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Joint toxicity of chlorpyrifos and cadmium on the oxidative stress and mitochondrial damage in neuronal cells

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ABSTRACT

Pesticides and heavy metals can be easily biomagnified in food chains and bioaccumulated in individuals, thus pose significant threat to human health. However, their joint toxicity for long-term exposure at low dose has not been thoroughly investigated. In the present study, we investigated the oxidative damages in brain of rats exposed subchronically to organophosphorus pesticide chlorpyrifos (CPF) and heavy metal cadmium (Cd), and their mixtures at the environmentally relevant doses. Rats were given different doses of CPF and Cd by oral gavage for three months. After treatment, brain tissues were subjected for biochemical analysis. Mitochondrial damage and reactive oxidative species were also measured in neuroblastoma SH-SY5Y cells treated with CPF, Cd and their mixtures. The results showed that CPF and Cd generated protein and lipid peroxidation, disturbed the total antioxidant capability, and altered mitochondria ultrastructure in the brain. Lipids and proteins were sensitive to the oxidative damage induced by CPF and Cd. CPF and Cd decreased mitochondrial potential and induced reactive oxygen species in SH-SY5Y cells. However, the mixture did not display higher toxicity than the sum of that of the individual treatments. Thus, CPF and Cd could have a potential antagonistic interaction on the induction of oxidative stress.

1. Introduction

Organophosphorus pesticides, which inhibit cholinesterase activity and induce cholinergic neurotoxicity, have been used widely in household and agriculture (Abou-Donia, 2003). Chlorpyrifos (CPF), which is an organophosphorus insecticide widely used to protect various crops against many types of pests, is known to have cholinergic neurotoxicity (Eaton et al., 2008). CPF accumulates in food, water, air, and soil (Guardino et al., 1998). CPF exposure is not only found in the rural residents, but also in people living in the cities due to the use of CPF for sanitary pest control (Rauh et al., 2006). The pesticide residues have also been found in the human blood (Liu and Pleil, 2002).

Cadmium (Cd), a heavy metal, has been widely used in industry and Cd can accumulate in the water, soil, and food (McLaughlin et al., 2007). Cadmium can accumulate in human body for a long time, which does serious harm to various organs (Waalkes, 2000; Satarug et al., 2002). Many studies have reported that Cd could mainly induce the damage in kidney, liver, and bone (Brzoska and Moniuszko-Jakoniuk, 2005; Johri et al., 2010; Arroyo et al., 2012). Besides, Cd also induces neurotoxicity in humans (Leal et al., 2012).

Since CPF and Cd can easily be accumulated in the environment, and humans can be exposed to CPF and Cd through food chains, they have been reported to be harmful to the public health (Satarug et al., 2002; Lemus and Abdelghani, 2000). Previous studies reported that simultaneous exposure of different pollutants could potentiate the toxicity of individual chemicals (Boobis et al., 2011). The combined effect of the chemical mixtures can usually be defined as additive (additive effect), greater than additive (synergistic effect), or less than additive (antagonistic effect) (Crain et al., 2008). In the current study, additive interaction is defined when the effect of the mixture equals to the sum of the individual effects, and the synergistic or antagonistic interaction means that the effect of the mixture is greater or less than the sum of their individual

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effects, respectively, according to early reports (Crain et al., 2008; Folt et al., 1999; Piggott et al., 2015). At present, although the acute toxicity of chlorpyrifos and cadmium are well understood (Acker and Nogueira, 2012; Spiazzi et al., 2013), their combined toxicity at environmentally relevant low doses upon long time exposure have not been thoroughly investigated.

In our previous study, the metabolomic changes in the CPF-, Cd-, and their mixtures-treated rat brain were characterized, which showed that energy and amino acids metabolism was disturbed by CPF and Cd treatment (Xu et al., 2015). In this study, we aimed to investigate the joint subchronic toxicity of CPF and Cd in the brain of rats as well as in the cultured neuroblastoma cells, focusing on the biochemical changes relevant to oxidative stress in the rat brain.

2. Materials and methods

2.1. Chemicals

Chlorpyrifos (purity > 96%) was obtained from Nantong Shuangma Fine Chemical Co., Ltd (Jiangsu, China). Fetal calf serum was obtained from Chuanye Biosciences (Tianjin, China). Superoxide dismutase (SOD) and catalase (CAT) were purchased from Beyotime (Shanghai, China). Diethylenetriaminepentacetic acid, bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM), hypoxanthine, Hoechst 33258, cadmium chloride, and xanthine oxidase were purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA). WST-1 was obtained from Dojindo Laboratories (Kumamoto, Japan). All other reagents were of analytical grade and obtained from commercial providers.

2.2. Animal experiment

Six-week old male SD rats were obtained from Beijing HFK Bioscience Co., Ltd (Beijing, China) and were housed individually in cages. Animals were acclimatized for at least 1 week prior to the study. The animal room was maintained at 22 ± 2 °C, 50%–60% humidity and a light/dark cycle of 12 h. Animals had free access to water and diet.

To study the joint effect of chlorpyrifos (CPF) and cadmium (Cd), we used a full-factorial design containing 16 treatment groups, with six rats in every group. Rats in the zero-, low-, mid-, and high-dose CPF-treatment groups received 0, 1.7, 5.0, and 15.0 mg/kg/day, respectively, and those in the corresponding Cd-treatment groups received 0, 0.7, 2.0, and 6.0 mg/kg/day, respectively. Low, medium and high doses in the CPF plus Cd treatment group were mixtures of the low, medium, and high dosages of both CPF and Cd (Table 1).

Chlorpyrifos and cadmium chloride were dissolved in corn oil and deionized water, respectively, and administered via oral gavage (0.5 ml/kg body weight for rats) each day for 90 days. The rats in control group received an equivalent volume of corn oil and water.

All animal procedures were performed in accordance with the applicable Chinese legislation and approved by the Animal and Medical Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences.

After the 90-day experimental treatment, one of the rats in each group was perfused by 4% paraformaldehyde. And then the prefrontal cortex of the brain was dissected and fixed in 2.5% glutaraldehyde at 4 °C for further processing of transmission electron microscopy examination (see section 2.3). All other rats were subject to anesthesia and decapitated. The whole brain was dissected and cut into small pieces, ground in liquid nitrogen, and then stored at −80 °C until used for biochemical assay (see section 2.4).

Table 1
Experimental design for joint effects of chlorpyrifos and cadmium.

Group	Chlorpyrifos (mg/kg/day)	Cadmium (mg/kg/day)
Control	–	–
CPF-L	1.7	–
CPF-M	5	–
CPF-H	15	–
Cd-L	–	0.7
Cd-M	–	2
Cd-H	–	6
CPF-L + Cd-L	1.7	0.7
CPF-M + Cd-L	5	0.7
CPF-H + Cd-L	15	0.7
CPF-L + Cd-M	1.7	2
CPF-M + Cd-M	5	2
CPF-H + Cd-M	15	2
CPF-L + Cd-H	1.7	6
CPF-M + Cd-H	5	6
CPF-H + Cd-H	15	6

Abbreviations: CPF, chlorpyrifos; Cd, cadmium; L, low dose; M, middle dose; H, high dose.

2.3. Transmission electron microscopy examination

After fixed in 2.5% glutaraldehyde, the prefrontal cortex samples of the brain were washed by phosphate buffer, dehydrated with ethanol (from 50 to 100%), and then embedded in epoxy resin. The ultrathin sections, which were sectioned with ultramicrotome, were stained with uranyl acetate and lead citrate before transmission electron microscopy (TEM) examination. When taking EM pictures, only cells with prominent neuronal cell morphology were chosen. Five fields from each of the three sections for one EM sample were examined.

2.4. Preparation of brain samples for biochemical assay

The whole brain tissues were homogenized in 10 volume of 50 mM phosphate buffer (PBS, pH 7.4). The homogenates were centrifuged for 20 min at 1000 g and supernatants were collected for biochemical assay (see sections 2.5–2.9). Protein concentrations were measured by the Bradford's method. Briefly, 0.1 ml of supernatant was added to 5 ml of Commassie brilliant blue reagent. The mixture was incubated at room temperature for 5 min and the absorbance was measured at 595 nm. The protein concentration was calculated based on a standard curve prepared by different concentrations of BSA solutions.

2.5. Superoxide dismutase activity assay

The activity of superoxide dismutase (SOD) in brain was measured based on the measurement of WST-1 (sodium salt of 4-[3-(4iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) reduction rate (Peskin and Winterbourn, 2000). The reaction mixture contained 19.3 ml assay buffer (sodium phosphate, 50 mM, pH 8.0, containing 0.1 mM diethylenetriamine pentaacetic acid and 0.1 mM hypoxanthine), 100 μl of 10 mM WST-1 solution, 100 μl of catalase solution (2 mg/ml) and 5 μl of xanthine oxidase (4.5 mU/ml final concentration). The prepared brain homogenate (20 μl) was transferred into the well of a 96-well plate. Then, reaction mixture (200 μl) was transferred into the sample well. The plate was mixed immediately and measured at 450 nm in a microplate reader at 37 °C for 5 min. The SOD activity was calculated by the SOD standard curve, which was made according to the method described above.

2.6. Catalase activity assay

Catalase (CAT) activity was determined according to the method of Sinha (1972). The prepared brain homogenate (0.1 ml) was added to the reaction mixture [0.5 ml of 0.01 M phosphate buffer (pH 7.0) and 0.4 ml of 0.2 M H_2O_2], and mixed immediately and reacted for exactly 1 min at 37 °C. Catalase activity was calculated as μmoles of H_2O_2/min/mg protein.

2.7. Measurement of malondialdehyde level

The level of malondialdehyde (MDA) in the brain was determined based on its ability to react with thiobarbituric acid (TBA) (Stocks and Dormandy, 1971). The prepared brain homogenate (1 ml) was precipitated with 0.5 ml of 20% trichloroacetic acid (TCA). The mixture was centrifuged at 800 g for 15 min after 2 h incubation in an ice bath. The supernatant (1 ml) was mixed with 0.1 ml of 0.1 M EDTA and 0.25 ml of 1% TBA, and then boiled for 20 min, cooled and determined at 532 nm. The concentration of MDA was calculated using extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$.

2.8. Measurement of protein carbonyl level

Protein carbonyl (PCO) content was measured based on its ability to react with 2, 4-dinitro-phenyl-hydrazine (DNPH) to form hydrazones (Levine et al., 1990). Briefly, The prepared brain homogenate (0.1 ml) was reacted with 0.4 ml of 20 mM DNPH solution for 60 min. Then, the mixture was added into 0.5 ml 20% (v/v) trichloroacetic acid and centrifuged at 12000 g for 15 min. The precipitate was washed three times with the mixture of ethanol and ethyl acetate (1:1 v/v), resuspended in 1 ml of 6 M guanidine hydrochloride and then measured at 370 nm. The level of PCO was calculated using extinction coefficient of 22.0 $mM^{-1}\ cm^{-1}$.

2.9. Measurement of acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was determined using the spectrophotometric method (Ellman et al., 1961). The rate of hydrolysis of acetylthiocholine was measured with 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) as the substrate. The absorbance at 412 nm was monitored continuously for 5 min. The AChE activities were defined as nanomoles of acetylthiocholine formed per minute per milligram of protein.

2.10. Cell culture and treatment

Human neuroblastoma cell line SH-SY5Y (CAMS Cell Center, Beijing, China) was maintained in Dulbecco's modified Eagle's medium (DMEM) complemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Incubations were carried out at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

2.11. Immunofluorescence analysis

SH-SY5Y cells seeded on the coverslips were treated with various concentrations of CPF, Cd, and their mixtures for 48 h. Then the cells were fixed in 4% paraformaldehyde in PBS for 15 min, washed three times with PBS, permeabilized with 1% Triton X-100 in PBS for 10 min and blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. Cells were incubated for 2 h with primary antibodies followed by washing with PBS three times. Then the slides were incubated with secondary antibodies for 1 h. Nuclei were stained with Hoechst 33258 for 15 min. The fluorescent pictures were observed and captured with the Carl Zeiss LSM710 confocal microscope (Oberkochen, Germany).

2.12. Flow cytometry

The SH-SY5Y cells subjected to various treatments were collected and rinsed twice by ice-cold PBS (0.1 M, pH 7.4). Then cells were re-suspended by 500 μl DMEM, and stained with 5 μl 10 mM Rhodamine 123 (Rh123, for measurement of mitochondrial potential) or 5 μl 20 mM DCFH-DA (for measurement of reactive oxygen species, ROS) for 30 min at 37 °C. Then the cells were washed with PBS twice and loaded on the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.13. Statistical analysis

SPSS 18.0 software (SPSS, Inc., Chicago, MI, USA) was employed for the statistical evaluation. Analysis of variance (ANOVA) test was performed to access significant difference among the groups, followed by post hoc Dunnett's test.

3. Results

3.1. Toxicological signs of the rats exposed to CPF, Cd, and their mixtures

No deaths of the treated rats were observed during the course of the present study. No signs of cholinergic side effects in the lower-dose groups of CPF were found at any point in the study. Rats exposed to high-dose of CPF displayed significant tremors and other signs of the toxicity such as piloerection and diarrhea. In addition, Cd induced no obvious toxic symptoms in rats. No significant differences in the body weight gain were observed between any treated group and vehicle control group (data not shown). However, the high dose CPF exposure caused a mild smaller body weight gain in the rats.

As expected, CPF inhibited acetylcholinesterase (AChE) activity in brain dose-dependently (Fig. 1). Cd had no effect on brain AChE activity. The mixture of CPF and Cd inhibited the AChE activity to a similar extent as CPF, indicating that Cd did not interfere with CPF's effect on AChE activity.

3.2. Effects of CPF, Cd, and their mixtures on MDA and PCO levels

In previous research, we measured metabolomic changes in the CPF-, Cd-, and their mixtures-treated groups, which revealed that energy and amino acids metabolism was disturbed by CPF and Cd

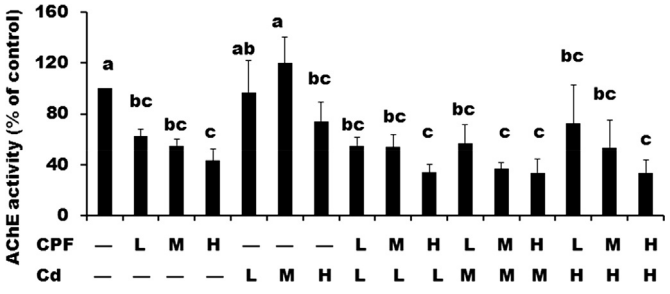

Fig. 1. The inhibition rate of acetylcholinesterase (AChE) activity in brain of rats treated with chlorpyrifos (CPF) and cadmium (Cd). The AChE activities of the control rats are 230.52 ± 7.39 nmol/mg/min. Different letters indicate a significant difference among groups ($P < 0.05$), while the same letters indicate no significant difference among groups ($P > 0.05$). Abbreviations: CPF, chlorpyrifos; Cd, cadmium; L, low dose of CPF and Cd (1.7 and 0.7 mg/kg/day); M, middle dose of CPF and Cd (5 and 2 mg/kg/day); H, high dose of CPF and Cd (15 and 6 mg/kg/day).

treatment (Xu et al., 2015). To further examine whether CPF, Cd, and their mixtures could induce the lipid peroxidation in brain, we determined the malondialdehyde (MDA) level, an indicator of lipid peroxidation. CPF and Cd induced increases in MDA level compared to the control in a dose-dependent manner (Fig. 2A). However, the effect of the mixtures was lower than the sum of the effects of individual treated groups, indicating that the joint effect of CPF and Cd on lipid peroxidation was antagonistic.

In addition, the protein peroxidation level, indicated by protein carbonyl (PCO) level, was also measured in brain of rats treated with CPF, Cd and their mixtures. We found that even low dose of CPF and Cd individual treatment induced significant increase of PCO compared to control (Fig. 2B). The effect of the mixtures was not higher than the sum of the effect of individual treated groups. In particular, the mixture of low dose CPF and Cd even did not significantly induce PCO level compared to control. This result suggested that CPF and Cd probably have an antagonistic interaction on protein oxidation.

3.3. Effects of CPF, Cd, and their mixtures on activities of catalase and superoxide dismutase

The activity of catalase (CAT) and superoxide dismutase (SOD) reflected the intracellular antioxidant capacity. Thus we measured the activity of CAT and SOD in the CPF-, Cd-, and their mixtures-treated rats. We found that the increases of CAT activity were significant in the rats from high dose CPF-treated group and low or middle dose Cd-treated groups, compared to control (Fig. 3A). Overall, the joint effect of CPF and Cd on CAT activity was an antagonistic effect.

However, the SOD activity was not altered by the individual CPF and Cd treatments. SOD activity was significantly reduced in high-dose Cd plus different doses of CPF groups (Fig. 3B).

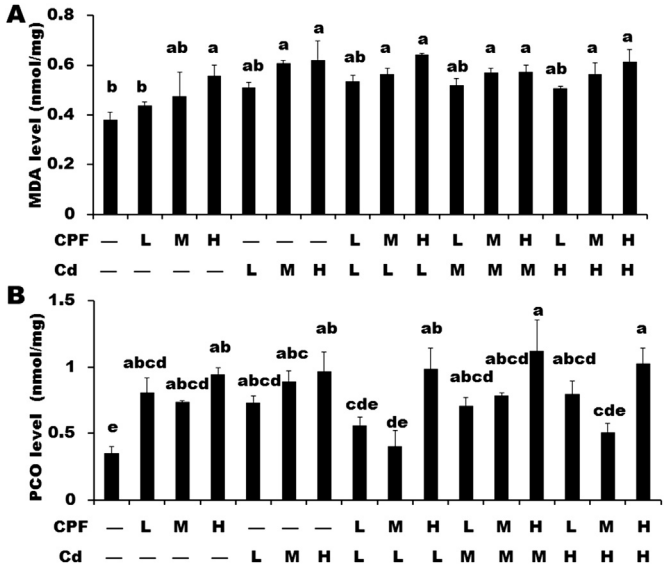

Fig. 2. Changes of malondialdehyde (MDA) level (**A**) and protein carbonyls (PCO) level (**B**) in brains of rats. Rats were administrated orally with chlorpyrifos (CPF), cadmium (Cd) at doses of 1.7 and 0.7 mg/kg/day (L), 5 and 2 mg/kg/day (M), 15 and 6 mg/kg/day (H), respectively, and their mixtures for 90 days. Data were expressed as mean ± SD and differences among different groups were evaluated by ANOVA. Different letters indicate a significant difference among groups ($P < 0.05$), while the same letters indicate no significant difference among groups ($P > 0.05$). Abbreviations: L, low dose; M, middle dose; H, high dose.

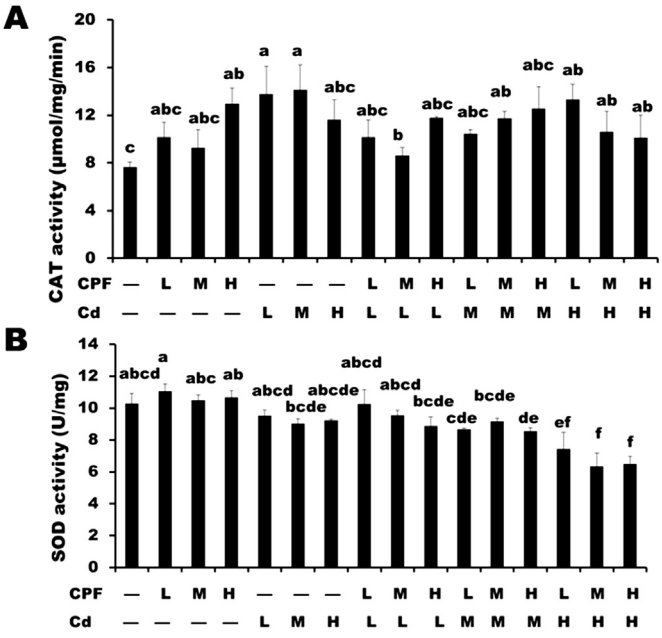

Fig. 3. Catalase (CAT) activity (**A**) and superoxide dismutase (SOD) activity (**B**) in brains of rats. Adult rats were administrated orally with chlorpyrifos (CPF), cadmium (Cd) at doses of 1.7 and 0.7 mg/kg/day (L), 5 and 2 mg/kg/day (M), 15 and 6 mg/kg/day (H), respectively, and their mixtures for 90 days. Data were expressed as mean ± SD and differences among different groups were evaluated by ANOVA. Different letters indicate a significant difference among groups ($P < 0.05$), while the same letters indicate no significant difference among groups ($P > 0.05$). Abbreviations: L, low dose; M, middle dose; H, high dose.

3.4. Changes of mitochondria in the brain of the chemicals-treated rats

Given that mitochondria damage usually played an important role in the oxidative stress, we detected the ultrastructures of mitochondria by TEM (Fig. 4). Mitochondria in control group had overall normal structure. However, mitochondria with abnormal appearance such as swelling, fragmentation, diminution, cristae disarrangement and decrease of electron density of mitochondrial matrix were found in the brain of rats exposed to high dose of CPF. Similar results were observed in the rats exposed to high dose of Cd and mixture of high dose CPF and Cd, respectively (Fig. 4). Thus the TEM observations results may suggest that CPF and Cd treatments induced mitochondria damage.

3.5. CPF and Cd induced mitochondrial damage in cultured neuroblastoma cells

In the previous study (Xu et al., 2015), we found that degenerating neuronal cells were significantly induced by CPF and Cd treatment. Thus, we sought to further investigate whether CPF and Cd could cause mitochondria damage in cultured neuroblastoma SH-SY5Y cells. As shown in Fig. 5, the mitochondria in untreated cells formed a reticular network surrounding the nucleus. CPF and Cd treatment, even at relative lower concentrations, induced the formation of puncture-like mitochondria structures, indicating a disintegration of the mitochondria network (Fig. 5).

Using flow cytometry, we detected a decrease of mitochondrial potential in both CPF- and Cd-treated SH-SY5Y cells in a dose-dependent manner (Fig. 6A). However, the mixture of CPF and Cd did not induce a further decrease of mitochondrial potential, indicating a potential antagonistic interaction between CPF and Cd.

In addition, ROS levels in SH-SY5Y cells were measured with

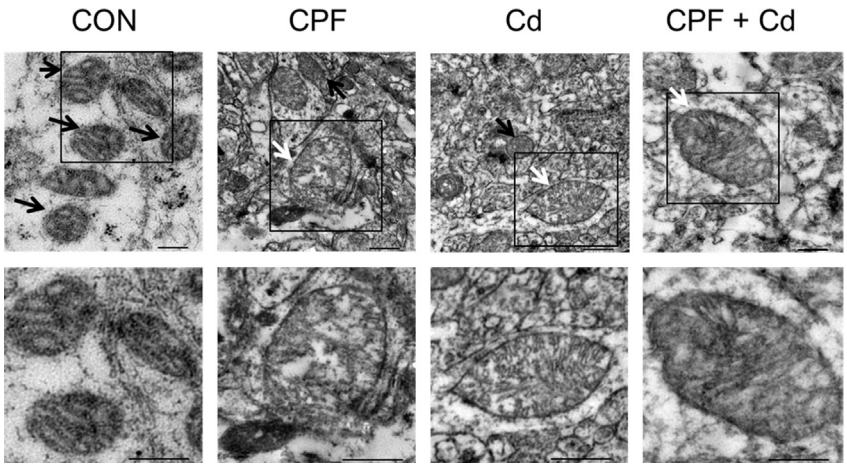

Fig. 4. Electron microscopy images of mitochondria in brain tissue of rats. The rats were treated with vehicle, CPF at high dose of 15 mg/kg/day, Cd at high dose of 6 mg/kg/day, and CPF plus Cd (CPF + Cd) at their high doses, respectively, for 90 days. The prefrontal cortex of the brain was dissected, fixed, sectioned, and then examined by transmission electron microscopy with five fields for each of the three sections of one sample. One representative image (with enlarged image for the framed region in the lower panel) was shown for each group. Only cells with prominent neuronal cell morphology were chosen. White arrows indicate probably damaged mitochondria, and black arrows indicate mitochondria with overall normal morphology. Scale bar = 500 nm. Abbreviations: CON, control (vehicle); Cd, cadmium; CPF, chlorpyrifos.

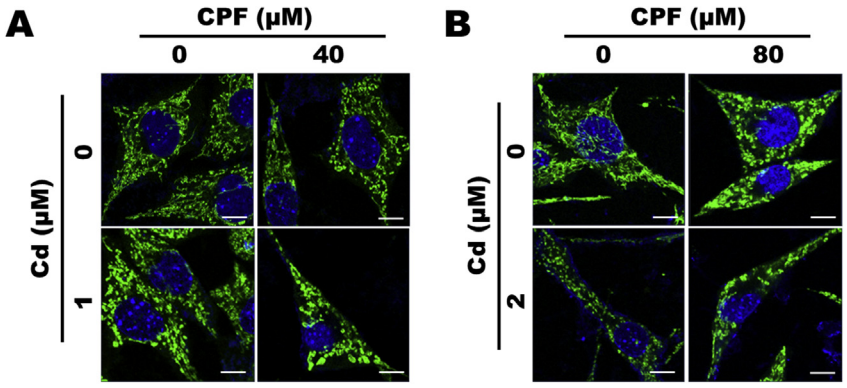

Fig. 5. Mitochondrial morphology was examined by visualizing the distributions of cytochrome c immunostaining (cyt C- green) after SH-SY5Y cells were treated for 48 h with either 40 μM CPF, 1 μM Cd, and their mixture (**A**), or 80 μM CPF, 2 μM Cd, and their mixture (**B**). Nuclei were counterstained with Hoechst 33258 (blue). Abbreviations: CPF, chlorpyrifos; Cd, cadmium. Scale bar = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

flow cytometry. As shown in Fig. 6B, consistently with *in vivo* results, ROS was induced by both CPF and Cd treatment. Although the mixture of CPF and Cd induced a higher increase of ROS, the difference between the mixture and individual compound treatment was not significant. Overall, the cell culture results suggest that CPF and Cd could induce ROS and mitochondria damage in neuroblastoma cells with a potential antagonistic interaction.

4. Discussion

Both chlorpyrifos (CPF) and cadmium (Cd) are common environmental pollutants and could induce neurotoxicity. In the present study, we investigated the combined toxicity of CPF and Cd in the brain of rats. The obvious toxic symptoms of CPF, but not Cd, were observed in rats treated with CPF at high-dose for 90 days. The dosage from the low- and middle-doses of CPF and Cd groups were too low to cause obvious toxic signs. In this study, we found that CPF, Cd and their mixtures induced the prominent oxidative damage in the rat brain. CPF and Cd, even at low dose, could induce the lipid peroxidation and protein oxidation. Although Cd did not affect the inhibition of acetylcholinesterase activity by CPF, these two compounds displayed a potential antagonistic interaction on the induction of oxidative stress in brain.

The low doses of CPF and Cd used in our study (1.7, 0.7 mg/kg/d, respectively) were chosen based on human occupational and environmental exposure to CPF and Cd. The calculation was described as below. It was reported that the maximum residue of CPF in various vegetables and fruits in some places of China was 3.47 mg/kg (Health Council of the Netherlands, 2003). In addition, atmospheric levels of chlorpyrifos (CPF) in airborne of the working environment ranged from 0.012 to 0.145 mg/m^3 (Yuan et al., 2014). It is known that one person breaths 20 m^3 air every day (European Medicines Agency, 2007). Therefore, the average level of CPF intake from air was about 0.077 mg/kg/d for humans with average 60 kg body weight (the average level of CPF = $[0.145 \times 20 + 3.47 \times 0.5] \div 60 = 0.077$ mg/kg/d). In China, food intake was one of the main sources of Cd exposure. The concentrations of Cd residue in the rice in some areas in China, which were grown in soil contaminated with Cd, were 22 times higher than the national standard (0.2 mg/kg) (Brigden et al., 2014). The amount of daily intake was about 0.5 kg of food (He and Zheng, 2010). Therefore, the average level of Cd intake was about 0.037 mg/kg/d for humans with average 60 kg body weight (the average level of Cd exposure = $22 \times 0.2 \times 0.5 \div 60 = 0.037$ mg/kg/d). According to guidelines for extrapolation of human doses to animal doses, animal dose (mg/kg) = human equivalent dose (mg/

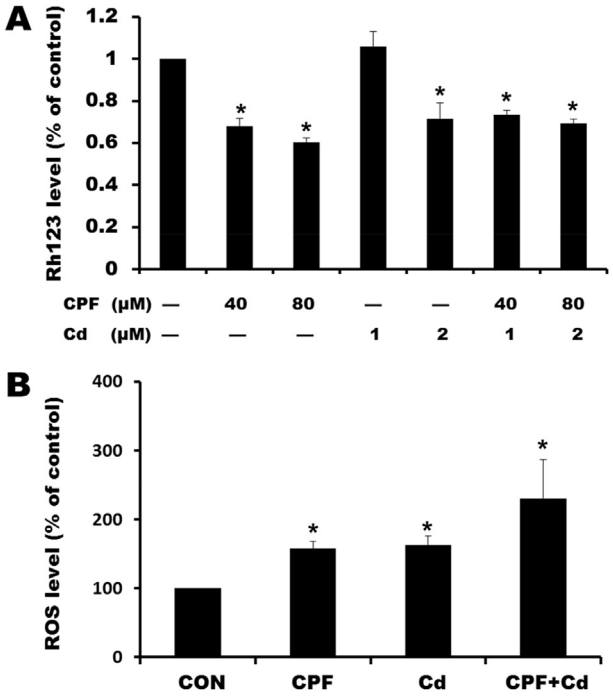

Fig. 6. The changes of mitochondrial membrane potential **(A)** and ROS levels **(B)** in SH-SY5Y cells. The cells were treated with 40 or 80 μM chlorpyrifos (CPF), 1 or 2 μM cadmium (Cd) and their responsive mixtures for 48 h. Then the cells were collected and then stained with Rhodamine 123 (Rh123, for measurement of mitochondrial potential) or DCFH-DA (for measurement of reactive oxygen species, ROS), respectively. The fluorescence intensity was measured by flow cytometer. The basal fluorescence intensity for mitochondria membrane potential and ROS levels in the cells treated with neither CPF nor Cd are 1223.1 ± 73.2 and 565.0 ± 69.9, respectively. * indicated significant difference from the control ($P < 0.05$).

kg) × (human Km/animal Km), where the Km value for a 0.25 kg rat and a 60 kg human is 6 and 37, respectively (National Health Research Institute, 2016). Therefore, the equivalent dose of CPF in rats = 0.077 × (37 ÷ 6) = 0.47 mg/kg/d. The equivalent dose of Cd in rats = 0.037 × (37 ÷ 6) = 0.23 mg/kg/d. Thus, the low doses of CPF and Cd used in our study (1.7, 0.7 mg/kg/d, respectively) are close to the human occupational and environmental exposure to CPF and Cd.

Oxidative damage plays an important role of tissue damages caused by a wide variety of toxicant (Berlett and Stadtman, 1997; Griendling and FitzGerald, 2003). Malondialdehyde (MDA) is the major product of peroxidized polyunsaturated fatty acids (Marnett, 2002). The increased level of MDA in the brain of rats treated with CPF, Cd and their mixtures indicated the lipid peroxidation in the brain. The plasma membranes of brain cells contained high level of oxidizable polyunsaturated fatty acids (Gupta, 2004). CPF, Cd and their mixtures were likely to cause oxidative stress to damage the polyunsaturated fatty acids and resulted in the lipid peroxidation. Protein peroxidation could diminish the protein functions (Berlett and Stadtman, 1997). Protein carbonyls (PCO), the marker of the protein peroxidation, were produced by the oxidation of the protein backbone, amino acid side-chains, or by reaction with alkenes (Levine et al., 1990). The increased PCO levels suggest that CPF, Cd, and their mixtures induced the protein peroxidation, which may result in neuron death. Thus lipids and protein in the brain may be the sensitive targets of both CPF and Cd. Although individual CPF and Cd could induce the damage of lipids and protein, the mixtures of CPF and Cd did not aggravate the damage of lipids and proteins in the brain compared to the individual chemical treatments.

CAT and SOD, the antioxidant enzymes, were highly expressed in the central nervous system (Mokni et al., 2007). Our results indicated that in the brains, there was a significant elevation of the CAT activity in the CPF-, Cd-, and their mixtures-treated rats, while SOD level was decreased in the rats exposed to the high-dose of Cd plus CPF. The increase in CAT activity is likely a compensatory effect in response to the increased oxidative damage induced by CPF, Cd, and their mixtures, because the compensatory up-regulation of CAT protected organisms against oxidative stress. SOD, which contains both copper and zinc, could convert O_2^- to H_2O. Cadmium could decrease the Zn^{2+} content in the Cu, Zn-SOD protein and change the protein conformation to decrease the SOD activity (Huang et al., 2006). CPF could produce ROS to decrease SOD activity, which was likely to increase the oxidative stress induced by the high dose of Cd.

ROS generated from mitochondria could cause further damage to mitochondrial components (Cadenas and Davies, 2000). When the structures of mitochondria were damaged, the function of mitochondrial respiratory chain was disrupted and then more serious oxidative damage was induced (Indo et al., 2007; Marchi et al., 2012). In the present study, we found mitochondria had structural damages such as swelling, fragmentation, diminution, cristae disarrangement and decrease of electron density of mitochondrial matrix in the brain cells of the treated rats. In addition, CPF and Cd could cause mitochondrial damage and induce ROS in the cultured neuroblastoma cells. These results suggest that CPF, Cd, and their mixtures could probably directly induce the damage of mitochondria and oxidative stress in brain neuronal cells.

In conclusion, our present study showed that CPF, Cd, and their mixtures generated oxidative damage and disturbed the total antioxidant capability in the brain. Lipids and proteins in the brain were sensitive targets of the CPF and Cd. The oxidative damage of CPF and Cd on the brain were relatively similar. The joint effect of CPF and Cd was lower than the sum of their individual effects on the lipid peroxidation and protein oxidation in rats' brain. However, the detailed mechanism underlying their antagonistic interaction merits further investigated.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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