Full length article

In vitro neurotoxic hazard characterization of different tricresyl phosphate (TCP) isomers and mixtures

Daniel J. Duarte¹, Joost M.M. Rutten¹, Martin van den Berg, Remco H.S. Westerink*

Neurotoxicology Research Group, Toxicology Division, Faculty of Veterinary Medicine, Institute for Risk Assessment Sciences (IRAS), Utrecht University, NL-3508 TD Utrecht, The Netherlands

A R T I C L E   I N F O

Article history:
Received 14 October 2015
Received in revised form 1 February 2016
Accepted 1 February 2016
Available online xxx

Keywords:
In vitro neurotoxicity
Tricresyl phosphates
Aerotoxic syndrome
Hazard characterization
Microelectrode array (MEA) recordings
Neurite outgrowth

A B S T R A C T

Exposure to tricresyl phosphates (TCPs), via for example contaminated cabin air, has been associated with health effects including the so-called aerotoxic syndrome. While TCP neurotoxicity is mainly attributed to ortho-isomers like tri-ortho-cresyl phosphate (ToCP), recent exposure and risk assessments indicate that ToCP levels in cabin air are very low. However, the neurotoxic potential of non-ortho TCP isomers and TCP mixtures is largely unknown. We therefore measured effects of exposure (up to 48 h) to different TCP isomers, mixtures and the metabolite of ToCP (CBDP: cresyl saligenin phosphate) on cell viability and mitochondrial activity, spontaneous neuronal electrical activity, and neurite outgrowth in primary rat cortical neurons.

The results demonstrate that exposure to TCPs (24–48 h, up to 10 μM) increases mitochondrial activity, without affecting cell viability. Effects of acute TCP exposure (30 min) on neuronal electrical activity are limited. However, electrical activity is markedly decreased for the majority of TCPs (10 μM) following 48 h exposure. Additional preliminary data indicate that exposure to TCPs (48 h, 10 μM) did not affect the number of neurites per cell or average neurite length, except for ToCP and the analytical TCP mixture (Sigma) that induced a reduction of average neurite length.

The combined neurotoxicity data demonstrate that the different TCPs, including ToCP, are roughly equipotent and a clear structure-activity relation is not apparent for the studied endpoints. The no-observed-effect-concentrations (1 μM) are well above current exposure levels indicating limited neurotoxic health risk, although exposures may have been higher in the past. Moreover, prolonged and/or repeated exposure to TCPs may exacerbate the observed neurotoxic effects, which argues for additional research.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

Tricresyl phosphate (TCP) is an organophosphate (OP) used as plasticizer, flame retardant, and oil additive to reduce wear and tear of the engine. TCP consists of one phosphate with three cresyl groups (methylphenyl) at the single bonded oxygen molecules (see Fig. 1 in de Reen et al. (2014) for schematic illustration). These cresyl groups can exist in three different forms: ortho, meta and para, determined by the position of methyl on the phenyl. TCP is thus a mixture consisting of different TCP isomers of which in particular the ortho-isomer (tri-ortho-cresyl phosphate; ToCP) is neurotoxic (Aldridge, 1954; Henschler, 1958).

ToCP can be metabolized to cresyl saligenin phosphate (2-(ortho-cresyl)-4H-1,2,3-benzodioxaphosphoran-2-one; CBDP) by multiple cytochrome P450 subtypes (Reinen et al., 2015). CBDP inhibits acetylcholine esterase (AChE) and butyrylcholinesterase (BuChE), leading to an excess of the neurotransmitter acetylcholine and subsequent cholinergic syndrome. In addition, CBDP can inhibit neuropathy target esterase (NTE), resulting in organophosphate-induced delayed neuropathy (OPIDN) (Honrado de Oliveira et al., 2002; Padilla and Veronesi, 1985; Barrett and Oehme, 1994; Carrington and Abou-Donia, 1988; Carletti et al., 2013).

Human occupational exposure to ToCP, for example via contaminated cabin air in aircraft, has received particular attention as it has been proposed to result in neurological complaints such as the so-called aerotoxic syndrome (Winder et al., 2002; Ross, 2008; Furlong, 2011; Liyasova et al., 2011; Abou-Donia et al., 2013). As a consequence of its neurotoxicity and the proposed association

* Corresponding author at: Neurotoxicology Research Group, Toxicology Division, Faculty of Veterinary Medicine, Institute for Risk Assessment Sciences (IRAS), Utrecht University, P.O. Box 80.177, NL-3508 TD Utrecht, The Netherlands.
Fax: +31 30 2515077.
E-mail address: r.westerink@uu.nl (R.H.S. Westerink).
¹ Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.neuro.2016.02.001
0161-813X/© 2016 Elsevier Inc. All rights reserved.

Please cite this article in press as: Duarte, D.J., et al., In vitro neurotoxic hazard characterization of different tricresyl phosphate (TCP) isomers and mixtures. Neurotoxicology (2016), http://dx.doi.org/10.1016/j.neuro.2016.02.001
with aerotoxic syndrome, the commercial use of ToCP has been strongly reduced over the last decades and ToCP now constitutes no more than 2% of the commercial TCP blends used in aircraft engine oil (SAE, 2005; DeNola et al., 2008, Table 1). Consequently, ToCP exposure in cabin air is nowadays below the limits for analytical detection (Houtzager et al., 2013; Schindler et al., 2013; but see also Cranfield, 2011; Murawski and Michaelis, 2011). Despite ongoing debate (de Boer et al., 2015), it is thus unlikely that exposure to ToCP is responsible for reported health complaints, such as the alleged aerotoxic syndrome (de Ree et al., 2014).

While the neurotoxicity of ToCP is relatively well-studied and mainly attributed to the inhibition of NTE, AChE and BuChE by its metabolite CBDP, it is possible that ToCP or its metabolite affect yet unknown cellular targets as observed previously for among others OP-induced acute and subchronic inhibition of voltage-gated calcium channels (VGCC; Meijer et al., 2014a; Meijer et al., 2014b; Meijer et al., 2015). Interestingly, ToCP was indeed recently shown to inhibit VGCC as well as glutamatergic calcium signaling in mouse embryonic neurons following 1–6 days of exposure (Hausherr et al., 2014). However, the neurotoxic potential of

Table 1
Isomer composition of the used analytical and commercial TCP mixtures.

<table>
<thead>
<tr>
<th>Isomer</th>
<th>m-m-m-TCP</th>
<th>p-p-p-TCP</th>
<th>o-o-o-TCP</th>
<th>m-m-p-TCP</th>
<th>p-p-p-TCP</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCP (Sigma)</td>
<td>22%</td>
<td>5%</td>
<td>2%</td>
<td>41%</td>
<td>25%</td>
<td>5%</td>
</tr>
<tr>
<td>TCP (TCI)</td>
<td>42%</td>
<td>31%</td>
<td>1–2%</td>
<td>n.p.</td>
<td>n.p.</td>
<td>25%</td>
</tr>
<tr>
<td>Disflamoll TKP-P (Lanxess)</td>
<td>33%</td>
<td>3%</td>
<td>0%</td>
<td>44%</td>
<td>20%</td>
<td>0%</td>
</tr>
<tr>
<td>Durad 125 (Chemtura)</td>
<td>13%</td>
<td>11%</td>
<td>0.3%</td>
<td>39%</td>
<td>36%</td>
<td>0.7%</td>
</tr>
<tr>
<td>Kronites TCP-S (Chemtura)</td>
<td>30%</td>
<td>3%</td>
<td>1.5%</td>
<td>40%</td>
<td>18%</td>
<td>7.5%</td>
</tr>
</tbody>
</table>

n.p. not provided by the manufacturer.

* likely mainly m-m-p-TCP and m-p-p-TCP as these were not specified by the manufacturer, but purity is reported to be >99%.

Please cite this article in press as: Duarte, D.J., et al., In vitro neurotoxic hazard characterization of different tricresyl phosphate (TCP) isomers and mixtures. Neurotoxicology (2016). http://dx.doi.org/10.1016/j.neuro.2016.02.001
commercial TCP mixtures and non-ortho TCP isomers that currently dominate these mixtures is largely unknown. Additional research to identify alternative mechanisms of ToCP is thus critical to improve human hazard and risk assessment of TCP. The current research therefore aims at revealing the (in vitro) neurotoxic potential of non-ortho TCP isomers and TCP mixtures compared to ToCP using primary rat cortical cultures. Primary cortical cultures consist of a mixed population of multiple types of (excitatory and inhibitory) neurons as well as supportive cells (e.g., astrocytes) that form functional neuronal networks. These primary cultures are well characterized, widely accepted, easily cultured and recapitulate many aspects of nervous system function (for review, see Johnstone et al., 2010; de Groot et al., 2013). While electrophysiological assessment of ion channel or neurotransmitter receptor function is ideally suited to investigate chemically-induced changes in neuronal function and transmission (de Groot et al., 2013), it may be too endpoint-specific and not taking into account the net effect on the neuronal network. We therefore selected three highly integrated endpoints for the in vitro hazard characterization of TCPs following acute (30 min) and subchronic (24 and 48 h) exposure. First, effects on cell viability and mitochondrial activity were assessed. Next, effects of TCPs on spontaneous neuronal electrical activity were assessed using microelectrode array (MEA) recordings as a non-invasive in vitro neurotoxicity screening method (Hogberg et al., 2011; McConnell et al., 2012; Valdivia et al., 2014; Nicolas et al., 2014). Lastly, effects on neurite outgrowth were measured as a specific, integrated measure of neurotoxicity that represents the effects of multiple underlying targets (Harrill et al., 2011).

2. Materials and methods

2.1. Chemicals

Tri-meta-cresyl phosphate (TmCP; CAS 563-04-2; purity: >95%), tri-para-cresyl phosphate (TpCP; CAS 78-32-0; purity: >98%), tri-ortho-cresyl phosphate (ToCP; CAS 78-30-8; purity: >96%) and an analytical TCP mixture (CAS 1330-78-5; purity: >99%) were obtained from TCI Europe (Eschborn, Germany). The commercial TCP mixture Disflamoll TKP-P (CAS 1330-78-5; purity: >97%) was kindly provided by Lannexx (Kallo, Belgium). The commercial TCP mixtures Kronitek TCP-S (CAS 1330-78-5; purity: >91%) and Durad-125 (CAS 1330-78-5; purity: ~99%) were kindly provided by Chemtura Corporation (West Lafayette, IN, USA). Cresyl saligenin phosphate (CBDP; CAS 1222-87-3) was a kind gift from Dr. Christoph van Thriel (Iudo-Dortmund, Germany). All other chemicals, including a second analytical TCP mixture (CAS 1330-78-5; purity: >93%), were obtained from Sigma–Aldrich (Zwijndrecht, Netherlands), unless otherwise noted. See Table 1 for isomer composition of the different TCP mixtures.

Stock solutions of TCP mixtures and isomers were prepared in dimethylsulfoxide (DMSO; ~99% purity). Stock solutions of CBDP were prepared in acetonitrile (ACN; ~99% purity). Final concentrations of DMSO and ACN were always kept below 0.1%.

2.2. Cell culture

Experiments were approved by the Ethical Committee for Animal Experiments of Utrecht University and were in accordance with Dutch law. Primary cultures of rat cortical neurons were prepared from postnatal day 0 to 1 Wistar rat pups as described previously (Nicolas et al., 2014; Meijer et al., 2015; de Groot et al., 2016), with minor modifications. Briefly, pups were decapitated and cortices were rapidly dissected on ice and minced into small pieces, which were mechanically dissociated by gentle trituration and filtered through a cell strainer (BD Falcon, 100 µm nylon). Cells were resuspended in dissection medium, i.e. Neurobasal-A supplemented with sucrose (14 g/500 mL), 200 mM l-glutamine (Life Technologies, Bleiswijk, The Netherlands), 2.5 mM glutamic acid, 10% fetal bovine serum (FBS, Life Technologies), and 1% of a solution containing 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin (Life Technologies). The cell-containing medium was centrifuged for 5 min at 800 rpm and supernatant was removed. Primary cortical cells were diluted in dissection medium and seeded on poly-l-lysine-coated (50 µg/mL) 96-well plates at a density of approximately 3 × 10^4 cells/well (i.e., approx. 1 × 10^5 cells/cm²) for measurements of cell viability or mitochondrial activity, on 48-well MEA plates (Axion Biosystems Inc., Atlanta, USA) at a density of approximately 1 × 10^5 cells/well (i.e., approx. 1 × 10^6 cells/cm²) for measurements of neuronal activity, and on 8-channel coverslips (Ibidi GmbH, Planegg, Germany) at a density of approximately 1 × 10^5 cells/well (i.e., approx. 1 × 10^6 cells/cm²) for immunostaining and measurements of neurite outgrowth.

Cells were cultured in a humidified incubator at 37 °C and 5% CO₂. At day in vitro (DIV) 1, the dissection medium was replaced by glutamate medium, i.e. Neurobasal-A supplemented with sucrose (14 g/500 mL), 200 mM l-glutamine, 2.5 mM glutamic acid, 2% B-27 (Life Technologies), and 1% penicillin/streptomycin. On DIV 4, glutamate medium was replaced by FBS culture medium, i.e. Neurobasal-A supplemented with sucrose (14 g/500 mL), 200 mM l-glutamine, 10% FBS, and 1% penicillin/streptomycin (Life Technologies).

2.3. Measurements of cell viability and mitochondrial activity

After 9 days in vitro (DIV 9), cells were exposed for 24 h or 48 h to the test compounds (final concentration 0.1–100 µM) dissolved in FBS culture medium. Cell viability was assessed at DIV 10 (24 h) and DIV 11 (48 h) using a combined CFDA-AM and neutral red assay, whereas mitochondrial activity was assessed using an alamar blue assay. The CFDA-AM (5-carboxylfluorescein diacetate acetoxyethyl ester) assay measures cytoplasmic esterase activity. CFDA-AM is hydrolysed into a fluorescent compound by cytoplasmic (intracellular) esterases and can therefore be used as a readout for cell viability. The neutral red assay uses the ability of cells to incorporate neutral red in their lysosomes as a measure of cell viability. The alamar blue assay measures the conversion of non-fluorescent resazurin to resorufin, which is the result of mitochondrial activity and thereby indirectly also for cell viability.

The different assays were performed as described previously (Heusinkveld and Westerink, 2012; Heusinkveld et al., 2013). Briefly, cells were incubated for 30 minutes with 12.5 µM alamar blue and 4 µM CFDA-AM in FBS at 37 °C. Resorufin was measured spectrophotometrically at 540/590 nm (Infinite M200 microplate; Tecan Trading AG, Männedorf, Switzerland), whereas hydrolyzed CFDA was measured spectrophotometrically at 493/541 nm. The ab/CFDA solution was then replaced by 200 µL neutral red solution (175 µM in PBS, Invitrogen, Breda, The Netherlands) for 1 h at 37 °C. Next, cells were incubated for 30 min with 200 µL extraction solution (1% glacial acetic acid, 50% ethanol, and 49% H₂O) during gentle shaking at room temperature (rt). After 30 min extraction, fluorescence was measured spectrophotometrically at 530/645 nm.

2.4. Measurements of neuronal electrical activity using microelectrode arrays (MEAs)

Primary rat cortical neurons were cultured on 48-well MEA plates, with each well containing 16 nano-textured gold micro-electrodes (40–50 µm diameter; 350 µm center-to-center spacing) with four integrated ground electrodes. Spontaneous...
electrical activity was recorded at DIV9–11 at a constant temperature of 37°C using a Maestro 768-channel amplifier with integrated heating system and temperature controller (Axion Biosystems Inc.) as described previously (Nicolas et al., 2014; de Groot et al., 2014). Axion’s Integrated Studio (AxIS 1.7.8) was used to manage data acquisition. Channels were sampled simultaneously with a gain of 1200× and a sampling frequency of 12.5 kHz/channel using a band-pass filter (200–5000 Hz), resulting in raw data files.

MEA plates were allowed to equilibrate in the Maestro for 5–10 min prior to recordings of electrical activity. At DIV9, a 30 min baseline recording of spontaneous activity was made. After this recording the cells were exposed by adding 5 μL of the test chemical (final concentration 0.1–10 μM) and a subsequent 30 min recording was performed directly following the onset of exposure to determine the acute effect of the test compounds compared to baseline spontaneous activity (paired comparison). At DIV10 and DIV11 neuronal activity was measured again to determine the effects of test compounds following 24 h and 48 h exposure, respectively. Next, effects of TCPs are normalized to time-matched DMSO controls to prevent confounding by changes in neuronal activity by ongoing development of the cortical cultures over time.

2.5. Measurements of neurite outgrowth using immunocytochemistry

At DIV9, cells were exposed for 48 h to the test compounds (final concentration 10 μM) dissolved in FBS culture medium. At DIV11 cells were fixed by incubating cells with 300 μL/well 4% paraformaldehyde (PFA; Electron microscopy sciences, Hatfield, USA) in 0.1 M phosphate buffer (pH 7.4) at rt for 30 minutes. After fixation, PFA was replaced by 250 μL Dulbecco’s Phosphate-Buffered Saline (DPBS) (Gibco, Paisley, Scotland, UK) and plates were stored at 4°C for 3 days. Subsequently, coverslips were quenched for PFA, permeabilized, and incubated with blocking buffer (2% bovine serum albumin and 0.1% saponin in DPBS (Sigma–Aldrich)) containing 20 mM NH₄Cl for 20 min at rt. Each of the subsequent wash and incubation steps was performed in blocking buffer. Coverslips were washed three times before incubation with 200 μL/well rabbit anti-β-III tubulin (final dilution 1:500, abcam, Cambridge, United Kingdom)) in blocking buffer for 24 h at 4°C. Coverslips were then washed 3 times with blocking buffer and incubated with 200 μL/well donkey anti-rabbit Alexa 488 (final dilution 1:100 (Life Technologies, Bleiswijk, The Netherlands)) as secondary antibody for 45 min at rt in the dark. Nuclear staining was performed by incubating the

---

**Fig. 2.** Effects on cell viability following 24 h (A) or 48 h (B) exposure to different TCP mixtures. Effects on cell viability were assessed using esterase activity (CFDA assay, top), lysosomal activity (neutral red assay, middle) and mitochondrial activity (alamar blue assay bottom) normalized to DMSO controls. Data are expressed as mean ± SEM of 17–55 wells (n) per condition from 6–11 plates (N) derived from at least 3 independent dissections (cultures). Gray-shaded areas indicate the minimal relevant effect size (MES) derived from the average standard deviation of control cells, whereas an asterisk indicates a statistically significant (p < 0.05) effect that exceeds the MES.

Please cite this article in press as: Duarte, D.J., et al., In vitro neurotoxic hazard characterization of different tricresyl phosphate (TCP) isomers and mixtures. Neurotoxicology (2016), http://dx.doi.org/10.1016/j.neuro.2016.02.001
coverslips with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies) at a concentration of 200 nM for 2 min at rt in the dark. Next, coverslips were washed three times with blocking buffer and coverslips were sealed with FluorSave (Calbiochem, San Diego, California) and stored at 4 °C in the dark until analysis.

Immunostained coverslips were visualized using a Leica SPEII confocal microscope (Leica DMi4000 equipped with TCS SPE-II) using a 20× oil immersion objective (N.A. 0.6) and images were captured using Leica Application Suite Advanced Fluorescence software (LAS AF version 2.6.0; Leica Microsystems GmbH, Wetzlar, Germany). See de Groot et al. (2016) for additional details of the procedure and an example of an immunostaining used to quantify effects on neurite length.

2.6. Data analysis and statistics

Cell viability and mitochondrial activity data, background-corrected using lysis values, have been obtained from 4–11 plates (N) derived from at least 3 independent dissections (cultures) and are expressed as percentage (mean ± SEM of 15–55 wells (n) per condition) of time-matched solvent controls.

For MEA data, raw data files were re-recorded to generate Alpha Map files for further data analysis in NeuroExplorer® software (Nex Technologies, Madison, USA). During re-recording, spikes were detected using the AxIS Spike Detector with dynamic threshold detection (Adaptive threshold crossing, Ada BandPft v2) set at seven times standard deviation of the internal noise level (rms) on each electrode. The spike count files (Alpha Map files) were loaded into NeuroExplorer for further analysis of the percentage of active wells (defined as ≥1 active electrode), the percentage of active electrodes (defined as ≥0.1 spikes/s) per well, and the average mean spike rate (MSR; spikes/s) per active electrode. Effects of test compounds were calculated as follows: MSR were averaged per well (22–94 wells (n) from at least four independent isolations) and effects of test compounds were calculated as percentage change compared to baseline. Next, the effect test compounds was expressed (mean ± SEM from n wells) normalized to control wells.

For analysis of neurite outgrowth, images were analyzed using High Content Analysis software (HCA-Vision version 2.1.6; CSIRO, Campbell, Australia). Next, neurites were manually traced from the HCA processed images using NeuronJ software (ImageJ plugin, version 1.4.2 Erik Meijering, Rotterdam, The Netherlands) to determine average neurite length and number of neurites. For each condition, data were obtained from 2–3 independent experiments and expressed as percentage of control cells (mean ± SEM of 6–24 cells (n) per condition).

For all data, wells (or cells) that showed effects two times standard deviation (SD) above or below average are considered outliers (~5%) and were excluded from further analysis. One-way analyses of variance (ANOVA), followed by Bonferroni post-hoc analyses were performed to investigate concentration- and/or time-dependence of changes in cell viability, mitochondrial activity, and spontaneous electrical activity after exposure to TCPs. Effects of TCP exposure on neurite outgrowth were tested for significance using unpaired two-sample t-tests. Effects were considered statistically significant if p-values < 0.05. Given the large number of measurements (and the associated risk of false positive results), a minimal effect size (MES) was calculated for the different assays. This MES was estimated based on the average SD of the solvents controls and amounted to 5%, 5%, 15%, 25% and 25% for the CFDA, neutral red, alamar blue, MEA and neurite outgrowth data, respectively. Effects are considered biologically relevant only if >MES and p < 0.05. Notably, the large MES for MEA and neurite outgrowth data (25%), resulting from the heterogeneity inherent to the use of cortical cultures, hampers detection of small effects.

3. Results

3.1. Cell viability and mitochondrial activity

Effects of different TCP isomers, mixtures and the ToCP metabolite CBDP (0.1–100 μM) on cell viability and mitochondrial activity were assessed following 24 h and 48 h exposure in primary cultured rat cortical neurons using CFDA-AM, neutral red and alamar blue assays. As illustrated in the supplemental data, exposure to TCP isomers did not induce overt morphological changes (Fig. S1).

Following 24 h exposure, none of the TCP isomers (Fig. 1A, top) or mixtures (Fig. 2A, top) at concentrations up to 100 μM induced an appreciable decrease in cytoplasmic esterase activity as measured using the CFDA-AM assay. The absence of cytotoxicity was confirmed in the neutral red assay, which also did not show appreciable effects of the TCP isomers (Fig. 1A, middle) or mixtures (Fig. 2A, middle) up to 100 μM. At increasing concentrations, CBDP induced a modest decrease in cytoplasmic esterase activity, amounting to 93 ± 2% and 79 ± 3% (compared to solvent control) at 10 and 100 μM, respectively (Fig. 1A, top). However, CBDP did

---

Fig. 3. Effects on mean spike rate (MSR) following acute (30 min, 0.1–10 μM, A) or prolonged (30 min–48 h, 10 μM, B) exposure to different TCP isomers and CBDP. Data are normalized to DMSO controls (ACN controls for CBDP) and expressed as mean ± SEM of 33–93 wells (n) per condition from at least 4 independent cultures (N). Gray-shaded areas indicate the minimal relevant effect size (MES) derived from the average standard deviation of control cells, whereas an asterisk indicates a statistically significant (p < 0.05) effect that exceeds the MES.

Please cite this article in press as: Duarte, D.J., et al., In vitro neurotoxic hazard characterization of different tricresyl phosphate (TCP) isomers and mixtures. Neurotoxicology (2016), http://dx.doi.org/10.1016/j.neuro.2016.02.001
not induce substantial changes in the neutral red assay (Fig. 1A, middle).

In contrast, all tested compounds showed clear effects in the alamar blue assay (Figs. 1A and 2A, bottom). The alamar blue assay, which measures mitochondrial activity, showed a decrease of ~50% for CBDP at 100 μM. On the other hand, all TCP isomers and mixtures showed a profound increase in mitochondrial activity at 10 μM, which was somewhat attenuated at 100 μM for TpCp, TmCP, the TCP mixture (Sigma), Disflamoll TPK-P, and Durad-125.

Following 48 h exposure, none of the TCP isomers at concentrations up to 100 μM induced cytotoxicity in the CFDA-AM assay (Fig. 1B, top). Similarly, the TCP mixture (TCI), Disflamoll TPK-P and Kronitex-TCP did not induce cytotoxicity (Fig. 2B, top), whereas only (very) limited cytotoxicity was observed for CBDP, the TCP mixture (Sigma) and Durad-125 at 100 μM. The absence of major cytotoxicity was confirmed in the neutral red assay, which indicated no or only (very) limited (ToCP and TpCP) cytotoxicity following 48 h exposure at 100 μM (Figs. 1B and 2B, middle).

Remarkably, following 48 h exposure, the decrease in mitochondrial activity (alamar blue assay) for CBDP was no longer prominent, whereas ToCP exposure still resulted in a profound increase in mitochondrial activity at 10 and 100 μM (Fig. 1B, bottom). For TmCP and TpCP (Fig. 1B, bottom) as well as the TCP mixtures (TCI and Sigma), Disflamoll TPK-P and Kronitex-TCP (Fig. 2B, bottom) the increase in mitochondrial activity was still apparent at 10 μM and attenuated at 100 μM. Although Durad-125 already induced a modest increased mitochondrial activity following 48 h exposure at 0.1 μM (but not at 1 μM), the increase at 10–100 μM was comparable to 24 h exposure.

3.2. Neuronal electrical activity

Since a small reduction in cell viability was observed for some test compounds at 100 μM, effects of acute (30 min) exposure to different TCP isomers, mixtures and the ToCP metabolite CBDP on neuronal electrical activity were assessed using multi-electrode array (MEA) recordings only at concentrations up to 10 μM.

Neuronal electrical activity of cortical cultures, expressed as mean spike rate (MSR) of baseline activity, was not altered to a degree that exceeded the MES upon acute exposure to any of the test compounds at concentrations up to 10 μM (Figs. 3A and 4A), except for TmCP and ToCP. At 10 μM, the MSR following acute TmCP exposure was reduced to 73 ± 4% compared to control, whereas the MSR for acute ToCP exposure showed a marked increase up to 130 ± 5% compared to control. Notably, acute exposure to CBDP did not affect MSR at concentrations up to 10 μM (Fig. 3A).

Since concentrations up to 1 μM did not affect MSR upon acute exposure, the effects of prolonged exposure (24 h and 48 h) were assessed only at the highest concentration (10 μM; Figs. 3B and 4B), while acute exposure to ToCP increased MSR, it was unaffected at 24 h and reduced to 66 ± 7% following 48 h exposure (Fig. 3B). Similarly, MSR was reduced following 48 h exposure to TpCP (63 ± 7%), TmCP (71 ±9%) TCP mixtures (TCI: 64 ± 8%; Sigma: 61 ± 7%) and Disflamoll TPK-P (61 ± 9%). On the other hand, 48 h exposure to CBDP, Kronitex-TCP and Durad-125 did not affect MSR to a degree that exceeded the MES (Figs. 3B and 4B).

3.3. Neurite outgrowth

Since several TCP isomers and mixtures were able to reduce neuronal activity following 48 h exposure at 10 μM, effects on neurite outgrowth were assessed as a potential underlying cause. Unfortunately, this analysis was hampered by the presence of non-neuronal cells (astrocytes) in our mixed neuronal cultures. As a result, analysis of neurite outgrowth had to be done manually and the number of cells analyzed is therefore limited. Consequently, the following data on neurite outgrowth is best considered as preliminary data.

The average number of neurites per cell amounted to 4.3 ± 1.9 (n = 16) in control cortical cultures exposed to DMSO for 48 h. While the number of neurites per cell (as percentage of DMSO control) fluctuated, it was not affected to a degree that exceeded the MES after 48 h exposure to any of the test compounds except for TmCP that (insignificantly) reduced the number of neurites per cell to 72 ± 8% compared to control (Fig. 5A). Similarly, the average neurite length per cell fluctuated strongly, but was not affected to a degree that exceeded the MES by any of the test compounds except for TmCP and the TCP mixture (Sigma), which reduced the average neurite length per cell to respectively 67 ± 6% and 74 ± 6% compared to DMSO controls (Fig. 5B).

4. Discussion

Both, the CFDA and neutral red assay indicate that exposure to individual TCP isomers or TCP mixtures had no or only (very) limited effects on cell viability (Figs. 1 and 2). On the other hand, the CFDA assay indicates that the ToCP metabolite CBDP induced a considerable reduction in cell viability. However, the results of this esterase-based assay should be interpreted with caution as CBDP is

![Fig. 4](image-url). Effects of mean spike rate (MSR) following acute (30 min, 0.1–10 μM, A) or prolonged (30 min–48 h, 10 μM, B) exposure to different TCP mixtures. Data are normalized to DMSO controls and expressed as mean ± SEM of 22–94 wells (n) per condition from at least 4 independent cultures (N). Gray-shaded areas indicate the minimal relevant effect size (MES) derived from the average standard deviation of control cells, whereas an asterisk indicates a statistically significant (p < 0.05) effect that exceeds the MES.

Please cite this article in press as: Duarte, D.J., et al., *In vitro neurotoxic hazard characterization of different tricresyl phosphate (TCP) isomers and mixtures*. Neurotoxicology (2016), http://dx.doi.org/10.1016/j.neuro.2016.02.001
an effective inhibitor of AChE and BuChE and may also inhibit non-
specific esterases. Consequently, the decrease observed in the
CFDA assay may reflect an inhibitory effect of CBDP on intracellular
esterases rather than a decrease in cell viability. Notably, the
absence of inhibitory effects of CBDP in the neutral red assay is in
line with limited effects on cell viability.

While profound cytotoxicity appears absent, the alamar blue
assay provides a clear indication for increased mitochondrial activity (Figs. 1 and 2). After 24 h exposure, all TCP isomers and mixtures induce a profound increase in mitochondrial activity at 10 µM. At 100 µM, the effects on mitochondrial activity are generally attenuated, possibly indicating mitochondrial uncoupling as cell viability is not affected according to the CFDA and neutral red assay. Notably, CBDP does not increase mitochondrial activity, but appears to induce mitochondrial uncoupling at 100 µM.

The effects on mitochondrial activity are generally attenuated at 48 h, which is suggestive for adaptive mitochondrial changes, since the CFDA and neutral red assay indicate no or (very) limited effects on cell viability. An alternative explanation for the attenuating effects at 48 h could involve a reduced availability of the TCPs, either due to metabolism, degradation and/or adsorption to culture materials. Despite the absence of overt cytotoxicity, the effects on mitochondrial activity could be considered neurotoxic as they may affect neuronal function and development. The observed changes in mitochondrial activity are in line with earlier in vivo/ex vivo studies showing ultrastructural changes in mitochondria, in mitochondrial permeability transition and in mitochondrial membrane potential of hen nerve cells following exposure to a high dose of ToCP (Xin et al., 2011).

Spontaneous neuronal electrical activity is a highly integrated readout for effects on processes underlying neuronal function and transmission. As such, MEA recordings allow for reproducible screening of neurotoxic compounds with high sensitivity and specificity (McConnell et al., 2012; Valdivia et al., 2014; Nicolas et al., 2014). Despite the marked increase in mitochondrial activity, MEA recordings demonstrated that neuronal activity of primary cortical cultures is largely unaffected upon acute (30 min) exposure to TCP isomers, mixtures and CBDP (Figs. 3A and 4A). Our data indicate that at 10 µM, only ToCP and TmpCP were able to significantly affect MSR. ToCP and TmpCP clearly have opposite effects on MSR, with ToCP resulting in a decreased spike rate and TmpCP resulting in a decreased spike rate. Consequently, ToCP and TmpCP may have different modes of action. So far, increases in MSR have been largely limited to glutamatergic agonists and GABAergic antagonists (McConnell et al., 2012; Mack et al., 2014). Since prolonged exposure to ToCP was recently reported to decrease glutamatergic signaling (Hausherr et al., 2014), it is tempting to speculate that the neurotoxicity of ToCP also involves modulation of glutamatergic receptors.

While only ToCP and TmpCP were able to affect MSR following acute exposure, all TCP isomers and most TCP mixtures reduced neuronal activity following 48 h exposure (10 µM; Figs. 3B and 4B). Although the underlying cause for this general reduction in MSR following prolonged exposure is unclear, it is unlikely to be due to changes in cell viability as these were not observed at 10 µM. There is also no clear relation between mitochondrial activity and the reduction in MSR. Alternatively, it may be related to neurotransmitter receptor mediated signaling pathways, such as decreased glutamatergic signaling as it was previously observed that 24 h exposure to ToCP (sub- and low µM range) reduced glutamate-mediated calcium influx and the number of glutamate responsive neurons (Hausherr et al., 2014).

The overall decrease in neuronal activity is unlikely to be due to a decrease in network connectivity as our preliminary data indicate TCP exposure (10 µM) does not significantly affect the number of neurites per cell and decreases in neurite length were observed only for TmpCP and the TCP (Sigma) mixture (Fig. 5). However, literature data indicates that different cell types show remarkable differences in sensitivity. Mouse embryonic cortical neurons show a reduction in neurite length of ~65% at 10 µM ToCP (Hausherr et al., 2014), which is comparable with the reduction in neurite length observed in rat PC12 (~65% reduction) and mouse N2A (~40% reduction) cells following exposure to ~3 µM TCP (Flaskos et al., 1998). On the other hand, neurite length of neonatal rat cortical cells (Fig. 3), rat C6 glioma cells (Flaskos et al., 1998) and human SH-SY5Y cells (Chen et al., 2013) appears to be largely insensitive to TCP exposure. The underlying cause for the difference in sensitivity between different cell types is unclear, but it is unlikely to be due to the absence of traditional TCP targets such as NTE and AChE (Carrrington and Abou-Donia, 1988), since even PC12, N2A, C6 and SH-SY5Y cells are all well known to express these proteins (Li and Casida, 1998; Hargreaves et al., 2006; Emerick et al., 2015).
Earlier studies demonstrated that TCP (most likely via CBDP) inhibits NTE and AChE at dose levels that convert to ~10 μM (Carrington and Abou-Donia, 1988; Cohen et al., 2006). Since the present study demonstrates that at comparable effect concentrations (10–100 μM) the different TCP isomers and mixtures are roughly equipotent in affecting cell viability, mitochondrial activity, neuronal electrical activity and neurite outgrowth, future hazard and risk assessment may need to focus on total TCP rather than on low level exposure to only TCP only. Extrapolation of our in vitro data obtained with primary rat cortical cultures exposed for maximally 48 h to human chronic exposure scenarios is difficult and hampered by a lack of information regarding the free TCP concentration and other unknowns. Nevertheless, it can be estimated that an NO observed effect concentration of 1 μM converts to an intake of ~1.75 mg (or ~25 μg/kg for a 70 kg person) assuming 100% bioavailability and a distribution restricted to the bodily fluids (5L). However, a recent exposure study indicated that total TCP intake via exposure to cabin air amounts to only ~6 ng/kg/d (Houtzager et al., 2013), comparable with other recently measured exposure values (Solbu et al., 2011; Schindler et al., 2013). While TCP exposure may have been higher in the past and may also be much higher during fume-events (DeNola et al., 2011) reported exposure values ~300× higher than those reported by (Houtzager et al., 2013), the effect concentrations reported here and by others (e.g. Hausherr et al., 2014) are well above recent exposure levels, indicating that the current neurotoxic risk of TCPs is rather limited for the studied endpoints.

In conclusion, our combined biochemical, morphological and electrophysiological data demonstrate that TCP isomers and mixtures induce no or only limited cytotoxicity at concentrations up to 100 μM (Figs. 1 and 2), but can affect mitochondrial activity (Figs. 1 and 2), neuronal electrical activity (Figs. 3 and 4) and neurite outgrowth (Fig. 5) at non-cytotoxic concentrations (10 μM). However, these reported effect concentrations are well above estimated systemic levels based on recent measurements of cabin air concentrations of TCPs. As such, other potential causes for the so-called aeroxic syndrome, including other chemical and physical exposures, may need to be considered. On the other hand, more sensitive neurotoxic endpoints may exist and it must be noted that some effects worsen following prolonged exposure. Given the general human (occupational) exposure scenario, additional research may thus also need to focus on prolonged and/or repeated exposure to total TCPs.

Funding

This work was supported by a grant from the Dutch Ministry of Infrastructure and the Environment (grant number 31099651) and the Faculty of Veterinary Medicine (Utrecht University).

Acknowledgements

We gratefully acknowledge the members of the Neurotoxicology Research Group for their helpful discussions, Aart de Groot for technical assistance and statistical advice, Gina van Kleef and Fiona Wijnolts for help with cortical cultures, and Martje de Groot and Rob Bleumink for help with immunocytochemistry. Conflict of interest: none.

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.neuro.2016.02.001.

References


