Characterization of the Interaction between Cadmium and Chlorpyrifos with Integrative Techniques in Incurring Synergistic Hepatotoxicity

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Abstract

Mixture toxicity is an important issue for the risk assessment of environmental pollutants, for which an extensive amount of data are necessary in evaluating their potential adverse health effects. However, it is very hard to decipher the interaction between compounds due to limited techniques. Contamination of heavy metals and organophosphoric insecticides under the environmental and biological settings poses substantial health risk to humans. Although previous studies demonstrated the co-occurrence of cadmium (Cd) and chlorpyrifos (CPF) in environmental medium and food chains, their interaction and potentially synergistic toxicity remain elusive thus far. Here we integrated the approaches of thin-layer chromatography and 1H NMR to study the interaction between Cd2+ and CPF in inducing hepatotoxicity. A novel interaction was identified between Cd2+ and CPF, which might be the bonding between Cd2+ and nitrogen atom in the pyridine ring of CPF, or the chelation formation between one Cd2+ and two CPF molecules. The Cd-CPF complex was conferred with distinct biological fate and toxicological performances from its parental components. We further demonstrated that the joint hepatotoxicity of Cd ion and CPF was chiefly due to the Cd-CPF complex-facilitated intracellular transport associated with oxidative stress.

Introduction

The co-existence of various pollutants in environment and food chains is considerably concerned due to the fused impact on environment and public health [1,2], especially if the joint toxicity of pollutants poses adverse health effects on humans [2]. Mounting evidence suggests that simultaneous exposure of pollutants on organisms can potentiate the toxicity of individual components [3,4]. Thus far, it has been difficult to study the joint toxicity of pollutants, in particular to determine the interaction between compounds, due to limited technical approaches [5]. Only limited interactions have been fully characterized, such as the interaction of chlorpyrifos (CPF) with methyl mercury [6].

The heavy metal cadmium (Cd) has broad industrial applications, such as battery production and electroplating, and it is substantially dispersed in the environment [7,8]. Excretion of Cd ion in human body is about 1–2 μg/day, and the half-life is 20–30 years. Exposure to Cd could cause serious diseases, such as itai-itai disease or even cancers [9]. As a typical environmental hazard, Cd is ranked eighth within the top 20 in the priority list of hazardous substances by the ATSDR (Agency for Toxic Substances and Disease Registry) [10]. CPF is one of the most widely used organophosphoric insecticides worldwide under agricultural and residential settings in the last few decades [11,12]. Although CPF has been banned for very long time, it still largely remains in water, air and soil, as well as in many dwellings. CPF was found in 100% of indoor air samples and 64–70% of blood samples from mothers and newborns [13]. Large amount of CPF can cause acute toxicity, and even a trace amount of CPF can induce neurological toxicity in fetuses and children [13]. Cd ion and CPF are often jointly present in the same environmental media and food chains, and are simultaneously exposed on organisms [14,15], leading to pronounced environmental and health problems [16]. They incur common sensitive targets of toxicity, such as carcinogenicity and hepatotoxicity [17,18,19,20], and oxidative stress is assumed to be the principal molecular basis underlying cytotoxicity caused by Cd and CPF [21]. Despite the co-occurrence of these two chemicals in environmental medium and food chains, their toxicity and human risk assessment were predominantly based on the toxicological performances of single chemical. The interaction of Cd ion with other organophosphorus pesticide (such as fenitrothion) has been suggested by other studies [22]; whereas the toxic effects of CPF have also been demonstrated to be modulated by metals, such as zinc [23]. However, the synergistic interaction between Cd ion and CPF has not been established thus far, and the corresponding molecular mechanism is largely unknown as well.
In the current study, to elucidate the reciprocal impact between CPF and Cd ions, we here addressed their joint hepatoxicity using a few in vitro assessments in a representative human hepatocyte cell line Hep G2. We embarked on their synergistic molecular interaction by integrating techniques, such as thin-layer chromatography (TLC) and 1H NMR. Overall, we demonstrated the formation of the Cd-CPF complex, which was conferred with distinct biological fate and toxicological performances from its parental chemicals.

Results and Discussion

To evaluate the potential synergistic effect, we first evaluated the joint cytotoxic effect of Cd2+ and CPF on Hep G2 cells by assessing the cell viability with the MTT assay. After 24 h, no toxicity was observed to Hep G2 cells treated with up to 32 μM Cd2+ and up to 1,280 μM CPF, respectively (Fig. 1a & b). To intensively study the synergistic effect, we chose the concentration of 10 μM for both Cd2+ and CPF, at which neither of them caused damage to cell viability (Fig. 1 & Fig. 2a). It should be noted that CPF was dissolved in DMSO and the concentration of 10 μM CPF in culture medium contained only 0.001% DMSO which caused no toxicity to cells compared to the blank control (data not shown). The concomitant exposure of the Cd2+ and CPF mix at 10 μM exerted great impairment to Hep G2 cells, causing approximately 50% reduction in cell viability, compared to the control or the individual treatment by Cd2+ or CPF (Fig. 2a, P<0.001). Moreover, remarkable morphological alternations representing cell death were observed for the cells upon the combined treatment, as these cells became rounder and smaller than the cells without treatment or treated with only one chemical (Fig. 2b). The FACS analysis with FITC-conjugated Annexin V and PI staining further validated cell death in Hep G2 cells, as largely increased apoptosis (>10 fold increase for the Annexin V+ cell population) was detected in cells treated with combined Cd2+ and CPF treatment compared to individual Cd2+ or CPF treatment (Fig. 2c, P<0.001). These results together demonstrated a strong synergistic cytotoxic effect of Cd2+ and CPF on Hep G2 cells.

Previous studies have documented that both Cd2+ and CPF could induce cytotoxicity through oxidative stress, such as reactive oxygen species (ROS) generation [21]. We thus assessed intracellular ROS content in cells upon the concomitant exposure. As shown in Fig. 3a, the ROS level was not significantly changed in Hep G2 cells upon treatment with single compound; however, the ROS level was elevated upon combined exposure compared to the single exposure and the vehicle control (P<0.01). In agreement with previous observations [24], the accumulation of intracellular ROS was likely responsible for the increased cell death caused by the concomitant exposure of Cd2+ and CPF. Lipid peroxidation is also an important index in characterizing oxidative stress [25], and malondialdehyde (MDA) is recognized as a marker for lipid peroxidation [26]. For instance, Cd2+ in vivo administration causes pronounced hepatic oxidative stress in animals, and leads to remarkable liver damage characterized by increased lipid peroxidation and altered antioxidant enzymatic activity [27,28]. The MDA content was not significantly changed in cells treated with individual component, whereas it was increased upon the joint exposure (Fig. 3b, P<0.05). In response to oxidative stress, the anti-oxidation system is normally enhanced to protect cells against oxidant damage in hepatocytes [27,29], of which the antioxidant enzyme glutathione peroxidase (GSH-Px) is an important ROS scavenger. The GSH-Px activity was significantly increased only in Hep G2 cells treated with both Cd2+ and CPF (P<0.05), compared to the control or the individual treatment by Cd2+ or CPF, but not in cells treated with single component in comparison to the control (Fig. 3c). Additionally, we assessed the level of lactate dehydrogenase (LDH), a soluble cytosolic enzyme released into culture medium due to damaged plasma membrane [30]. As shown in Fig. 3d, the LDH level in the culture supernatant was...
proportional to the results of cell viability as described in Fig. 1 and Fig. 2, as the LDH release was significantly increased in cells upon the binary exposure of Cd²⁺ and CPF only (P<0.01), compared to the control or the individual treatment by Cd²⁺ or CPF. These results together suggested that the concomitant treatment of Cd²⁺ and CPF led to synergistic impairment to Hep G2 hepatocytes linked to oxidative stress, while Cd²⁺ or CPF alone at the same concentration could not bring harm to these cells.

To substantiate the role of oxidative stress in the conduction of the synergistic toxicity by Cd²⁺ and CPF, we pre-treated the cells with N-acetyl cysteine (NAC), an antioxidant [31]. As shown in Fig. 4a, induction of ROS was significantly undermined by the pre-treatment of NAC in cells treated with Cd²⁺+CPF, compared to the cells without the pre-treatment of NAC (P<0.001). Therefore, the alterations to the cell morphology were greatly ameliorated upon the pre-treatment of NAC, as the number of the rounder and smaller cells decreased compared to the cells without the pre-treatment of NAC (Fig. 4b). To this end, oxidative stress is demonstrated to be the primary modulator of synergistic impairments mediated by Cd²⁺ and CPF, coupled with reduced cell viability and cell death.

To investigate the mechanism of the synergistic toxicity conducted by Cd²⁺ and CPF, we characterized the molecular interaction between these two compounds. The TLC results indicated the existence of CPF and the CPF-Cd complex (Fig. 5a). The CPF-Cd complex presented a separate band as the pink arrow indicated on the TLC plate, while the CPF alone did not. The UV-vis absorption spectrum analysis of the reaction mix also revealed distinct absorption peaks around 320–350 nm between CPF and CPF+Cd, suggesting the formation of CPF-Cd complex (Fig. 5b). Furthermore, the NMR spectroscopy was employed to illustrate the precise site of binding between CPF and Cd. As the the NMR spectra shown in Fig. 5c and Table 1, Cd ions induced great chemical shift of Ha in the pyridine ring of CPF, resulting in decreased electron density of nitrogen atom and sulphur atom (the molecular formula of CPF was presented in Fig. 6a). This observation demonstrated that a new coordination mode was formed between Cd²⁺ and CPF due to the nephelauxetic effect that refers to a decrease in the Racah interelectronic repulsion parameter. To study the proportion of CPF and Cd²⁺ in the complex, we performed the 1H NMR spectra using different ratios of CPF to Cd²⁺, i.e. 10:1, 2:1, 1:1, 1:2, 1:5 at molar concentrations. A significant change in chemical shift was detected at the ratio of 2:1 for CPF/Cd²⁺ (Fig. 5c and Table 1), suggesting that CPF and Cd²⁺ tended to form a complex with two CPF molecules to one Cd²⁺. We also carried out the NMR spectroscopy at different time points, 6 h, 24 h and 48 h, and no difference in chemical shift of hydrogen atom was observed along time course (Table 1), implying that the complex was quickly formed and stably existed. Similar to this finding, a previous study using the approach of NMR also demonstrated that Hg²⁺ could associated with the two sulfur atoms in the Demeton S side chain, resulting in stabilizing...
the Demeton S molecule, another organophosphorus pesticide [32]. These data collectively confirmed the formation of a novel complex between Cd ion and CPF.

Previous studies demonstrated that the formation of a complex between chemicals could often accelerate their transport across cell membrane and increase the intracellular accumulation [6,33], likely resulting in cytotoxicity that might not happen to a single chemical [2,34]. A representative interaction of CPF was identified with methyl mercury, and the formation of this complex significantly enhanced the bioaccumulation of methyl mercury and induced greater toxicity [6]. We thus assessed the intracellular Cd content in Hep G2 cells upon Cd²⁺ or Cd²²⁺ CPF exposure both at 10 mM for 24 h. The ICP-MS data indicated that the intracellular Cd content in Hep G2 cells upon Cd²⁺ or Cd²²⁺ CPF exposure both at 10 mM for 24 h. The ICP-MS data indicated that the intracellular Cd concentration in Cd²⁺+CPF-treated cells was increased by >20% (Fig. 6b, P<0.05), suggesting facilitated transport of Cd²⁺ into cells aided by the complex. CPF is a lipophilic molecule that could readily permeate and penetrate the lipid bilayer membrane [35], whereas the transport of Cd ions is rather difficult, and limited transportation mainly relies on two paths, the cell surface sulfhydryl ligand and the calcium channel [36]. Metallothionein (MT) was reported to be induced by Cd and sequesters intracellular free Cd ions via formation of an MT-Cd complex [10]. MT is also an important intracellular component as ROS scavenger, whose suppression could lose the ability to protect cells from ROS-mediated impairment. Meanwhile, Cd exposure was demonstrated to incur a significant reduction of MT content [37]. Since ROS is the predominant cause of apoptosis, MT reduction caused by CPF might enhance ROS-induced cytotoxicity [38]. Whether CPF-Cd complex also diminishes MT warrants detailed investigation. To this end, we could speculate that the formation of CPF-Cd complex therefore potentiated their localization and retention inside cells with the aid of CPF-mediated transportation through diffusion crossing the lipophilic membrane, which presumably accounted for the increased oxidative stress and reduced cell viability.

Organisms are typically exposed to a mix of chemicals, where the toxicological action for each individual chemical might be altered by the co-occurring ones. Both Cd ions and CPF are serious environmental pollutants worldwide and they are often found to co-exist in the environment, food, wild organisms and even in human specimens [14,15]. In the current study, we mechanistically demonstrated a novel interaction between Cd ion and CPF through the binding of Cd²⁺ to the pyridine ring of CPF. One possibility is the bonding between Cd²⁺ and nitrogen atom in the pyridine ring of CPF (Fig. 6c–i); meanwhile the other possibility is the chelation among one Cd²⁺ and nitrogen atom and sulphur atom from two CPF molecules (Fig. 6c–ii). The formation of Cd-CPF complex largely changed their biological fate and toxicological performance with facilitated cellular uptake and increased toxicity to hepatocytes via oxidative stress. These data together verify the molecular mechanism underlying the Cd/CPF-conducted joint hepatoxicity.

This report presents novel results on the toxic effects of a binary mixture of Cd and CPF on HepG2 cells. The binary mixture has barely been studied yet, and not in HepG2 cells in any case. This study is therefore novel in this sense. In deed, these data, for these chemicals that are indeed widely present in the environment, are very useful. The data indicates that CPF may complex with Cd to facilitate its entry into cells, thereby increases the level of Cd in the cells and also its toxic effect through oxidative stress. This mechanism could occur widely for more combinations of other chemicals. Additionally, we addressed the interaction between Cd ions and CPF by integrating the techniques, such as TLC and 1H
NMR, which would pave the way for future studies in addressing the synergistic interaction between pollutants.

Materials and Methods

Chemicals, Reagents and Cell Culture

Chlorpyrifos (CPF) was purchased from Shuangma Fine Chemical Co., Ltd, Nantong City with the purity of more than 99.99%, and Cd2+ (in CdCl2) was purchased from Sigma. The human hepatoma Hep G2 cells (purchased from the Shanghai Cell Bank of Type Culture Collection of CAS) were cultured in 1640 medium (Hyclone), supplemented with 10% fetal bovine serum (Gibco) and 100 U/mL penicillin/streptomycin (Gibco) in a humidified atmosphere with 5% CO2 at 37°C. The stock solutions of Cd2+ (in CdCl2) and CPF were made in sterile ddH2O and DMSO, respectively, and then filtered through Minisart filters (0.45 μm).

Cell Survival Assay

Cell survival was assessed by the MTT assay following the instructions from the manufacturer (Roche). Briefly, Hep G2 cells were serum starved for 12 h, and were then inoculated into 96-well plates at a concentration of 5.0×10^3 cells/well upon different treatments. Cells were cultured for another 24 h, and 20 μL MTT (5 mg/mL) was added to each well followed by incubation for 4 h. Thereafter, 200 μL DMSO was added into each well, and the 96-well plates were read at 490 nm on a microplate reader (Thermo) after shaking.

Figure 4. The amelioration of the synergistic hepatotoxicity conducted by Cd2+ and CPF by the pre-treatment of NAC. Hep G2 cells were treated individually or jointly with Cd2+ and CPF both at 10 μM for 6 h, and then the intracellular ROS generation (a) was assessed (n = 4). (b) The representative phase-contrast images. The original magnification was 200×. doi:10.1371/journal.pone.0059553.g004

ROS Detection and LDH Leakage Assay

Hep G2 cells were seeded in 6-well plates overnight and these cells were treated individually or jointly with Cd2+ and CPF both at 10 μM for 6 h. The generation of intracellular ROS was spectrophotometrically measured using dichlorofluorescein-diacetate (DCF-DA, Sigma) as described previously [39]. Relative fluorescence intensity was recorded using a fluorescent plate reader (Thermo) at an excitation wavelength of 485 nm and emission was measured at a wavelength of 530 nm. The fluorescence intensity was assayed, which was proportional to the amount of intracellular ROS concentration. For experiments with NAC, cells were pre-treated with 500 μM NAC (Sigma) 1 h prior to the treatment of Cd2+CPF both at 10 μM, and cultured for another 6 h in the presence of NAC. The CytoTox-ONE Homogeneous Membrane Integrity Assay Kit (Promega) was used to assess LDH release, according to the manufacturers’ instructions. This assay was on the basis of the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD.

The Cell Death Analysis

Hep G2 cells were seeded in 6-well plates at a density of 3.0×10^4 cells per well for 24 h. Cells were treated with Cd2+ and/or CPF for 6 h, and were then collected after wash with PBS. The proportions of apoptosis and necrosis were determined by the flow cytometry analysis after Annexin V and propidium iodide (PI) staining (BD Biosciences) as previously described [39,40].
Assays for MDA Level and GSH-Px Activity

Hep G2 cells were treated individually or jointly with Cd\textsuperscript{2+} and CPF both at 10\textmu M for 6 h, and thereafter cells were collected into RIPA lysis buffer after wash with PBS. The MDA level and the GSH-Px activity in the Hep G2 cells were assessed according to the manufacturer’s instructions (both from Wuhan Xinqidi Biological Technology Co., LTD, China). Briefly, supernatants of cell lysates were added into pre-coated GSH-Px or MDA monoclonal antibody microelisa wells followed by the conventional procedure as described previously [41].

Cd Determination through Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Hep G2 cells in 10 cm-plates (1.0\times10^6) were treated with Cd\textsuperscript{2+} or Cd\textsuperscript{2+} plus CPF both at 10 \textmu M for 24 h. These Hep G2 cells were washed repeatedly with PBS before the collection into digestive solution. The intracellular Cd mass was measured using

| Time (h) | CPF/Cd | Ha (ppm) | Hb (ppm) | Hc (ppm) | Ha (ppm) | Hb (ppm) | Hc (ppm) | Ha (ppm) | Hb (ppm) | Hc (ppm) | Ha (ppm) | Hb (ppm) | Hc (ppm) |
|----------|--------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| 0        | 1/0    | 8.577     | 4.313     | 1.335     | 8.577     | 4.313     | 1.335     | 8.577     | 4.313     | 1.335     | 8.577     | 4.313     | 1.335     |
|          | 10/1   | 8.635     | 4.320     | 1.346     | 8.634     | 4.322     | 1.347     | 8.634     | 4.320     | 1.347     | 8.634     | 4.308     | 1.346     |
|          | 2/1    | 8.637     | 4.320     | 1.330     | 8.634     | 4.322     | 1.347     | 8.637     | 4.330     | 1.348     | 8.620     | 4.315     | 1.337     |
|          | 1/1    | 8.621     | 4.318     | 1.338     | 8.636     | 4.319     | 1.348     | 8.619     | 4.307     | 1.337     | 8.620     | 4.315     | 1.337     |
|          | 1/2    | 8.627     | 4.314     | 1.342     | 8.628     | 4.319     | 1.343     | 8.627     | 4.313     | 1.343     | 8.627     | 4.313     | 1.343     |
|          | 1/5    | 8.551     | 4.274     | 1.296     | 8.549     | 4.270     | 1.294     | 8.548     | 4.270     | 1.294     | 8.549     | 4.270     | 1.294     |

Table 1. Time-dependent \textsuperscript{1}H chemical shift changes of CPF with various ratios of added Cd\textsuperscript{2+}.
the ICP-MS method according to the protocol described in a previous study [42]. Briefly, samples were quantified by volume and digested with strong oxidation-acid solution (a mix of nitric acid and hydrogen peroxide with a proportion of 3:2) overnight. Then, the primarily digested samples were digested thoroughly at 180°C for 20 mins by microwave assisted digestion (MAD, Mars5 HP500, CEM Corporation, USA). Cd concentrations in these samples were finally quantified using ICP-MS (Agilent 7500, USA).

Thin-layer Chromatography

Chemical-chemical interaction assessment between CPF and Cd2⁺ was performed with the approach of TLC. CPF (0.01 M) and Cd2⁺ (0.01 M) were allowed to react in ethyl acetate or deionized water under slow mixing for 24 h. The mixture was resolved on silica plates using 30% ethyl acetate/70% hexane, and was visualized by exposure to iodine and ultraviolet (UV) light.

The 1H Nuclear Magnetic Resonance (NMR) Spectroscopy Analysis

The CPF and Cd2⁺ were diluted with d6-DMSO in 5 mm precision NMR tubes. The 1H chemical shifts were referenced internally to the solvent resonance. After completely dissolved, samples were placed in the spectrometer for hydrogen spectrum analysis. 1H NMR was measured on the 400 MHz spectrometers (NMR in d6-DMSO). Chemical shifts (δ) were given in ppm relative to residual solvent (d6-DMSO; δ 2.50 for 1H NMR).

Statistical Analysis

One-way analysis of variance (ANOVA) was used to analyze the mean differences among groups compared to the control. Two-tailed Student’s t test was used to analyze experimental data between two groups. Data were shown in mean ± SD. P<0.05 was considered statistically significant.

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Author Contributions

Conceived and designed the experiments: SL. Performed the experiments: LC GQ XS SZ LW JL SL. Analyzed the data: LC GQ XS JL SL. Contributed reagents/materials/analysis tools: GQ NS YD JL. Wrote the paper: SL.

References

1. Smith E, Gancarz D, Rofe A, Kempson IM, Weber J, et al. (2012) Antagonistic effects of cadmium on lead accumulation in pregnant and non-pregnant mice. J Hazard Mater 198: 453–456.

2. Kortenkamp A, Faust M, Scholze M, Baekhuys T (2007) Low-level exposure to multiple chemicals: reason for human health concerns? Environ Health Perspect (Suppl 1): 106–114.
3. Hass U, Scholze M, Christiaensen S, Dalgaard M, Vingaard AM, et al. (2007) Combined Exposure to Anti-androgens Exacerbates Disruption of Sexual Differentiation in the Rat. Environ Health Perspect 115: 122–128.

4. DelRazo NJ, Foy BD, Garrahan JM, Frazier JM (2003) Cadmium uptake kinetics in rat hepatocytes: Correction for albumin binding. Toxicol Sci 72: 19–30.

5. Boobis A, Budinski R, Collie S, Crofton K, Embry M, et al. (2011) Critical analysis of literature on low-dose synergy for use in screening chemical mixtures for risk assessment. Crit Rev Toxicol 41: 369–383.

6. Stevens JA, Benson WH (1999) Toxicological interactions of chlorpyrifos and methyl mercury in the amphipod, Hyalella azteca. Toxicol Sci 52: 167–177.

7. Angenard G, Muczynski V, Colliguy H, Pairault C, Duquenne C, et al. (2010) Cadmium Increases Human Fetal Germ Cell Apoptosis. Environ Health Perspect 118: 331–337.

8. Ragunathan N, Dairou J, Sanfins E, Busi F, Noll C, et al. (2010) Cadmium-fenitrothion interaction in the spider Pardosa lugubris and the fruit fly Drosophila melanogaster. B Environ Contam Toxicology 102: 285–299.

9. Tuzmen N, Candan N, Kaya E, Demiryas N (2008) Biochemical effects of acute dietary As, Cd, Cr, and Pb intake on the development of male rat brain. Brain Res 1202: 146–156.

10. Mansour SA, Belal MH, Abou-Arab AAK, Gad MF (2009) Monitoring of arsenic, cadmium, chromium, and lead on gene expression regulated by a battery of 13 different promoters in recombinant HepG2 cells. Toxicol Appl Pharmacol 238: 79–90.

11. Klaassen CD, Liu J, Diwan BA (2009) Metallothionein protection of cadmium toxicity. Toxicol Appl Pharmacol 238: 215–220.

12. Markovis M, Cupar S, Durovic R, Milovsic J, Kligje P (2010) Assessment of Heavy Metal and Pesticide Levels in Soil and Plant Products from Agricultural Area of Belgrade, Serbia. Arch Environ Con Tox 58: 341–351.

13. Rusyniak DE, Nanagas KA (2004) Organophosphate poisoning. Semin Neurol 24: 197–204.

14. Rash VA, Garfinkel R, Perera FP, Andrews HF, Hoeper L, et al. (2006) Impact of Prenatal Chlorpyrifos Exposure on Neurodevelopment in the First 3 Years of Life Among Inner-City Children. Pediatrics 118: e143–e159.

15. Manussar SA, Belal MH, Abu-Arab AAK, Gad MF (2009) Monitoring of pesticides and heavy metals in cucumber fruits produced from different farming systems. Chromatogr 75: 601–609.

16. Fatta D, Canna-Michaudou S, Michael C, Demetriou Georgio E, Christodoulou M, et al. (2007) Organochlorine and organophosphoride insecticides, herbicides and heavy metals residue in industrial wastewaters in Cyprus. J Hazard Mater 145: 169–179.

17. Hsieh CJ, Hsieh WS, Su YN, Liao HF, Jeng SF, et al. (2011) The Taiwan Birth Panel Study: a prospective cohort study for environmentally related child health. BMC Res Notes 4: 291.

18. Tuzmen N, Candan N, Kaye E, Demiray N (2006) Biochemical effects of chlorpyrifos and deltamethrin on altered antioxidative defense mechanisms and lipid peroxidation in rat liver. Cell Biochem Funct 26: 119–124.

19. Oh SH, Lim SC (2006) A rapid and transient ROS generation by cadmium triggers apoptosis via caspase-dependent pathway in HepG2 cells and this is inhibited through N-acetylcysteine-mediated catalase upregulation. Toxicol Appl Pharm 212: 212–223.

20. Padilla S, Marshall RS, Hunter DL, Oxendine S, Moser VC, et al. (2005) Neurochemical Effects of chronic dietary and repeated high-level acute exposure to Chlorpyrifos in rats. Toxicol Sci 80: 161–171.

21. Rai A, Mavara SK, Khare P, Srivastava A, Bandopadhaya S (2010) Characterization of Developmental Neurotoxicity of As, Cd, and Pb Mixture: Synergistic Action of Metal Mixture in Gliad and Neuronal Functions. Toxicol Sci 118: 595–601.

22. Bucio L, Souza V, Albores A, Sierra A, Chavez E, et al. (1995) Cadmium and ethanol: neurochemical effects of chronic dietary and repeated high-level acute exposure. Toxicol Lett 13: 257–262.

23. Goel A, Chahain DP, Dhillon DK (2000) Protective effects of zinc in chlorpyrifos induced hepatotoxicity: a biochemical and trace elemental study. Biol Trace Elem Res 74: 171–183.

24. Baran CP, Zeiger MM, Triandafilpani S, Marsh CB (2004) The role of ROS and RNS in regulating life and death of blood monocytes. Curr Pharm Design 10: 855–866.

25. Keller JN, Mark RJ, Bruce AJ, Blanc E, Rothstein JD, et al. (1997) 4-hydroxyestrone, an aldehydic product of membrane lipid peroxidation, impairs glomerular transport and mitochondrial function in synaptosomes. Neuroscience 89: 685–696.

26. Draper HH, Hadley M (1990) Malealdehyde Determination as Index of Lipid-Peroxidation. Method Enzymol 168: 421–431.

27. Goel A, Dani V, Dhillon DK (2005) Protective effects of zinc on lipid peroxidation, antioxidant enzymes and hepatic histrarchitecture in chlorpyrifos-induced toxicity. Chemico-Biological Interactions 156: 131–140.

28. Dondono F, Placentin L, Banni M, Rebolo M, Burlando B, et al. (2005) Quantitative PCR analysis of two melosan metallothionein genes unveils differential expression and regulation. Gene 345: 259–270.

29. Ediz L, Hiz O, Ozkol H, Gulcu E, Toprak M, et al. (2011) Relationship between Anti-CCP Antibodies and Oxidant and Anti-Oxidant Activity in Patients with Rheumatoid Arthritis. Int. J. Mech Sci 8: 139–147.

30. Arun P, Spadaro J, John J, Gharavi RB, Bentley TB, et al. (2011) Studies on blast traumatic brain injury using in-vitro model with shock tube. Neuroreport 22: 379–384.

31. Jayalakshmi K, Sairam M, Singh SB, Sharma SK, Ilaivazhagan G, et al. (2005) Neuroprotective effect of N-acetyl cysteine on hypoxia-induced oxidative stress in primary hippocampal culture. Brain Res 1046: 73–75.

32. Pelkonen SO, Judeh ZMA (2005) Mechanism of interactions between Hg(II) and demeton S: an NMR study. Environ Sci Technol 39: 2386–2391.

33. Moto FC, Alegian SV, Dunston SK, Pessah IN (1995) The Delta-Isomer of Hexachlorocyclohexane Increases Rapid Release of the Myo-Inositol-1,4,5-Trisphosphate-Sensitive Ca2+ Store and Blocks Capacitative Ca2+ Entry in Rat Basophilic Leukemia-Cells. Mol Pharmacol 48: 312–322.

34. Djian MP, Ludke JL (1975) Studies on combined effects of organophosphates and heavy metals in birds. I. Plasma and brain cholinesterase in coturnix quail fed methyl mercury and orally dosed with parabulin. Bull Environ Contam Toxicol 13: 257–262.

35. Veldman TP, Post RS, Rick DL, Marty MS, Matson JO, et al. (2009) The Effect of Plasma Lipids on the Pharmacokinetics of Chlorpyrifos and the Impact on Interpretation of Blood Biomonitoring Data. Toxicol Sci 108: 258–272.

36. Shahid ZA, Blazka ME, Endo T (1995) Metal transport in cells: cadmium uptake by rat hepatocytes and renal cortical epithelial cells. Environ Health Perspect (Suppl 1): 73–75.

37. Goel A, Dani V, Dhillon DK (2006) Chlorpyrifos-induced alterations in the activities of carbohydrate metabolizing enzymes in rat liver: The role of zinc. J Hazard Mater 163: 125–214.

38. Klaassen CD, Liu J, Diwan BA (2009) Metallothionein protection of cadmium toxicity. Toxicol Appl Pharmacol 238: 215–220.

39. Liu S, Li S, Du Y (2010) Polychlorinated biphenyls (PCBs) enhance metastatic properties of breast cancer cells by activating Rho-associated kinase (ROCK). PLoS One 5: e11272.

40. Liu S, Goldstein RH, Scapansky EM, Rosenblatt M (2009) Inhibition of rho-associated kinase signaling prevents breast cancer metastasis to human bone. Cancer Res 69: 6742–6751.

41. Liu S, Suragani RN, Wang F, Han A, Zhao W, et al. (2007) The function of b-catenin-regulated eIF2alpha kinase in murine iron homoeostasis and macrophage maturation. J Clin Invest 117: 3296–3305.

42. Yang RS, Chang I, Wu JP, Tsai MH, Wang HJ, et al. (2007) Persistent tissue kinetics and redistribution of nanoparticles, quantum dot 705, in mice: ICP–MS quantitative assessment. Environ Health Perspect 115: 1319–1343.