

Comparative Toxicity of Pentachlorophenol with its Metabolites tetrachloro-1,2-hydroquinone and Tetrachloro-1,4-benzoquinone in HepG2 Cells

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Abstract: The organochlorine compound, pentachlorophenol (PCP), is classified as a hazardous substance. Its metabolite, tetrachloro-1,2-hydroquinone (TCHQ), has been detected in occupationally-exposed subjects and can readily be converted to tetrachloro-1,4-benzoquinone (TCBQ) under physiological conditions. Hazard characterization has previously identified the liver as the target organ of PCP toxicity in rats and dogs and as the liver is the major site of metabolism of the parent compound, this raises concern for the effects that the metabolites of PCP may have on the liver. Although the hepatotoxic effects of PCP have been described, less is known about the effects of its metabolites on hepatocyte function. Studying the effects of these metabolites on hepatocytes may provide valuable information regarding the effects that these compounds could exert on the liver itself and allude to the clinical manifestations of toxicity that can be expected. The aim of this study was therefore to assess the effect of PCP, TCHQ and TCBQ on the following cellular parameters: cell viability, mitochondrial membrane potential and intracellular ROS formation, as indicators of hepatocyte homeostasis. Both PCP and its metabolites, TCHQ and TCBQ decreased cell viability with IC_{50} of 68.05, 129.40 and 144.00 μ M, respectively. All three compounds caused mitochondrial depolarization, with the effect being more profound following exposure to TCHQ and TCBQ. PCP did not induce any ROS generation, whereas TCHQ and TCBQ produced extensive ROS. Findings from this study suggest that in hepatocytes the mechanism of toxicity of PCP differs from that of its metabolites, TCHQ and TCBQ.

Keywords: Hepatocytes, HepG2, Mitochondria, Pentachlorophenol, ROS, Tetrachloro-1,4-benzoquinone, Tetrachloro-1,2-hydroquinone.

1. INTRODUCTION

The low cost and efficacy of organochlorine pesticides led to their widespread use in agriculture as insecticides and to control vectors of disease to ensure public health. In the 1960's the harmful effects of organochlorine pesticides were established and their use was banned in many countries. However, they are still used in some developing countries today and, due to their resistance to degradation, remain ubiquitous in the environment [1]. Organochlorine pesticides can be characterised into sub groups according to their chemical structure [2]. These sub groups include monocyclic derivatives such as lindane, cyclodienes such as dieldrin, aldrin, endrin, heptachlor, endosulphan and chlordane, as well as biphenyl derivatives such as dichlorodiphenyltrichloroethane (DDT) and its analogues, methoxychlor, chlorobenzylate and dicofol [2]. The chlorophenols are a sub-group of organochlorine pesticides consisting of chlorinated monocyclic hydrocarbons. There are five types of chlorinated phenols including mono-, di-, tri-, tetra- and pentachlorophenol (PCP). Within these five sub-groups there

are a total number of 19 chlorophenols [3]. PCP has been extensively used as a wood preservative, herbicide, insecticide, defoliant, fungicide, molluscicide, algacide, disinfectant, germicide and as an ingredient in anti-fouling paints [4,5]. Although the use of PCP has been restricted, it is still currently used as a heavy duty wood preservative [6], thus persisting in the environment. Hexachlorobenzene, a widely used pesticide, constitutes another source of PCP exposure as PCP is one of the principal metabolites of hexachlorobenzene, a highly lipophilic compound that accumulates in human tissues after ingestion [7].

Organochlorine compounds are reported to be responsible for a variety of effects including effects on the immune system [5, 8], neurotoxic effects and impairment of thyroid function [8-10] in mammals. It is also linked to various cancers [11, 12], and in animals has been shown to cause hepatocellular carcinoma [5, 13]. Exposure to excessive concentrations of chlorophenols is known to cause convulsions, which is inversely related to the degree of chlorination [14]. This group of chemical entities also uncouple mitochondrial oxidative phosphorylation, producing hyperthermia *in vivo* [14]. Unlike convulsions, the hyperthermic effect has been shown to positively correlate with the degree of chlorination [14]. Industrial surveys and epidemiological studies have suggested that, in humans, PCP can have adverse effects on the liver, kidney, skin, blood,

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lungs and central nervous system [15]. Furthermore, PCP toxicity in humans has been said to cause permanent visual and central nervous system damage [15].

In mammals, the liver contains the highest concentration of biotransforming enzymes necessary for xenobiotic metabolism and foreign compounds usually find their fate in it [16]. Even though xenobiotic metabolism usually allows for elimination of most toxic compounds, it is capable of modifying the pharmacological properties of substances, thereby activating inert chemicals into biologically reactive species [17-19]. Little is known about the metabolism of PCP in humans and initial research demonstrated that after a single dose the majority of PCP is excreted via the urine either unchanged or as its glucuronide derivative [20]. These same studies initially suggested that PCP does not undergo any biological transformation other than conjugation, which was later disputed as it was found that human liver fractions are capable of metabolizing PCP, at a rate comparable to that of rat liver microsomal extract [21]. In rodents PCP is metabolized to the quinols: tetrachloro-1,2-hydroquinone (TCHQ) and tetrachlorocatechol [22]. Only TCHQ has ever been detected in man [23], but it has been reported that other mammals, especially rodents, can further metabolize TCHQ and tetrachlorocatechol to yield their respective semiquinones and finally quinones, tetrachloro-1,4-benzoquinone (TCBQ) and tetrachloro-1,2-benzoquinone [22,24,25]. TCHQ has been detected in the urine of subjects that were occupationally exposed to PCP [12]. Literature suggests that the reason why some studies failed to detect TCHQ in toxicokinetic experiments is due to the instability of TCHQ in urine samples [26]. Although TCBQ has not been detected in man, research shows that under physiological conditions TCHQ can readily autoxidize to form the semiquinone radical intermediate to finally produce TCBQ [27]. Hazard characterization identified the liver as the target organ of PCP toxicity in rats and dogs [28] and as the liver is the major site of metabolism of the hepatotoxic parent compound, this raises concern for the effects that the metabolites of PCP may have on the liver.

Oxidative stress, the result of a disturbance in the pro-oxidant / anti-oxidant balance of the cell, is an important mechanism of hepatotoxicity [17]. Oxidative stress has been implicated in the progression of a number of liver diseases, like alcoholic liver disease, non-alcoholic steatohepatitis and hepatitis C, into liver cirrhosis and hepatocellular carcinoma [29, 30]. Oxidative stress is also believed to be involved in chronic hepatic inflammation and fibrosis through the activation of Kupffer cells. The link is prominent in that certain antioxidants have been studied as possible treatments for the prevention of liver fibrosis [31]. Reactive oxygen species (ROS) generation implicated in alcoholic liver disease and non-alcoholic steatohepatitis are believed to originate from mitochondria [32, 33]. A common mechanism of hepatotoxicity is mitochondrial dysfunction [34, 35]. Due to the central role of mitochondria in both energetic metabolism and thus cellular homeostasis, they are a target for toxic substances. Mitochondrial dysfunction may be caused by direct disruption of mitochondrial metabolism, which includes uncoupling of oxidative phosphorylation and alterations of the components of the electron transport chain which would lead to changes in mitochondrial membrane potential (MMP) [17, 34]. Changes in MMP results in loss of

structural and functional integrity [17, 34]. These changes may occur due to oxidative damage. Damage to mitochondrial DNA also affects mitochondrial function [17]. Mitochondrial dysfunction, in turn, results in the impairment of the cellular energy metabolism which may lead to oxidative stress due to the formation of ROS [34, 35-37].

Although the hepatotoxic effects of PCP have been described [28], less is known about the effects of its metabolites on hepatocyte function. Studying the effects of these metabolites on ROS generation, mitochondrial homeostasis and cell viability may provide valuable information regarding the effects that these compounds could exert on the liver itself and allude to the clinical manifestations of toxicity that can be expected. In addition, such an investigation may also shed some light on the mechanism of toxicity of the metabolites of PCP in hepatocytes. The aim of this study was therefore to assess the effect of PCP, TCHQ and TCBQ on the following cellular parameters: cell viability, mitochondrial membrane potential and intracellular ROS formation, as indicator of effect on hepatocyte function using the HepG2 cell line as model. HepG2 cells were chosen since it is a well-established cell line that has been used previously to study the effects of chemical entities on cytotoxicity [38], mitochondrial homeostasis [39, 40] and oxidative stress [40].

2. MATERIALS AND METHODS

2.1. Cells, Cell Maintenance and Seeding

HepG2 cells were purchased from American Tissue Culture Collection (ATCC catalogue no. HB-8065). Cells were maintained in Eagle's modified essential medium (EMEM), supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, and 10% heat-inactivated fetal calf serum at 37°C in a 5% CO₂ atmosphere. Cell maintenance was carried out following standard operating procedures in this laboratory [41]. At confluency, cells were harvested using trypsin-EDTA and 100 µl was seeded into relevant wells on 96-well microplates at a density of 2×10⁴ cells/well and allowed to attach and acclimatize for 48 h prior to exposure.

2.2. Preparation of Test Compounds for Exposure

Stock solutions of PCP, TCHQ and TCBQ were made up to 30 mM in DMSO and stored at -70°C until use. For cell viability determination, the compounds were diluted with culture medium to obtain working solutions with concentrations of 10, 20, 100, 200 and 300 µM. For the mitochondrial membrane potential and ROS determination assays, the compounds were diluted with phosphate buffered saline (PBS) to obtain working solutions with concentrations of 10, 20, 100, 200 and 300 µM. Stock solutions of the positive controls, tamoxifen and 2,2'-azobis-2-methylpropanimidamide dihydrochloride (AAPH), were made up to 30 mM in DMSO and stored at -70°C until use. In keeping with the test compounds, positive controls were diluted to 300 µM working solutions with either culture medium or PBS.

2.3. Endpoint Assays

2.3.1. Cell Viability

Cell viability was assessed using the neutral red uptake (NRU) assay adapted from Fotakis and Timbrell [42]. To

initiate exposure, 100 μl of the working solutions of the positive control, PCP, TCHQ or TCBQ was added to the respective wells and plates to obtain final exposure concentrations of 5, 10, 50, 100 and 150 μM . Exposure concentrations were based on solubility considerations and pilot experiments aimed at acquiring a concentration range that yielded points above and below the calculated concentration of PCP that would produce 50% decrease in cell viability (IC_{50}) (data not shown). Cells were then exposed to the test compounds for 24 h. Vehicle controls were exposed to 0.5% (v/v) DMSO in culture medium. After exposure, medium was removed and cells incubated with 100 $\mu\text{g}/\text{ml}$ neutral red dye dissolved in EMEM (pH 6.4) for 2 h. Cells were then washed with PBS and dried over night, after which 100 μl of elution buffer consisting of ethanol/acetic acid/ H_2O at a ratio of 49:1:50 (v/v/v) was added. The plates were put on an orbital shaker for 30 min to aid dye dissolution and spectrophotometrically read at 540 nm using a BioTek EL_X 800 universal plate reader. Tamoxifen, which is known to produce cell death in HepG2 cells [43, 44], was used as positive control. Cell viability was determined and expressed as the percentage of vehicle controls. Tests were carried out in duplicate on nine separate occasions (n = 18).

2.3.2. Mitochondrial Membrane Potential

Mitochondrial membrane potential (MMP) was determined with a fluorescent, ratiometric dye according to the method of Nuydens *et al.* [45], with minor modifications. Cells were loaded with 20 μM 5,5,8,6,6,8-tetrachloro-1,18,3,3,38-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) for 1 h. Thereafter, 50 μl of PBS was immediately added to each well of the microplate to keep cells hydrated. This was followed by the addition of 50 μl of the working solutions of the positive control, PCP, TCHQ or TCBQ to the respective wells and plates to obtain final exposure concentrations of 5, 10, 50, 100 and 150 μM . Concentrations were selected to coincide with cytotoxicity experiments. Plates were then incubated for 2 h before the fluorescence was monitored at excitation 485 nm and emission 520 nm for the monomeric form of JC-1 and excitation 544 nm and emission 590 nm for the aggregate form of JC-1 using a BMG Labtech FluoStar Optima fluorescent plate reader. Tamoxifen, which is known to affect the MMP of HepG2 cells [46], was used as positive control. Vehicle controls were exposed to 0.5% (v/v) DMSO in PBS. MMP was determined and expressed as the percentage of vehicle controls. Tests were carried out in duplicate on nine separate occasions (n = 18).

2.3.3. Intracellular Reactive Oxygen Species

This assay was performed using the method of Zhang *et al.* [47] with slight modifications. Cells were preloaded with 6 μM 2',7'-dichlorodihydrofluorescein diacetate (DFCH-DA) for 1 h. Thereafter, 50 μl of PBS was immediately added to each well of the microplate to keep cells hydrated. This was followed by the addition of 50 μl of the working solutions of the positive control, PCP, TCHQ or TCBQ to the respective wells and plates to obtain final exposure concentrations of 5, 10, 50, 100 and 150 μM . Concentrations were selected to coincide with cytotoxicity experiments. Cells were then exposed for 3 h to allow ROS to develop from exposure to

the relevant test compound, whilst attempting to limit the time for cells to metabolise the parent compound. Fluorescence was then monitored at excitation and emission wavelengths of 485 nm and 520 nm, respectively, using a BMG Labtech FluoStar Optima fluorescent plate reader. AAPH, which is known to produce ROS in HepG2 cells [41], was used as positive control. Vehicle controls were exposed to 0.5% (v/v) DMSO in PBS. Levels of intracellular ROS was determined and expressed as the percentage of vehicle controls. Tests were carried out in duplicate on nine separate occasions (n = 18).

2.4. Statistical Analysis

Grubb's test for outliers was performed followed by the Kolmogorov-Smirnoff test to assess the normality of the data distributions. Significant differences between the means of the various concentration groups were detected by performing either unpaired *t*-tests or Mann-Whitney tests, depending on the normality of the data. GraphPad Prism v5.0 and the freeware package R v2.13.1 were used for all statistical manipulations. All tests were carried out in duplicate on nine separate occasions (n = 18). Relevant blanks were included to account for background signal. All results are expressed as mean% \pm SEM% of the respective vehicle control. Significant deviations from the relevant vehicle control mean are indicated by: * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

3. RESULTS AND DISCUSSION

3.1. Cell Viability

Viability was measured to determine at which concentrations the compounds affected cell survival. The IC_{50} value for PCP in the HepG2 cells was calculated to be $68.05 \pm 1.25 \mu\text{M}$ (Fig. 1). PCP-induced cytotoxicity demonstrated a dose-response relationship, with cell viability gradually decreasing when exposed to increasing concentrations of PCP following 24 h exposure (Fig. 2). IC_{50} values previously reported in HepG2 cells, following 24 h exposure to PCP, are 88.46 μM [48] and 107.12 μM [49]. The decrease in toxicity observed in the other studies, compared to the present study, may be attributed to the difference in assay method used. Even though PCP is an uncoupler of oxidative phosphorylation, it has been shown that the NRU assay is more sensitive to detect PCP toxicity than the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [50]. It has been suggested that this sensitivity is due to alterations in physical properties of membranes caused by PCP, which leads to disturbance in lysosomal membrane integrity, pore formation and ion pumps thus resulting in disruption of lysosomal acidity [50, 51].

A few studies have determined the effect of PCP on liver cells *in vitro*. In AML-12 mouse hepatocytes a 40% reduction in cell viability was reported after treatment with 58.20 μM of PCP [52]. *Carassius carassius* hepatocytes have been found to be more sensitive with a 50% decrease in cell viability being obtained at a PCP concentration of 10 μM [5]. Chang liver cells have been found to be more resistant to the toxic effects of PCP, only showing a significant decrease in cell viability when exposed to concentrations > 500 μM [53].

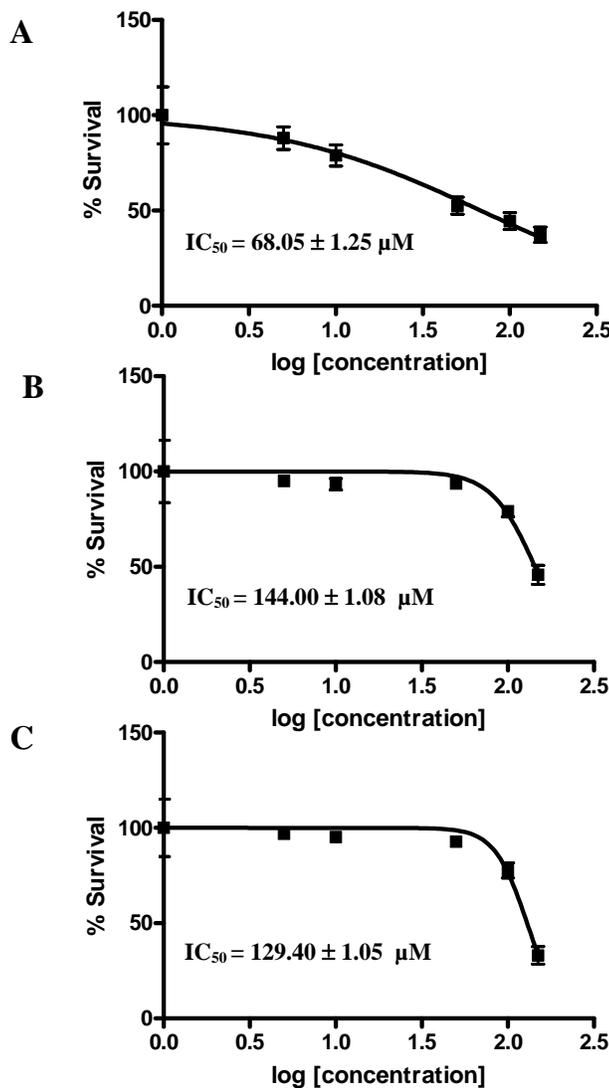


Fig. (1). Dose-response curves of the viability of HepG2 cells following 24 h exposure to (A) PCP, (B) TCHQ or (C) TCBQ. Curves were obtained by fitting four parameter Hill equations to the observed data. The following constraints were used when fitting the curves: Top = 100; Bottom = 0. IC_{50} refers to the calculated concentration of the test substance that would result in a 50% decrease in cell viability relative to vehicle-treated controls. X-axis = concentration of the test substance (logarithmic scale). Y-axis = percentage of viable cells relative to the vehicle-treated controls.

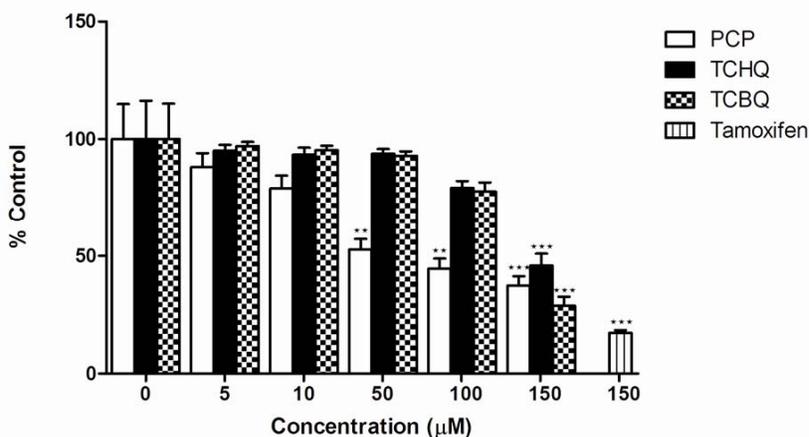


Fig. (2). Bar chart of the cell viability in HepG2 cells exposed for 24 h to PCP, TCHQ, TCBQ and Tamoxifen, the positive control (n = 18). Statistically significant deviations from the vehicle control means are indicated by ** and ***, representing p < 0.01 and P < 0.001, respectively. X-axis = concentration of the test substance. Y-axis = percentage of viable cells relative to the vehicle-treated controls. PCP = pentachlorophenol; TCHQ = tetrachlorohydroquinone, TCBQ = tetrachlorobenzoquinone.

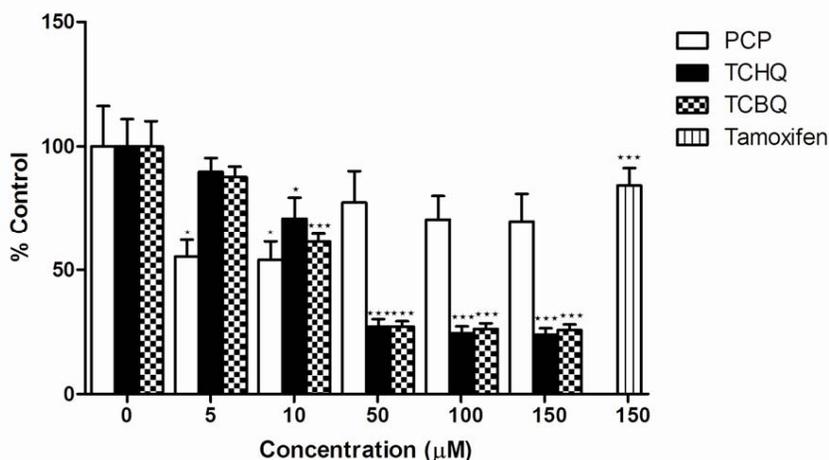


Fig. (3). MMP in HepG2 cells exposed for 24 h to PCP, TCHQ, TCBQ and Tamoxifen, the positive control (n = 18). Statistically significant deviations from the vehicle control means are indicated by * and ***, representing $p < 0.05$ and $p < 0.001$, respectively. X-axis = concentration of the test substance. Y-axis = ratio of JC-1 red/green fluorescence (used as an indication of mitochondrial membrane potential) expressed as percentage of the ratio observed with vehicle-treated controls. PCP = pentachlorophenol; TCHQ = tetrachlorohydroquinone, TCBQ = tetrachlorobenzoquinone.

With regards to other cell lines, PCP has proven toxic to monkey kidney fibroblasts [54], rat cellular granule neurons [55], mouse embryonic fibroblasts [54], rat sertoli cells [56], Jurkat T-cells [53], HeLa cells [54], as well as human lymphocytes and fibroblasts [57, 58].

Exposure to TCHQ and TCBQ produced IC_{50} values of $129.40 \pm 1.08 \mu\text{M}$ and $144.00 \pm 1.05 \mu\text{M}$, respectively (Fig. 1). These compounds showed typical dose-dependent cytotoxicity profiles with viability only starting to decrease at concentrations $\geq 50 \mu\text{M}$. For both metabolites significant decreases in viability were only detected at concentrations $\geq 150 \mu\text{M}$ (Fig. 2). A single study was found where the effect of TCHQ relative to PCP in HepG2 cells was determined [59]. These authors' results were contradictory to the present findings where the cells were found to be 5 times more sensitive to TCHQ than PCP. The differences in results could be attributed to the methodology used. Wang *et al.* [59] used the trypan blue exclusion method, which is based on the assumption that viable cells present with an intact plasma membrane that will exclude the trypan blue dye [60]. The NRU assay, used in the present study, is based on the active transport of neutral red dye from the surrounding medium into cytosolic lysosomes [42]. It was previously reported that PCP causes disruption of lysosomes prior to inducing apoptosis in mammalian cells and that this may form part of its mechanism of toxicity [57]. For this reason it can be argued that the NRU assay would be more sensitive than the trypan blue exclusion test to the effects of PCP because the organelle of interest for the NRU assay is directly targeted by PCP. The same may not be true in the case of TCHQ. This can possibly point to different mechanisms of toxicity between PCP and TCHQ, with PCP targeting lysosomes and TCHQ more selectively affecting the cellular plasma membrane. This may explain conflicting results between what was observed in the present study and what was reported by Wang *et al.* [59]. To our knowledge this study is the first to report the effects of TCBQ on hepatocyte viability *in vitro*.

3.2. Mitochondrial Membrane Potential

MMP was determined as it is an important indicator of mitochondrial homeostasis. The latter has an immediate effect on energy homeostasis and therefore also cell viability [35, 61]. Furthermore, MMP across the inner membrane has been linked to a variety of mitochondrial functions including: ATP synthesis, Ca^{2+} homeostasis, metabolite transport and the import of mitochondrial proteins [62], thereby being an indicator of the health of the organelle in cells exposed to a variety of toxic compounds. PCP is a well-known uncoupler of oxidative phosphorylation and its main mechanism of toxicity has been attributed to the resulting increase in aerobic metabolism and increasing heat production (hyperpyrexia) [63]. PCP has also been classified as a protonophoric uncoupler, which depletes the proton gradient by translocating protons from the intermembrane space into the mitochondrial matrix [64]. PCP caused significant ($p < 0.05$) mitochondrial depolarization (decreased red/green ratio of the dye JC-1) in HepG2 cells at 5 and 10 μM concentrations (Fig. 3). Higher concentrations did not show the same degree of depolarization. It has been shown that translocation of protons across the inner membrane space results in a compensatory increase in respiratory electron flow [64]. Increased electron flow results in an increase of protons being pumped into the intermembrane space. This could result in a compensatory increase in MMP. This compensatory increase in mitochondrial potential may have occurred in the present study in the cells exposed to higher concentrations of PCP. Decreases in MMP following PCP exposure have also been reported in rat sperm mitochondria when exposed to increasing concentrations of PCP (0.1 and 10 μM) for 30 min [65]. Significant dose-dependent decreases in MMP have also been reported in liver microsomes of *Cyprinus carpio* (common carp) exposed to 2.0 mg/L (approximately 7.5 μM) PCP for 24 h and 2.0, 4.0 and 6.0 mg/L for 72 h (approximately 7.5, 15.0 and 22.5 μM , respectively) [66]. In primary cultures of *Carassius carassius* (crucian carp) hepatocytes, a significant dose-dependent decrease in MMP

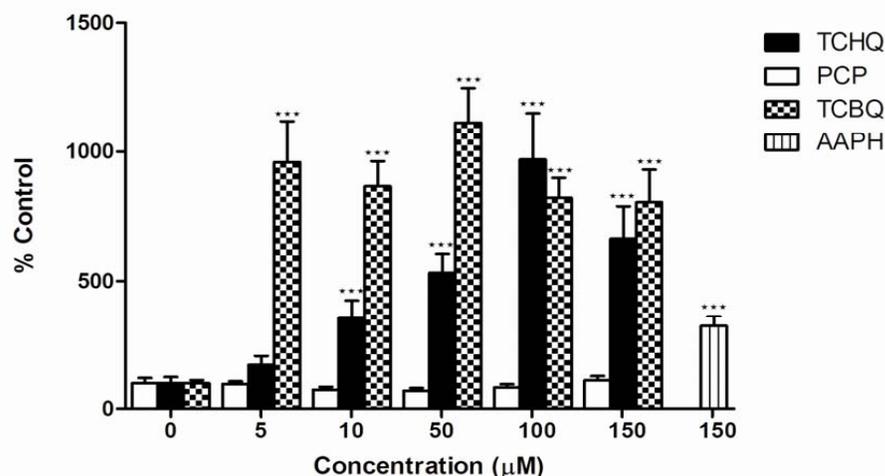


Fig. (4). ROS generation in HepG2 cells exposed for 24 h to PCP, TCHQ, TCBQ and AAPH, the positive control (n = 18). Statistically significant deviations from the vehicle control means are indicated by *** representing $p < 0.001$. X-axis = concentration of the test substance. Y-axis = Intracellular levels of ROS expressed as a percentage of ROS observed in the vehicle-treated controls. PCP = pentachlorophenol; TCHQ = tetrachlorohydroquinone, TCBQ = tetrachlorobenzoquinone; AAPH = 2,2'-azobis-2-methyl-propanimidamide dihydrochloride.

was noted when exposed to 1, 10 and 100 µM PCP for 8 h [5]. An increase in the number of human lymphocytes, which is characterized by a decrease in MMP was reported when cells were exposed to 225 ppm (approximately 93.9 nM) PCP [67]. Although in these studies different cells, different exposure times and different concentrations of PCP were used, these findings support the results of the present study in that PCP causes mitochondrial depolarization.

Significant, dose-dependent mitochondrial depolarization also occurred in HepG2 cells exposed to both TCHQ and TCBQ (Fig. 3). Compared to PCP, higher concentrations of the metabolites were necessary to decrease the MMP. However, TCHQ and TCBQ were more efficacious in decreasing MMP. Compared to PCP, both caused highly significant decreases in MMP at higher concentrations (> 10 µM). ROS is known to cause mitochondrial permeability transition, which leads to an influx of protons ultimately resulting in mitochondrial depolarization [68]. The large amounts of ROS generated in cells exposed to TCBQ and TCHQ may have resulted in mitochondrial depolarization. A single study was found where the effect of TCHQ on MMP was investigated. Lin *et al.* [69] reported a decrease in 3,3'-dihexyloxycarbocyanine iodide fluorescence intensity after treatment with increasing concentrations of TCHQ in NIH3T3 cells (mouse embryonic fibroblasts). This finding correlates with the results of the present study. To our knowledge this is the first report of the effect of TCHQ and TCBQ on MMP in intact, cultured hepatocytes.

3.3. ROS Generation

ROS generation was determined because oxidative stress is an important mechanism of hepatotoxicity and frequent mediator of cell death [70]. The metabolism of PCP has been shown to generate ROS [24, 25, 53]. Auto-oxidation and/or enzyme-mediated oxidation of the quinol metabolites of PCP, such as TCHQ, to its respective semi-quinones and quinones, namely TCBQ followed by reduction of these

quinones causes a redox cycling cascade which generates ROS mainly in the form of H_2O_2 (Fig. 5) [24, 53]. This mechanism supports the findings of the present study where no ROS generation was seen in cells exposed to PCP whilst extensive ROS generation was seen in cells exposed to TCHQ and especially TCBQ (Fig. 4).

Contrary to the findings in the present study, Dong *et al.* reported an increase in ROS generation in primary cultures of *Carassius carassius* hepatocytes exposed to 1, 10 and 100 µM of PCP for 8 h [5]. This may be explained by the different cell lines which were used for testing. It is possible that the primary hepatocyte cultures of *Carassius carassius* were able to metabolise PCP to its metabolites, as opposed to the perpetual cell line used in the present study, and that the quinol, semi-quinone and quinone metabolites of PCP, rather than the parent compound itself, were the actual causes of the ROS observed by Dong *et al.* [5]. This further suggests that HepG2 cells are either not able to metabolise PCP or do so at a reduced rate. This argument is supported by the fact that HepG2 cells, compared to primary hepatocytes, express very low levels of CYP2E1 [71], which would be the major CYP responsible for the metabolism of monocyclic aromatic compounds [72, 73].

A dose-dependent increase in ROS was observed in cells exposed to increasing concentrations of TCHQ up to 100 µM, after which the rate of ROS generation decreased. Although this decrease in ROS may be ascribed to a loss in cell viability, it is not very plausible as the incubation time for this assay was only 3 h. No literature regarding TCHQ- or TCBQ-induced ROS generation in cultured hepatocytes was found for comparison. To our knowledge this study is the first to report the effects of TCHQ and TCBQ on hepatocyte ROS generation *in vitro*. A single *in vitro* cell study reported a time-dependant increase in intracellular ROS levels in NIH3T3 (a mouse embryonic fibroblast cell line) treated with 25 µM TCHQ [59]. In other tests such as the salicylate hydroxylation assay, TCHQ and TCBQ have

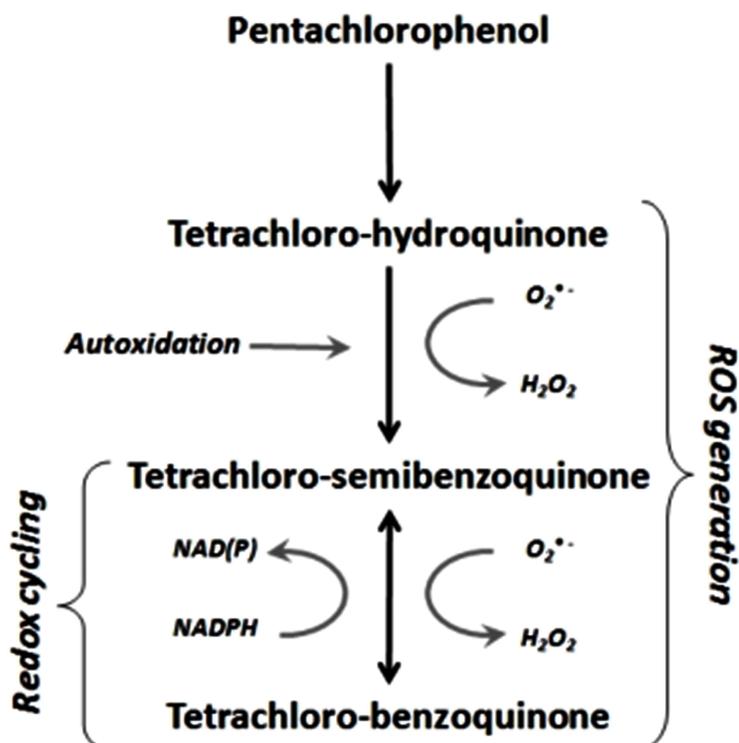


Fig. (5). Redox cycling and ROS generation in human hepatocytes during the metabolic conversion of PCP to TCHQ, which can produce TCBQ following autoxidation at physiological conditions.

been reported to produce the hydroxyl radical [74]. It has also been proposed that ROS-mediated DNA damage is the predominant type of damage induced by TCHQ and TCBQ [59] and that oxygen species may be involved in the mechanism of TCHQ toxicity [59]. TCBQ, which is also known as *p*-chloranil, is a strong oxidizing agent and is often used in chemical synthesis as an electron acceptor during aromatization steps [75]. This oxidizing characteristic supports the excessive intracellular ROS generation observed in the present study after exposure to TCBQ. The results of the aforementioned studies are in agreement with the findings of the current study.

4. ON THE MECHANISM(S) OF TOXICITY OF THE TEST COMPOUNDS

The present study observed that PCP caused mitochondrial depolarization. This is supported by the fact that PCP is a well-known uncoupler of oxidative phosphorylation that depletes the proton gradient, by translocating protons from the intermembrane space into the mitochondrial matrix [63], and induces aerobic metabolism and heat production [59].

It has been reported that ROS mediated DNA damage is the predominant type of DNA damage induced by TCHQ and TCBQ in HeLa cells [24] and that the mechanism of TCHQ toxicity in Balb/c mice involved oxidative stress by GSH depletion through TCHQ-GSH conjugation [53]. Moreover, TCHQ and TCBQ have previously been reported to form adducts to macromolecules such as DNA and proteins [27, 76]. Some researchers suggest that toxic effects exerted by TCHQ are, at least in part, due to the formation of hydroxyl radicals from autoxidation [77]. Others attribute TCHQ toxic effects to the semiquinone intermediate between TCHQ and TCBQ [78]. Since TCHQ can readily be

converted to TCBQ under physiological conditions [79], it is possible that TCBQ is also partially responsible for many of the toxic effects previously attributed to TCHQ itself. Reactive entities like TCHQ and TCBQ that produce excessive intracellular ROS generation and formation of macromolecule adducts may result in haptization *in vivo*, which may elicit an immune response and lead to fibrotic liver injury [80].

As TCBQ was the most potent inducer of ROS generation and had the lowest IC_{50} value of the two tested metabolites, it indicates that excessive ROS generation may play a role in the mechanism of toxicity of this metabolite. Although PCP was the most cytotoxic of the three tested compounds, the excessive elevations in ROS levels produced by its metabolites could possibly prove more toxic in an *in vivo* setting where more than one physiological system is present, as opposed to the present study. These findings suggest that the mechanism of hepatocyte injury induced by PCP differs from that of its metabolites in that it may not involve ROS. Since oxidative stress has been implicated in the progression of a number of liver diseases, concern is raised over the long term effects that the metabolites of PCP may have on the liver, especially in occupationally-exposed subjects.

5. CONCLUSION

Although the hepatotoxic effects of PCP have been described, less is known about the effects of its metabolites on hepatocyte function. This study provides valuable information regarding the effects that these compounds exert on cultured hepatocytes and raises concern around excessive ROS generation following exposure to the metabolites but not PCP. Results suggest that the mechanism of toxicity of PCP differs from that of its metabolites. PCP was found to

be more cytotoxic than its metabolites. All the test compounds disrupt mitochondrial homeostasis, which has an immediate effect on energy homeostasis and thus cell viability. This study provides initial insight into the mechanism of toxicity of TCHQ and TCBQ with regards to the liver where virtually no data is currently available.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

CYP	=	cytochrome P450
DCFH-DA	=	2',7'-dichlorodihydrofluorescein diacetate,
GSH	=	glutathione
MMP	=	mitochondrial membrane potential
MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PCP	=	pentachlorophenol
ROS	=	reactive oxygen species
TCBQ	=	tetrachloro-1,4-benzoquinone
TCHQ	=	tetrachloro-1,2-hydroquinone

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