ELSEVIER

Toxicology Letters 122 (2001) 157–169

Toxicology Letters

www.elsevier.com/locate/toxlet

Oxidative stress and liver toxicity in rats and human hepatoma cell line induced by pentachlorophenol and its major metabolite tetrachlorohydroquinone

Ying-Jan Wang [a], Ching-Chang Lee [a], Wen-Ching Chang [a], Hung-Bin Liou [a], Yuan-Soon Ho [b]

[a] *Department of Environmental and Occupational Health, National Cheng Kung University, Medical College, Tainan, Taiwan, People's Republic of China*

[b] *Institute of Biomedical Technology, Taipei Medical University, No. 250 Wu-Hsing St., Taipei, People's Republic of China*

Received 9 January 2001; received in revised form 19 April 2001; accepted 27 April 2001

Abstract

Pentachlorophenol (PCP) is a pesticide used worldwide in industrial and domestic applications. It is used extensively as biocide and wood preservatives. Metabolic studies carried out in rodents and human liver homogenates have indicated that PCP undergoes oxidative dechlorination to form tetrachlorohydroquinone (TCHQ). Free radical catalyzed tissue injury is thought to play a fundamental role in human disease. In the present study, we examined the effects of PCP and TCHQ on the induction of lipid peroxidation and liver injury in rats. In addition, the cytotoxic dose, cell death mechanisms and related gene expressions induced by PCP and TCHQ were also determined for human hepatoma cell line (Hep G2). The results indicated that more toxic effects could be observed both in rats and human hepatoma cell line treated with TCHQ than its parent compound, PCP. Oxygen species may be involved in the mechanism of TCHQ intoxication since the urinary 8-epi-PGF2α and AST, ALT activities can be induced by TCHQ and attenuated by vitamin E treatment. Apoptosis features were found in cells treated with TCHQ but not PCP. TCHQ-induced cell damage may issue signals for the induction of HSPs, the decrease of the bcl/bax protein ratio and the decrease of *CAS* gene, whereas the PCP-induced damage may not. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Pentachlorophenol; Tetrachlorohydroquinone; Lipid peroxidation; Liver injury; Cell death mechanisms; Gene expressions

1. Introduction

Pentachlorophenol (PCP) is a pesticide used worldwide in industrial and domestic applications. It is used extensively as biocide and wood preservatives (Mycroft and Schlag, 1986; Wang

* Corresponding author. Tel.: + 886-2-23562213; fax: + 886-2-27393422.

E-mail address: hoyuansn@mail.tmc.edu.tw (Y.-S. Ho).

0378-4274/01/$ - see front matter © 2001 Elsevier Science Ireland Ltd. All rights reserved.

PII: S0378-4274(01)00361-7

and Lin, 1995). The persistence of this chemical in the environment has resulted in its widespread existence throughout the food chain. The average amount of excreted PCP in the general population of Canada was determined to be 4.3 nmol/day (Treble and Thompson, 1996). Metabolic studies carried out in rodents and human liver homogenates have indicated that PCP undergoes oxidative dechlorination to form tetrachlorohydroquinone (TCHQ) (Juhl et al., 1985; Renner and Hopfer, 1990). In the presence of oxygen, superoxide radicals can be produced by the cycle of autoxidation and reduction between TCHQ and its corresponding semiquinone radical under certain physiological conditions (Carstens et al., 1990). Thus, PCP could present a potent source of reactive oxygen species (ROS) during metabolization.

Free radical catalyzed tissue injury is thought to play a fundamental role in human disease (Cross et al., 1987). Particular constraints in addressing this hypothesis have been the inability to assess free radical generation in vivo and the lack of information on drugs or vitamins that act as effective antioxidants in vivo. Isoprostanes are a family of prostaglandin (PG) isomers that are produced from oxidative modification of polyunsaturated fatty acids through a free radical catalyzed mechanism (Pratico, 1999). One of the compounds that can be produced in abundance by such a mechanism is 8-epi-PGF2α, a potent vasoconstrictor and a chemically stable end product of lipid peroxidation. Monitoring of this compound has been shown to be a useful index of in vivo lipid peroxidation (Wang et al., 1995; Pratico, 1999).

In our previous studies, we have shown that DNA strand breakage in mammalian cells, glutathione conjugate formation and the depletion of glutathione content in liver tissue can be induced by TCHQ (Wang and Lin, 1995; Wang et al., 1997). In addition, protein adducts and oxidative DNA lesions have also been reported by other investigators (Dahlhaus et al., 1996; Lin et al., 1999; Umemura et al., 1999). Studies have shown that PCP exerts a promoting, but not an initiating effect on liver carcinogenesis, and the promoting effect is related to oxidative stress and compensatory hepatocellular proliferation (Umemura et al., 1999). Thus, hepatotoxicity generated through oxidative damage is believed to play an important role during the pathophysiological process of liver disease induced by PCP.

Necrosis and apoptosis are distinct mechanisms of cell death with very different characteristics. Necrosis is caused by catastrophic toxic or traumatic events; in contrast, apoptosis is an active process of cell destruction with specific defining morphologic and molecular features (Ledda-Columbano et al., 1989; Kerr, 1991; Bursch et al., 1992). It is generally accepted that identification of the genes involved in the process of cell death is an important area of future research. The best studied example of a cell death-associated gene is probably the *bcl*-2 gene. Expression of the proto-oncogene *bcl*-2 has been shown to inhibit cell death in several cell types and in response to a wide variety of inducers (Huie and Padmaja, 1993). The ratio between bcl-2 and bax protein determines cell survival or death. The cellular apoptosis susceptibility (*CAS*) gene may have a dual function in mammalian cells, one in apoptosis and another in cell proliferation. Heat shock protein (HSPs) have been connected to protective mechanisms against a variety of injuries (Hirvonen et al., 1996). HSPs function as molecular chaperones and are essential to cell survival under stressful conditions.

In the present study, we examined the effects of PCP and TCHQ on the induction of lipid peroxidation and liver injury in rats. Monitoring of 8-epi-PGF2α in urine was served as an index of oxidant stress in vivo. Hepatocellular damage was measured via the biochemical estimates of the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Fesus et al., 1987). We also examined the protective effect of vitamin E, a chain-breaking antioxidant with the particular function of preventing lipid peroxidation in membrane systems, against the TCHQ-induced oxidative damage. In addition, the cytotoxic doses of and cell death mechanisms induced by PCP and TCHQ were determined for human hepatoma cell line (Hep G2). The results indicated that apoptotic cell death can be induced in human hepatoma cells by TCHQ but not PCP.

How cell injury can trigger the sequence of events that results in apoptosis or necrosis is not well understood. Finally, this study used western blotting analysis to further examine some death-related gene expressions (*hsp*-70, *bcl*-2, *bax* and *CAS*) during the process of apoptosis or necrosis induced by PCP or TCHQ.

2. Materials and methods

2.1. Materials

PCP, TCHQ and α-tocopherol acetate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The purity of the PCP and TCHQ is approximately 98%. Both of them were dissolved in absolute ethanol in concentrated form. The PCP used in the present study was the original compound and not the sodium salt form. Quantities of *hsp*-70 (W27), *bcl*-2 (clone 100) and *CAS* (C42920) mouse monoclonal antibodies and *bax* polyclonal antibody were obtained from Transduction Laboratories. DMEM medium, cell culture supplements and fetal calf serum were ordered from Life Technologies (Gibco BRL, NY, USA). All other chemicals were commercially available products of analytical grade.

2.2. Animal and treatment

Male Sprague–Dawley rats (250–350 g) purchased from the Animal Center of National Cheng Kung University were housed in a metabolic cage in a temperature and humidity controlled room with 12 h light/dark cycles. Feed and tap water were provided ad lib. Rats were given a single i.p. injection of 40 mg/kg PCP or 15 mg/kg TCHQ dissolved in 0.5 ml corn oil. For vitamin E pretreatment, 100 mg/kg α-tocopherol acetate was injected 2 h prior to TCHQ treatment. The control group received the same amount of corn oil only or 100 mg/kg vitamin E. Urine samples were collected from the metabolic cage at 24, 48 and 72 h after treatment. Blood samples were collected from the heart and abdominal aorta at 24, 48 and 72 h after treatment. Serum was removed from the blood samples. Both the serum and urine samples were stored immediately at −70°C until analysis.

2.3. Determination of urinary 8-epi-PGF2α and serum transaminases

Urine samples were thawed and centrifuged at 1500 *g* for 15 min. Aliquot of 0.5 ml supernatant was applied to the 8-Isoprostane Affinity Column (Cayman Chemical Company) for purification of samples. The affinity column purification procedures were validated and showed that averaged recoveries were greater than 90% with a variance of less than 20%. Each purified sample was assayed in a 96-well plate coated with mouse *anti*-rabbit IgG monoclonal antibody to 8-epi-PGF2α (Cayman Chemical Company. 8-Isoprostane EIA Kit). An 8-epi-PGF2α tracer bound to acetyl cholinesterase was used to compete with free 8-epi-PGF2α for binding sites until the maximum binding wells read 0.8 A.U. Each sample underwent assay in duplicate. There was less than a 15% discrepancy between the duplicate measurements of each sample. Urine creatinine was used as a denominator for urine 8-epi-PGF2α levels and was measured in each sample.

Serum alanine transaminase (ALT) and aspartate transaminase (AST) were assayed using a biochemical analyzer (EKTACHEM DT-II system, Kodak, USA).

2.4. Cell culture, drug treatments and cytotoxicity assay

The cell line Hep G2 (HB 8065; American Type Culture Collection) was derived from a human hepatocellular carcinoma. Hep G2 cells were maintained in Eagle's minimum essential medium, supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum at 37°C in a 5% carbon dioxide atmosphere. For exposure to PCP and TCHQ, the reagents were added in concentrated form to the culture medium and mixed gently. The cultures were then incubated for the times indicated in the figures. Cell viability was determined at the indicated times based on the trypan blue exclusion method. Trypsin was added to the control cells and the treated cells.

The cells were then centrifuged and suspended. Each cell suspension (0.1 ml) was mixed well with 0.1 ml trypan blue solution (0.2% in phosphate-buffered saline, PBS). After 1 or 2 min, each solution was placed on a hemocytometer where the blue stained cells were counted as nonviable.

2.5. DNA agarose gel electrophoresis

The control and treated cells were grown in 10 cm Petri dishes. Both attached and unattached cells were harvested, washed twice with ice-cold PBS, suspended in TNE (10 mM Tris–HCl, pH 7.6; 140 mM sodium chloride; and 1 mM EDTA) and lysed at 37°C in 4 ml of extraction buffer (10 mM Tris–HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 20 μg/ml pancreatic RNase; and 0.5% SDS). After 2 h, proteinase K was added to a final concentration of 100 μg/ml, and the mixture was incubated for another 3 h at 50°C. The DNA was extracted twice with equal volumes of phenol and once with chloroform–isoamyl alcohol (24:1 v:v). The DNA was then precipitated with 0.2 volumes of sodium acetate, pH 4.8, and 2.5 volumes of ethanol at −20°C overnight then pelletted with 13 000 *g* for 1 h. The samples under went electrophoresis in a 1.5% agarose gel. The DNA was made visible by ethidium bromide staining.

2.6. Hsp-70, bcl-2, bax and CAS, western blotting analysis

Cells were washed with PBS and lysed in an ice-cold RIPA buffer (Tris–HCl pH 7.2, 25 mM; SDS 0.1%; Triton X-100 1%; sodium deoxycholate 1%; NaCl 0.15 M; EDTA 1 mM) containing 1 mM of phenyl methyl sulfonyl fluoride (PMSF), 10 μg/ml of aprotinin, 1 mM of sodium orthovanadate and 5 μg/ml of leupeptin. Protein concentrations were determined with the BCA method (Pierce, Rockford, IL, USA). Protein (50 μg) was resolved on 12.5% polyacrylamide gels and blotted onto nitrocellulose sheets using the semidry blot system (TE 70; Hoefer Scientific Instruments, San Francisco, CA) at 2 mA/cm^2 for 60 min in 25 mM Tris–HCl, pH 8.3; 192 mM glycine; and 20% methanol. The membrane was blocked overnight at room temperature with a blocking reagent (20 mM Tris, pH 7.4; 125 mM NaCl; 0.2% Tween 20; 4% nonfat dry milk; and 0.1% sodium azide). Then it was incubated for 1 h with the mouse *anti*-human *hsp*-70 (W27), *bcl*-2 (100) and *CAS* (C42920) monoclonal antibodies or the rabbit *anti*-human *bax* polyclonal antibody. The proteins were washed three times, then incubated with alkaline phosphatase-conjugated rabbit *anti*-mouse or goat *anti*-rabbit antibody in PBS and 0.5% Tween 20 for another 45 min with gentle shaking. After three final washes, the proteins were made visible by the Bio-Rad NBT-BCIP color development system.

2.7. Statistical analysis

Data are expressed as mean ± SD. Statistical significance was performed using the Student's *t*-test for comparison between the means. Difference was considered significant at a *P* value of less than 0.05.

3. Results

3.1. Determination of 8-epi-PGF2α in urine samples collected from rats treated with PCP, TCHQ and TCHQ/vitamin E

Lipid peroxidation was estimated by measurement of the 8-epi-PGF2α in urine. As shown in Fig. 1, the basal level of 8-epi-PGF2α in control rats was about 0.53 ng/mg creatine. When rats were treated with a single dose of 100 mg/kg vitamin E for 1 day, the level of 8-epi-PGF2α in their urine was lower than that of the control rats but with no significant difference. When rats were treated with a single dose of 40 mg/kg PCP or 15 mg/kg TCHQ for up to 3 days, significantly higher amounts of urinary 8-epi-PGF2α were found in the treated rats. A roughly 2.5- to 6-fold increase of urinary 8-epi-PGF2α was found in rats treated with PCP at day 2 and 3. The increase of urinary 8-epi-PGF2α in rats treated with TCHQ from day 1 through day 3 was about 3- to 11-fold. Additionally, when the rats were treated with TCHQ and vitamin E simultaneously, the amount of urinary 8-epi-PGF2α was significantly lower than that of the TCHQ-treated rats.

3.2. Effects of PCP, TCHQ and TCHQ/vitamin E on serum transaminase activities in rats

Hepatic toxicity was monitored by quantitative analysis of the ALT and AST activities which were used as the biochemical markers of liver injury. As shown in Table 1, serum ALT and AST levels in normal rats were 42.3 ± 15.5 and 76.0 ± 8.9 U/l. In PCP-treated rats, serum ALT and AST elevated to about two-fold on day 1 and decreased to normal levels by day 3. In TCHQ-treated rats, serum ALT elevated to about five-fold on day 1 and also decreased significantly at day 2 and 3, respectively. A more than 10-fold increase in AST was found in TCHQ-treated rats on day 1; the amount of AST on days 2 and 3 was still two- to three-fold higher than in the control rats. Among the rats treated with vitamin E, a slightly increased serum AST was found. However, there was no significant difference when compared to the control rats. Vitamin E treatment can decrease the hepatic toxicity induced by TCHQ. The protection of vitamin E was significant but not complete.

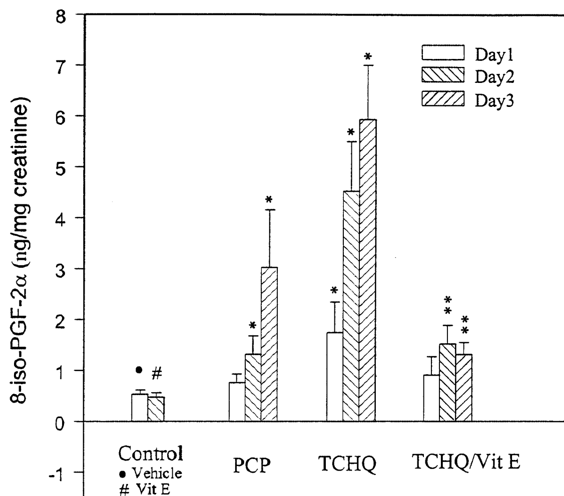


Fig. 1. Effects of PCP, TCHQ and TCHQ/Vit E on urinary 8-epi-prostaglandin F2α in rats. Five rats per group were injected intraperitoneally with PCP, TCHQ and combined TCHQ/Vitamin E at doses of 40, 15 and 15 plus 100 mg/kg. Urine samples were collected from the metabolic cages at 24, 48 and 72 h after treatment. Each value represents the mean ± SD for five animals. * $P < 0.05$ vs control. ** $P < 0.05$ vs TCHQ treated only.

3.3. Cytotoxicity effects and cell death mechanisms of PCP and TCHQ in Hep G2 cells

As seen in Fig. 2A, PCP at levels greater than 250 μm showed a significant toxicity to Hep G2 cells. At 24 and 48 h after exposure to 250 μm PCP, the viability of Hep G2 cells was less than 70 and 50%, respectively. Similar results were also observed in cells treated with TCHQ but with a higher rate of toxicity. After treatment with 100 μm TCHQ for 24 and 48 h, the cell viability was decreased by 41 and 66% in Hep G2 cells. Cell death mechanisms were monitored in Hep G2 cells with the same treatment as in the cytotoxic assays. To analyze DNA fragmentation, which is characteristic of apoptosis, both attached and detached cells were harvested at the indicated times after treatment with PCP and TCHQ. The dose-dependent experiments showed that DNA fragmentation could be induced at 48 h after exposure to doses greater than 20 μm TCHQ in Hep G2 cells (Fig. 2B). When cells were exposed to 0.1–1 mM PCP for 48 h, agarose gel electrophoresis showed no DNA fragmentation but a slight smear of DNA due to random cleavage of base pairs during the necrosis process (Fig. 2B).

3.4. Death-related gene expressions during PCP and TCHQ treatments

As seen in Fig. 3, when Hep G2 cells were treated with 200 μm TCHQ, *hsp*-70 concentrations began to increase after 12 h of incubation, with further increases at 24 and 36 h after exposure. A roughly three-fold increase in the concentration of *hsp*-70 was found in treated cells compared to the control cells. No significant change was found in the amount of *hsp*-70 in the cells treated with 0.5 mM PCP from 0 to 36 h. The expression levels of *bcl*-2 slightly increased in cells treated with PCP, when treated with TCHQ, *bcl*-2 also increased slightly at 6 and 12 h and then decreased at 24 and 36 h of incubation. The expression of *bax* gene increased after 6 h of incubation and persisted to 36 h in cells treated with PCP and TCHQ. No significant change of *CAS* gene expression was found in cells treated with PCP, however, when cells treated with

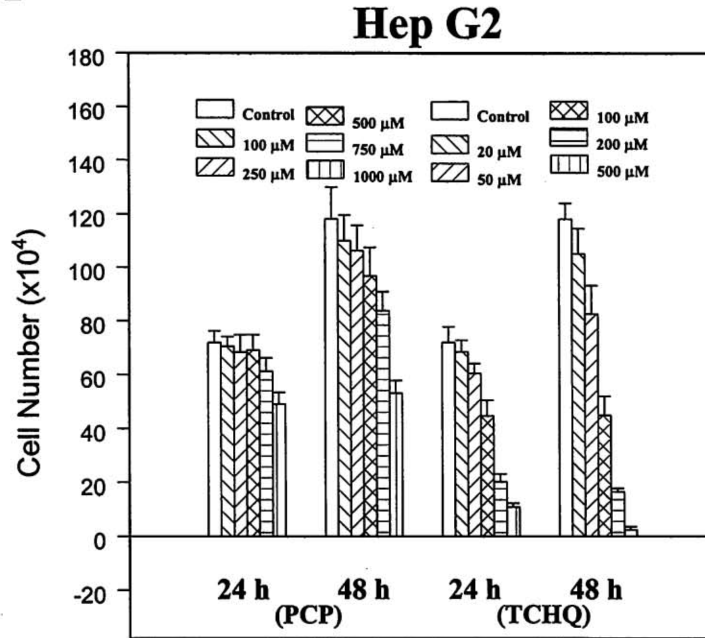
Table 1
Time-course effect of PCP, TCHQ and TCHQ/Vit E on serum ALT and AST activities in rats

	Control	PCP 1	PCP 2	PCP 3	TCHQ 1	TCHQ 2	TCHQ 3	Vit E	TCHQ 1/Vit E	TCHQ 2/Vit E	TCHQ 3/Vit E
ALT (U/L)	42.3 ± 15.5	90.0 ± 35.8	60.0 ± 3.6	47.7 ± 12.7	202.0 ± 10.4*	76.3 ± 6.4*	58.3 ± 11.0	41.7 ± 8.1	93.3 ± 13.3**	59.7 ± 13.3	66.0 ± 10.4
AST (U/L)	76.0 ± 8.9	177.7 ± 27.7*	90.3 ± 4.9	82.3 ± 9.2	477.3 ± 92.2*	183.3 ± 17.5*	148.0 ± 55.0	106.0 ± 25.1	228.3 ± 51.7**	129.7 ± 18.3**	148.3 ± 68.2

Rats were injected intraperitoneally with PCP, TCHQ and combined TCHQ/Vit E at doses of 40, 15 and 15 plus 100 mg/kg. Blood samples were collected from the heart and the abdominal aorta at 24, 48 and 72 h after treatment. Control rats were injected with vehicle or Vit E only for 24 h. Each value represents the mean ± SD for five animals. PCP 1, PCP 2, PCP 3 means treated with PCP for 1, 2, and 3 days. TCHQ 1, TCHQ 2, TCHQ 3 means treated with TCHQ for 1, 2, and 3 days.

* $P<0.05$ vs control.

** $P<0.05$ vs TCHQ treated only.



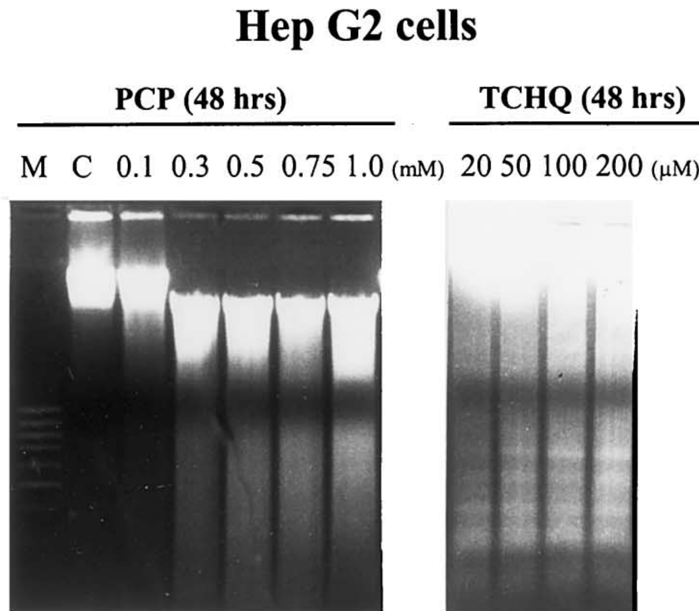


Fig. 2. (A) Effects of varying concentrations of PCP and TCHQ on cell death of Hep G2 cells. Cell viability was determined at 24 and 48 h after PCP and TCHQ treatment based on the trypan blue exclusion method. Data are expressed as means ± SD; (B) Agarose gel analysis of DNA fragmentation in Hep G2 cells. After treatment with 0.1–1 mM of PCP for 48 h, cells did not display the DNA ladder, suggesting that the cells died by necrosis. After treatment with 20–200 µM of TCHQ for 48 h, cells produced a DNA ladder characteristic of apoptosis.

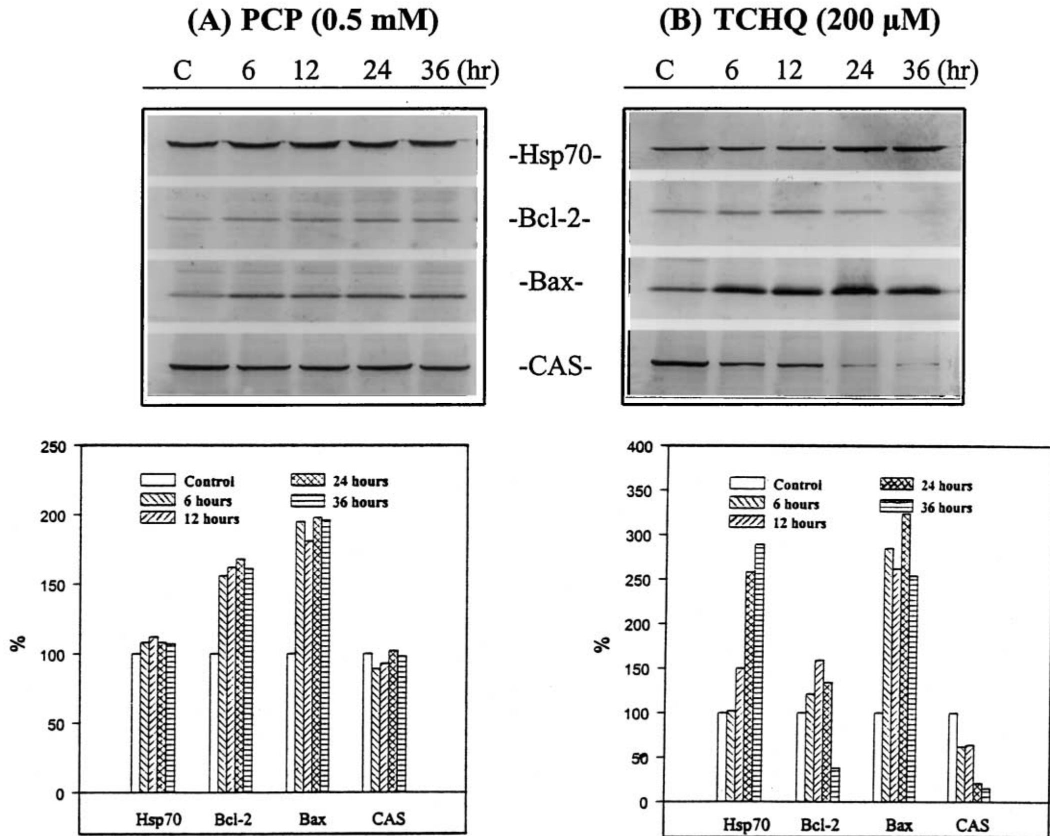


Fig. 3. Immunoblot analysis of the genes expression of *hsp*-70, *bcl*-2, *bax* and *CAS* in PCP and TCHQ-treated Hep G2 cells. Cells from each time point following 0.5 mM PCP or 200 μM TCHQ treatment were analyzed. Proteins recognized by each antibody are indicated on the central site. A typical protein blot was cut into two halves at the position of 46 KDa marker. The upper half blot was probed with antibodies which recognize *hsp*-70 and *CAS*, the lower half blot probed with antibodies which recognize *bcl*-2 and *bax*. Graphs are normalized quantitative results of the relative proteins.

TCHQ, *CAS* gene expression decreased significantly after 6 h of incubation, and further decreased during prolonged incubation. Only trance amounts of the *CAS* remained in the cells at 36 h of incubation.

4. Discussion

PCP and its metabolite TCHQ were evaluated for effects on hepatotoxicity in rats and cell death mechanisms in human hepatoma cell line. Measurements of 8-epi-PGF2α in urine and ALT, AST activities in serum served as markers of lipid peroxidation and liver damage. In this study, we found that significant increase of urinary 8-epi-PGF2α in rats could be induced by both PCP and TCHQ. The more intense toxic insult was found in rats treated with TCHQ rather than PCP, although the dose of TCHQ used was only one-third that of PCP. In in vitro tests, PCP uncouples oxidative phosphorylation, inactivate respiratory enzymes and causes damage to mitochondria (Deichman and Keplinger, 1981). Although covalent binding of PCP to DNA has been reported, PCP does not seem to produce real DNA damage (Van

Ommen et al., 1989). In contrast to PCP itself, TCHQ both binds to DNA and causes strand breaks in DNA in vitro (Smith, 1985; Wang and Lin, 1995; Wang et al., 1997). Oxygen radicals can be produced by the cycle of autoxidation and reduction between TCHQ and its corresponding semiquinone radicals or quinones, a process that is referred to as redox cycling (Carstens et al., 1990). Thus, oxygen radicals might play a central role in the generation of lipid peroxidation in rats treated with PCP and TCHQ.

The leakage of certain intracellular enzymes suggested irreversible damage in hepatocytes. In this case, serum ALT and AST activities frequently served as an index of liver injury. After one single dose of PCP and TCHQ, a significant increase in ALT and AST activities was observed on day 1, then declined on day 2 and 3. One day after i.p. injection may not be the optimal sampling time, and the peak value of acute liver toxicity needs to be further investigated. The elevated amounts of both 8-epi-PGF2α and ALT, AST activities in TCHQ-treated rats were higher than in the PCP-treated rats. These may be due in part to the metabolic pathway of PCP in vivo. It has been claimed that PCP is mainly excreted unchanged in the urine (Braun et al., 1977). The rats in this study eliminated approximately 74% of the dose as PCP and an additional 12% as PCP glucuronide. In contrast, other investigators indicate that PCP is extensively metabolized to PCP glucuronide in humans (Uhl et al., 1986; Reigner et al., 1992). The mean peak plasma concentration occurred 4 h after administration, and the enterohepatic recirculation was considered as important (Braun et al., 1977). An average half-life of 16 days was found in workers occupationally exposed to PCP. No accumulation of PCP could be observed in the enterohepatic circulation (Uhl et al., 1986; Pekari et al., 1991). Acute liver toxicity after a high dose injection of PCP and TCHQ might occur during the first several hours, with rapid recovery following. This may partly explain why the increased ALT, AST activities occurred on day 1 and decreased on day 2 and 3. In addition to the liver tissue, the extrahepatic tissues were also exposed continuously. Measurements of urinary 8-epi-PGF2α served as an index of oxidative stress in the whole body and not only the liver. The levels of urinary 8-epi-PGF2α increased persistently from day 1 through day 3. The protective effect of vitamin E against lipid peroxidation and hepatotoxicity in rats treated with TCHQ was investigated. Lipid peroxidation has been implicated as a possible mechanism for hepatic injury in acute TCHQ poisoning. The present study showed that the lipid peroxidation, as well as the activities of ALT and AST, was suppressed by administration of vitamin E.

8-Epi-PGF2α is a major F_2-isoprostane that has been shown to increase during free radical-mediated arachidonic acid oxidation in experimental animal studies and in human studies (Davi et al., 1999; Sodergren et al., 2000). However, there are only a few earlier animal studies investigating the effect of vitamin E on F_2-isoprostanes (Basu, 1998, 1999). Many different biomarkers of lipid peroxidation could be used to evaluate the oxidative damage. Different responses are represented by different biomarkers because they reflect different stages of the lipid peroxidation process. The noninvasive measurement of 8-epi-PGF2α is a promising approach for studies, investigating the possible roles of lipid peroxidation under normal conditions and in the pathology of human disease. Basal levels of 8-epi-PGF2α in vitamin E treated rats (100 mg/kg i.p.) were lower than those of the control rats prospectively. Nevertheless, the AST activities were slightly higher in the treated animals than in the control rats. In safety studies of vitamin E intake, adverse effects were rarely observed with dosages up to 2 g/kg body weight in rats (Kappus and Diplock, 1992). Whether vitamin E has other functions, apart from the antioxidant properties exhibited by the scavenging of free radicals and reactions with active forms of oxygen, has not yet been established. The cause for the effects of vitamin E on AST levels in the rats is unclear.

Lipid peroxidation is a well known example of oxidative damage in cell membranes and other lipid-containing structures. The present in vivo study shows that both PCP and TCHQ can induce lipid peroxidation in rats, and the induction potency was found stronger for TCHQ than PCP. Similar results were reported in previous studies

in vitro. It has been shown that slightly increased lipid peroxidation could be induced by PCP in isolated rat hepatocytes (Suzuki et al., 1997). Furthermore, oxidative damage such as single-strand breakage in isolated DNA and genotoxicity in human fibroblasts could be induced by TCHQ (Witte et al., 2000). An aspect of related interest that is under intensive investigation is lipid peroxidation mediated stress signaling, which may evoke a variety of cellular responses, ranging from induction of antioxidant enzymes to apoptotic death (reviewed in Girotti, 1998). Further investigation of these processes will facilitate the understanding of disease states associated with peroxidative stress.

Cytotoxicity, cell death mechanisms and related genes expression were investigated in human hepatoma cells treated with PCP and TCHQ. Using the trypan blue exclusion method to detect cytotoxicity, exposure to relatively mild insults (levels below 100 μM PCP and 50 μM TCHQ) led to slower onset and less overall cell death than exposure to more intense insults (levels greater than 500 μM PCP and 200 μM TCHQ). Failure to exclude trypan blue reflects a loss of plasma membrane integrity associated with necrosis. Thus, lysis occurred earlier and in a greater percentage of the cells exposed to more intense excitotoxic insults. The concentration of PCP required to cause 50% cell death was about five-fold greater than that required for TCHQ in Hep G2 cells. This may be due in part to their different toxic mechanisms in which oxygen radicals are believed to play an important role. The concentration ranges used to induce cell death in the experiments are higher than the general population under nonoccupational exposure encounters (Treble and Thompson, 1996). However, acute poisonings by accidents have occurred in many workers who were exposed while working in wood plants (Jorens and Schepens, 1993). In toxicological studies, the doses selected are usually larger than those humans come into contact with. Extrapolation of the observed dose–response relationship to an expected response at much lower doses could be achieved by consideration of other relevant data.

Cell death mechanisms induced by PCP and TCHQ were investigated in Hep G2 cells. The results suggest that TCHQ mediated apoptosis and DNA laddering in Hep G2, but PCP induced cell death more characteristic of necrosis than apoptosis. It is an unexpected finding that even high doses of PCP in Hep G2 cells cannot induce significant apoptotic features, since PCP is assumed be metabolized to TCHQ in hepatocytes. This may be due in part to the inhibiting effects of PCP on metabolizing enzymes. It has been shown that the metabolizing process of PCP could be inhibited by itself (Arrhenius et al., 1977; Juhl et al., 1985). Besides, the solubility of PCP in a culture medium should be taken into consideration, especially when treated with a higher concentration. The PCP used in the present study is the original compound but not its sodium salt form. It is indicated that the solubility of PCP in water is limited. The distinction between apoptosis and necrosis in cell culture systems can be confused because of the lack of scavenging cells; thus, the phagocytic step after apoptosis may not occur. It is important to look for features of apoptosis at various time points after the insult and not to unduly delay these observations, as secondary necrosis may intervene and therefore obfuscate the true nature of the injury to the compromised cell (Bonfoco et al., 1995). The selection of incubation time for treatment of Hep G2 cells with TCHQ is based on a time-course pilot study, and the results showed that 48 h is optimal for observing DNA fragmentation (data not shown). As seen in Fig. 2B, a dose-dependent DNA laddering could be observed in Hep G2 cells treated with different concentrations of TCHQ for 48 h, indicating that TCHQ is a strong inducer that can trigger apoptotic cell death in Hep G2 cells.

PCP has been proposed to be a promoting agent (McConnel, 1989; Sai et al., 1998). The cell proliferation resulting from toxicity may selectively induce enhanced replication of an already damaged genome in the initiated cell population. We cannot exclude the possibility that promutagenic lesions can also be produced by PCP in vivo, since free radicals could be generated during the metabolizing process of PCP. There is abun-

dant evidence suggesting that mutagenic DNA damage can be derived from the oxidative attacks of free radicals (Dedon et al., 1998; Nunoshiba, et al., 1999; Ruiz-Laguna et al., 2000). The ability of PCP to induce cell proliferation in mouse livers has been reported (Umemura et al., 1996). In our recent report, both PCP and TCHQ induced necrotic cell death in chang liver cells (Wang et al., 2000). The major difference between these two liver cell lines is that chang liver cells were derived from human epithelial origins and is normal, however, the Hep G2 cells were derived from human hepatocyte origins and were transformed. Transformed cell lines might be more susceptible than normal cell lines when treated with cytotoxic agents. It has been reported that the cytotoxic drugs commonly used in cancer therapy can induce tumor cell death by apoptosis (Martin and Green, 1994; Schmitt and Lowe, 1999; Lowe and Lin, 2000).

In this study, it was found that the expression of the *hsp*-70 gene increased significantly (threefold) in cells treated with TCHQ. However, no significant change was found in the cells treated with PCP. Obviously, the increased amount of *hsp*-70 in the Hep G2 cells is not sufficient to protect them against the lethal metabolic alterations brought about by TCHQ. These results suggest that TCHQ-induced cell damage may issue a profound intracellular signal for the induction of HSPs, whereas the PCP-induced damage may not issue this signal. The best example of a cell death-associated gene is probably the *bcl*-2 gene. The bcl-2 protein is a suppressor of programmed cell death that homodimerizes with itself and forms heterodimers with bax (Oltvai et al., 1993). Bax protein is considered a promoter of cell death, which is functionally neutralized by heterodimerization with bcl-2. The ratio of bcl-2/bax protein might account for the protective mechanism of bcl-2 in cells. In the present study, a significant decrease in the bcl-2/bax protein ratio was found in cells treated with TCHQ but not PCP. It was suggested that bcl-2 acts proximally, rather than distally, on the apoptotic effector which is thought to be calcium-activated endonuclease. In addition, the net cellular production of reactive oxygen species might also be regulated by bcl-2. *CAS* gene is a cytoplasmic antigen that is highly expressed in proliferating cells but only at low levels in cells that do not proliferate (Hirvonen et al., 1996; Scherf et al., 1996). *CAS* also plays a role in apoptosis, because *CAS* is involved in nuclear transport of apoptosis-associated proteins. Expression of the *CAS* gene decreased significantly in cells treated with TCHQ but not PCP, indicating that the *CAS* gene participates in an important regulating role during the process of apoptosis. However, the real mechanisms need to be further investigated. Gene expression measurement in this study included *hsp*-70, *bcl*-2, *bax* and *CAS*. The major concern of the selection is that the selected genes may have some relation to both apoptosis and necrosis. So, apoptosis-related gene expression is not the only concern in this study.

In summary, more toxic effects can be observed both in rats and human hepatoma cell lines treated with TCHQ than with its parent compound, PCP. Oxygen species may be involved in the mechanism of TCHQ intoxication since the urinary 8-epi-PGF2α and AST, ALT activities can be induced by TCHQ and attenuated by vitamin E treatment. Apoptosis features were found in cells treated with TCHQ but not PCP. TCHQ-induced cell damage may issue signals for the induction of HSPs, the decrease of the bcl/bax protein ratio and the decrease of *CAS* gene expression, whereas the PCP-induced damage may not.

Acknowledgements

This study was supported by the National Science Council (NSC 89-2320-B-006-023 and NSC 89-2314-B-006-170).

References

Arrhenius, E., Renberg, L., Johnsson, L., 1977. Subcellular distribution, a factor in risk evaluation of pentachlorophenol. Chem. Biol. Interact. 18, 23–34.

Basu, S., 1998. Metabolism of 8-iso-prostaglandin $F_{2\alpha}$. FEBS Lett. 428, 32–36.

Basu, S., 1999. Oxidative injury induced cyclooxygenase activation in experimental hepatoxicity. Biochem. Biophys. Res. Commun. 254, 764–767.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., Lipton, S.A., 1995. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc. Natl. Acad. Sci. USA 92, 7162–7166.

Braun, W.H., Young, J.D., Blau, G.E., Gehring, P.J., 1977. The pharmacokinetics and metabolism of pentachlorophenol in rats. Toxicol. Appl. Pharm. 41, 395–406.

Bursch, W., Oberhammer, F., Schulte-Hermann, R., 1992. Cell death by apoptosis and its protective role against disease. Trends Pharmacol. Sci. 13, 245–251.

Carstens, C.P., Blum, J.K., Witte, I., 1990. The role of hydroxy radicals in tetrachlorohydroquinone induced DNA strand break formation in PM2 DNA and human fibroblasts. Chem. Biol. Interact. 74, 305–314.

Cross, C.E., Haliwell, B., Borish, E.T., Proyor, W.A., Ames, B.N., Saul, R.L., McCord, J.M., Harman, D., 1987. Oxygen radicals and human disease. Ann. Intern. Med. 107, 526–545.

Dahlhaus, M., Almstadt, E., Henschke, P., Luttgert, S., Appel, K.E., 1996. Oxidative DNA lesions in V79 cells mediated by pentachlorophenol metabolites. Arch. Toxicol. 70, 457–460.

Davi, G., Ciabattoni, G., Consoli, A., Mezzetti, A., Santarone, S., Pennese, E., Vitacolonna, E., Bucciarelli, T., Costantini, F., Capani, F., Patrono, C., 1999. In vivo formation of 8-iso-prostaglandin $F_{2\alpha}$ and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation. Circulation 99, 224–229.

Dedon, P.C., Plastaras, J.P., Rouzer, C.A., Marnett, L.J., 1998. Indirect mutagenesis by oxidative DNA damage: formation of the pyrimidopurinone adduct of deoxyguanosine by base propenal. Proc. Natl. Acad. Sci. USA 95, 11 113–11 116.

Deichman, W.B., Keplinger, M.L., 1981. Phenols and phenolic compounds. In: Clayton, G.D., Cayton, F.E. (Eds.), Patty's Industrial Hygiene and Toxicology. John Wiley and Sons, New York, pp. 2567–2627.

Fesus, L., Thomazy, V., Falus, A., 1987. Induction and activation of tissue transglutaminase during programmed cell death. FEBS Lett. 224, 104–108.

Girotti, A.W., 1998. Lipid hydroperoxide generation, turnover, and effector action in biological systems. J. Lipid Res. 39, 1529–1542.

Hirvonen, M.-R., Brune, B., Lapetina, E.G., 1996. Heat shock proteins and macrophage resistance to the toxic effects of nitric oxide. Biochem. J. 315, 845–849.

Huie, R.E., Padmaja, S., 1993. The reaction of NO with superoxide. Free Rad. Res. Commun. 18, 195–199.

Jorens, P.G., Schepens, P.J., 1993. Human pentachlorophenol poisoning. Hum. Exp. Toxicol. 12, 479–495.

Juhl, U., Witte, I., Butte, W., 1985. Metabolism of pentachlorophenol to tetrachlorohydroquinone by human liver homogenate. Bull. Environ. Contam. Tox. 35, 596–601.

Kappus, H., Diplock, A.T., 1992. Tolerance and safety of vitamin E: a toxicological position report. Free Radic. Biol. Med. 13, 55–74.

Kerr, J.F.R., 1991. In Apoptosis: The Molecular Basis of Cell Death: Current Communications on Cell and Molecular Biology, vol. 3. Cold Spring Harbor Lab. Press, Plainview, NY, pp. 5–29.

Ledda-Columbano, G.M., Columbano, A., Coni, P., Faa, G., Peni, P., 1989. Cell deletion by apoptosis during regression of renal hyperplasia. Am. J. Pathol. 135, 657–662.

Lin, P.H., Waidyanatha, S., Pollack, G.M., Swenberg, J.A., Rappaport, S.M., 1999. Dose-specific production of chlorinated quinone and semiquinone adducts in rodent livers following administration of pentachlorophenol. Toxicol. Sci. 47, 126–133.

Lowe, S.W., Lin, A.W., 2000. Apoptosis and cancer. Carcinogenesis 21, 485–495.

Martin, S.J., Green, D.R., 1994. Apoptosis as a goal of cancer. Curr. Opin. Oncol. 6, 616–621.

McConnel, E.E., 1989. Toxicology and carcinogenesis studies of two pentachlorophenol technical-grade mixtures in B6C3F1 mice. U.S. National Toxicology Program. Department of Health and Human Services, NIH Publication No. 89-2804.

Mycroft, F.J., Schlag, R., 1986. Pentachlorophenol (PCP). Hazard Rev. 3, 1–3.

Nunoshiba, T., Obata, F., Boss, A.C., Oikawa, S., Mori, T., Kawanishi, S., Yamamoto, K., 1999. Role of iron and superoxide for generation of hydroxyl radical, oxidative DNA lesions, and mutagenesis in Escherichia. J. Biol. Chem. 274, 34 832–34 837.

Oltvai, Z.N., Milliman, C.L., Korsmeyer, S.J., 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74, 609–619.

Pekari, K., Luotamo, M., Jarvisalo, J., Lindroos, L., Aitoi, A., 1991. Urinary excretion of chlorinated phenols in saw-mill workers. Int. Arch. Occ. Env. Health 63, 57–62.

Pratico, D., 1999. F2-isoprostanes: sensitive and specific non-invasive indices of lipid peroxidation in vivo. Atherosclerosis 147, 1–10.

Reigner, B.G., Bois, F.Y., Tozer, T.N., 1992. Assessment of pentachlorophenol exposure in humans using the clearence concept. Hum. Exp. Toxicol. 11, 17–26.

Renner, G., Hopfer, C., 1990. Metabolic studies on pentachlorophenol (PCP) in rat. Xenobiotica 20, 573–582.

Ruiz-Laguna, J., Prieto-Alamo, M.J., Pueyo, C., 2000. Oxidative mutagenesis in Escherichia coli strains lacking ROS-scavenging enzymes and/or 8-oxoguanine defenses. Environ. Mol. Mutagen. 35, 22–30.

Sai, K., Upham, B.L., Kang, K.S., Hasegawa, R., Inoue, T., Trosko, J.E., 1998. Inhibitory effect of pentachlorophenol on gap junctional intercellular communication in rat liver epithelial cells in vitro. Cancer Lett. 130, 9–17.

Scherf, U., Pastan, I., Willingham, M.C., Brinkmann, U., 1996. The human CAS protein which is homologous to the CSE1 yeast chromosome segregation gene product is associated with microtubules and mitotic spindle. Proc. Natl. Acad. Sci. USA 93, 2670–2674.

Schmitt, C.A., Lowe, S.W., 1999. Apoptosis and therapy. J. Pathol. 187, 127–137.

Smith, M.T., 1985. Quinones as mutagens, carcinogens, and anticancer agents: introduction and overview. J. Toxicol. Environ. Health 16, 665–672.

Sodergren, E., Cederberg, J., Basu, S., Vessby, B., 2000. Vitamin E supplementation decreases basal levels of F_2-isoprostanes and prostaglandin $F_{2\alpha}$ in rats. J. Nutr. 130, 10–14.

Suzuki, T., Komatsu, M., Isono, H., 1997. Cytotoxicity of organochlorine pesticides and lipid peroxidation in isolated rat hepatocytes. Biol. Pharm. Bull. 20, 271–274.

Treble, R.G., Thompson, T.S., 1996. Normal values for pentachlorophenol in urine samples collected from a general population. J. Anal. Toxicol. 20, 313–317.

Uhl, S., Schmid, P., Schlatter, C., 1986. Pharmacokinetics of pentachlorophenol in man. Arch. Toxicol. 58, 182–186.

Umemura, T., Sai, K., Takagi, A., Hasegawa, R., Kurokawa, Y., 1996. Oxidative DNA damage and cell proliferation in the livers of B6C3F1 mice exposed to pentachlorophenol in their diet. Fund. Appl. Toxicol. 30, 285–289.

Umemura, T., Kai, S., Hasegawa, R., Sai, K., Kurokawa, Y., Williams, G.M., 1999. Pentachlorophenol (PCP) produces liver oxidative stress and promotes but not initiate hepatocarcinogenesis in B6C3F1 mice. Carcinogenesis 20, 1115–1120.

Van Ommen, B., Adang, A., Muller, F., Van Bladeren, P.J., 1989. The microsomal metabolism of pentachlorophenol and its covalent binding to protein and DNA. Chem. Biol. Interact. 60, 1–11.

Wang, Y.J., Lin, J.K., 1995. Estimation of selected phenols in drinking water with in situ acetylation and study on the DNA damaging properties of polychlorinated phenols. Arch. Environ. Contam. Toxicol. 28, 537–542.

Wang, Y.J., Ho, Y.S., Chu, S.W., Lien, H.J., Liu, T.H., Lin, J.K., 1997. Induction of glutathione depletion, p53 protein accumulation and cellular transformation by tetrachlorohydroquinone, a toxic metabolite of pentachlorophenol. Chem. Biol. Interact. 105, 1–16.

Wang, Y.J., Ho, Y.S., Jeng, J.H., Su, H.J., Lee, C.C., 2000. Different cell death mechanism and gene expression in human cells induced by pentachlorophenol and its major metabolite, tetrachlorohydroquinone. Chem. Biol. Interact. 128, 173–188.

Wang, Z., Ciabattoni, G., Creminon, C., Lawson, J., Fitzgerald, G.A., Patrono, C., Maclouf, J., 1995. Immunological characterization of urinary 8-epi-prostaglandin $F_{2\alpha}$ excretion in man. J. Pharmacol. Exp. Ther. 275, 94–100.

Witte, I., Zhu, B.Z., Lueken, A., Magnani, D., Stossberg, H., Chevion, M., 2000. Protection by desferrioxamine and other hydroxamic acids against tetrachlorohydroquinone-induced cyto- and genotoxicity in human fibroblasts. Free Radic. Biol. Med. 28, 693–700.