The IC_{50} was 168 μM, with a 95% confidence interval of 160–177 μM (gray bracket). (D) Changes in cell toxicity were measured in cultures with continuous exposure to increasing concentrations of TDCPP for 24 hours. The mean ± SEM from 18 independent experiments is shown and fit to a sigmoidal dose-response function. The IC_{50} was 171 μM, with a 95% confidence interval of 162–181 μM (gray bracket).

100 μM. Analysis of 18 independent experiments indicated that IC_{50} for TDCPP effect on cell viability was 168 μM (160–177 μM, 95% confidence interval). Thus, cell viability was not altered by TDCPP until approximately 5- to 10-times higher concentration needed to alter cell growth. Longer exposure times did result in a small shift in the cell viability response to TDCPP, but did not approach the IC_{50} of cell growth even after 96 hours (Supplemental Fig. 1B). It was therefore determined that 24 hours was sufficient to measure changes in cell physiological parameters after TDCPP exposure.

3.4. Effects of TDCPP on HK-2 cell toxicity

To determine the cause of TDCPP-induced cell growth inhibition, HK-2 cell cultures were also exposed to increasing TDCPP levels to measure the effect on cell toxicity (Fig. 1D). Similar to the effect on viability, 24-hour TDCPP exposure did not alter cell toxicity until over 100 μM. Analysis of 18 independent experiments indicated that the IC_{50} for TDCPP effect on cell toxicity was 171 μM (162–181 μM, 95% confidence interval). Thus, lower concentrations of TDCPP inhibited cell growth (cytostasis) of HK-2 cells without detectable changes in cell viability or toxicity.

3.5. Effects of TDCPP on HK-2 cell protein synthesis

Inhibition of macromolecular synthesis, including protein synthesis, is a known cause of cytostasis, so total protein levels were surveyed in HK-2 cell cultures exposed to increasing TDCPP levels for 24 hours (Supplemental Fig. 2A). The data was fit to a sigmoidal dose-response function, but would not converge to provide an IC_{50}. There was no significant change in protein content until TDCPP concentrations exceeded 250 μM, suggesting that the inhibition of cellular protein synthesis was not a major factor in TDCPP-induced cytostasis.

3.6. Effects of TDCPP on HK-2 cell cycle kinetics

Another potential cause of cytostasis is the activation of cell cycle checkpoints. Therefore, cell cycle kinetics were evaluated in HK-2 cell cultures exposed to increasing TDCPP levels for 24 hours (Supplemental Fig. 2B). Analysis of 4 independent experiments indicated a significant increase in G1 phase cells with a complementary significant decrease in G2/M phase cells at 100 μM TDCPP, suggesting a partial G1 arrest. Cells exposed to lower concentrations of TDCPP did not demonstrate significant change in cell cycle kinetics. Therefore, cell cycle checkpoints might play an important role in TDCPP-induced toxicity but not cytostasis. Additionally, a sub-G1 cell population was evident at 150–250 μM TDCPP, which is suggestive of apoptotic cells, consistent with elevated cell death at higher TDCPP concentrations.

3.7. Effects of NAC on TDCPP toxicity in HK-2 cells

Another potential cause of cytostasis is the cellular response to increased oxidative stress. Therefore, antioxidants were tested in HK-2 cell cultures either before or after TDCPP treatment. One antioxidant was NAC, a derivative of cysteine that has direct antioxidant activity but also can stimulate the levels of the physiological antioxidant glutathione [30]. First, HK-2 cells were treated with 1–10 mM NAC for 1, 3, or 24 hours prior to exposure to increasing concentrations of TDCPP (Fig. 2, top row). NAC pre-treatment had no effect on TDCPP inhibition of cell toxicity. Then, HK-2 cells were treated with 1–10 mM NAC for 1, 3, or 24 hours after exposure to increasing concentrations of TDCPP (Fig. 2, bottom row). NAC post-treatment demonstrated a dose-dependent attenuation TDCPP inhibition of cell toxicity. Other types of antioxidants, including lipoic acid (25–100 μM) and ascorbic acid (1–25 mM), did not show similar protective effects in this cell model (data not shown).

4. DISCUSSION

TDCPP is a chlorinated analog of tris(2,3-dibromopropyl)phosphate (Tris), a flame retardant compound that was widely used until it was shown to be mutagenic and carcinogenic [31,32]. Unlike Tris, TDCCP is currently not listed as a carcinogen by most regulatory agencies and is still used commercially. Numerous studies have documented the penetrance of TDCPP into the average home and workplace, and

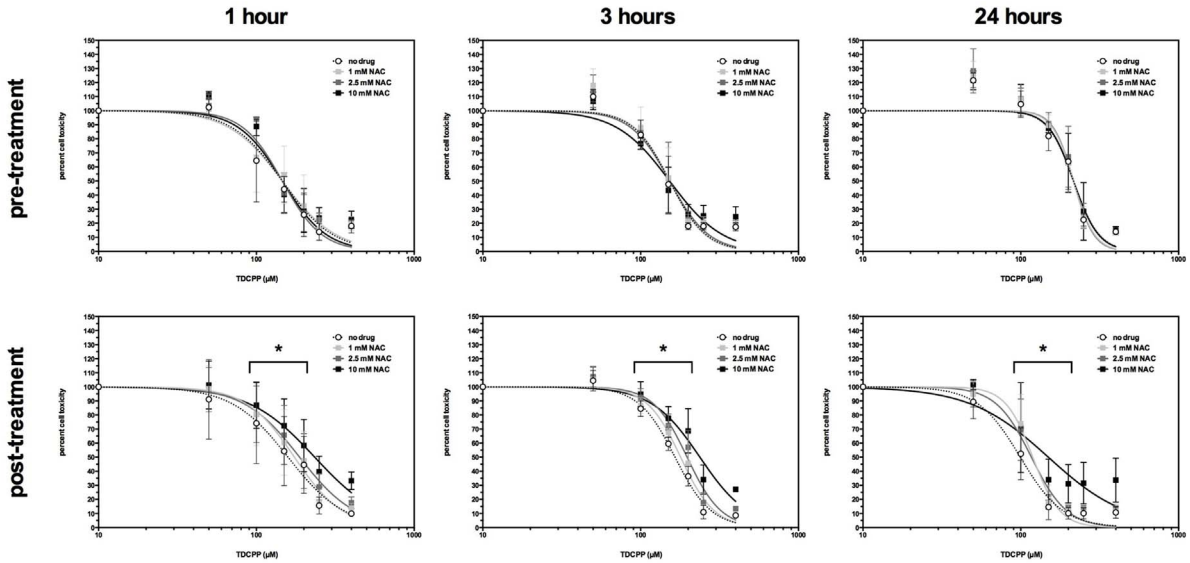

Fig. 2. NAC reverse the effect of TDCPP on cellular toxicity in HK-2 cells in a dose-dependent manner. Cells were treated with increasing concentrations of NAC before (top row) or after (bottom row) increasing concentrations of TDCPP for 24 hours. NAC was provided for 1 hour (first column), 3 hours (middle column), or 24 hours (last column) and at 3 different doses of 1 mM, 2.5 mM, or 10 mM (increasing gray shading). The mean ± SEM from 2–5 independent experiments is shown and fit to a sigmoidal dose-response function. Best-fit values for each curve were tested for significant difference based on the Extra sum-of-squares F test using the $LogIC_{50}$ of each curve; asterisks indicate significant difference between curves ($p < 0.05$).

significant levels of TDCPP have been detected in the population [9]. Moreover, certain groups have higher exposure to flame retardant compounds due to occupation, including firefighters involved in the knockdown and overhaul of structure fires [33,34]. Understanding the health effects of exposure to these chemicals will help define their risk to the average consumer and address their particular health threats to our first responders.

In this study, a kidney-derived culture model was used to characterize the toxicity of acute TDCPP exposure at the cellular level. TDCPP caused inhibition of cell growth at low micromolar concentrations, whereas the effects on cell viability and toxicity occurred at 5- to 10-times higher concentrations. This suggested that low dose TDCPP had cytostatic activity. Compared to reports from biomonitoring studies, this level of TDCPP is still significantly higher than the range of TDCPP levels reported in the average person. For example, one study reports the range of TDCPP in adipose tissue in a small group of US participants as 0.001–0.25 μM [3]. However, the effects described in this study are limited to acute TDCPP exposures; chronic TDCPP exposure may result in drug accumulation and toxicity at lower concentrations. Additionally, the body burden of TDCPP may be higher in the kidneys due to drug accumulation prior to excretion. And as previously mentioned, certain groups like firefighters are likely to have higher body burden of TDCPP compared to the average person [33,34].

This study appears to be the first report of flame retardant toxicity in HK-2 cells, which were derived from proximal tubule tissue in the human kidney. This cell line has been used widely to study renal toxins and subsequent changes in kidney-specific markers [35–37], although other studies have shown the utility of these cells to be more limited [38]. In the only other report of TDCPP in kidney-derived cells, 100 μM TDCPP (but not lower concentrations) decreased cell growth and viability in TR-transfected HEK 293 cells, although the results in non-transfected cells were not shown [20]. Several reports have characterized the effects of TDCPP in non-kidney human cell types [17–19,21]. Most of these reports address disruption in endocrine pathways, with Zhang and colleagues also providing a measure of growth in MCF-7 human breast adenocarcinoma cells after 5 days of TDCPP exposure [19]. More recently, Li and colleagues studied TDCPP effects in SH-SY5Y human neuroblastoma cells and showed evidence of increased oxidative stress and apoptotic cell death [21]. Interestingly, they also found that short-term pre-treatment with NAC could protect attenuate the effects of TDCPP in this cell type. Additional data is available from non-human cell models. Dishaw and colleagues showed cell growth inhibition (via reduced DNA synthesis) with 20–50 μM TDCPP exposure for 24 hours, similar to HK-2 cells [39]. However, cell growth (via protein synthesis) was not reduced when TDCPP exposure was extended to 4-6 days. Dishaw and colleagues also found evidence of increased oxidative stress after 4-day exposure to 50 μM TDCPP (but not 10–20 μM). Increased oxidative stress was suggested in TDCPP-exposed HK-2 cells as well, based on NAC attenuation of TDCPP toxicity. In contrast, another study in PC12 cells conducted by Ta and colleagues showed changes in morphology, viability, apoptosis, and key protein targets at concentrations as low as 5 μM TDCPP [40]. No direct measure of cell growth or oxidative stress was provided for comparison in this study. Crump and colleagues found TDCPP caused cell death in 2 avian hepatocyte lines with an IC_{50} of approximately 30–60 μM TDCPP after 36-hour exposure [41], which is similar to the response in HK-2 cells. Taken together, there are some commonalities among the effects of TDCPP across different cell types, but a comprehensive description is hampered by too few studies and differing exposure protocols. Additionally, a greater attention to lower TDCPP doses for longer time frames will be important to model the toxicity of TDCPP under more relevant circumstances.

In summary, low micromolar concentrations of TDCPP in HK-2 cell cultures caused cytostasis, which can disrupt cell physiology and increase the risk for carcinogenesis. Higher levels of TDCPP caused cell toxicity, which could be partially reserved by the antioxidant NAC. Strengths of this study include the use of human cells, as most previous studies evaluated TDCPP toxicity in whole animals and non-human cell models. Additionally, this study tests TDCPP in a cell line from the kidney, which is a major target of flame retardant toxicity. Furthermore, this appears to be the first study to describe a specific countermeasure to attenuate TDCPP toxicity, but this should be evaluated in future work. Weaknesses of this study include the limits in translation inherent in all cell culture studies, which must always be verified in whole organisms. Because the cell line used in this study was already transformed, the carcinogenic potential of TDCPP could not be studied in this model. Finally, most household products and building materials have a range of different types of flame retardants, which could have synergistic toxic activities when exposures are combined. Future studies should address these concerns with biological models

that reflect real world chemical exposures.

Conflicts of interest

The authors have no conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2017.05.003.

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