Alteration of mice cerebral cortex development after prenatal exposure to cypermethrin and deltamethrin

Junnan Guo⁎, Jinzhong Xu⁎, Junshi Zhang⁎, Lei An⁎,a,c

⁎ Corresponding authors at: Department of Neurology, Huaibe Hospital of Henan University, Kaifeng, 475000, Henan, China
a Department of Neurology, Huaibe Hospital of Henan University, Kaifeng, 475000, Henan, China
b Pediatric Surgery, Children’s Hospital of Kaifeng City, Kaifeng, 475000, Henan, China
c Translational Medicine Center, Huaibe Hospital of Henan University, Kaifeng, 475000, Henan, China

ARTICLE INFO

Keywords:
Pyrethroids
Cerebral cortex
Prenatal
Cell cycle
Progenitor

ABSTRACT

Pyrethroids, a group of insecticides with high efficiency, low toxicity and wide spectrum, are used for pest control in agriculture. Here, we administered two representative pyrethroids (cypermethrin and deltamethrin) and an equal volume of vehicle (corn oil) to the pregnant ICR mice. This study investigated the effects of cypermethrin and deltamethrin on cerebral cortex development in mice as well as possible mechanisms in proliferation and differentiation. The results showed that histopathologic change did not occurred in the cerebral cortex using Hematoxylin and Eosin staining, however, the observation of fetuses exposed to cypermethrin and deltamethrin revealed reduction of neuronal proliferation, maturation and differentiation. Moreover, cypermethrin/deltamethrin-induced apoptosis of nerve cell was significantly higher in treated groups than that in control group by using flow cytometry, Western blot and TUNEL. It was worth mentioning that the newborns exposed to cypermethrin and deltamethrin did not showed abnormal neuronal distribution. These findings suggested that prenatal cypermethrin and deltamethrin exposure impaired corticogenesis.

1. Introduction

Chemically synthesized pyrethroids, similar to the natural pyrethroids because of their chemical structure, can affect the function of organs through neurotoxicity (Crago and Schlenk, 2015), endocrine disruption (Jin et al., 2015), abnormal development (Jin et al., 2009) and reproductive toxicity (Wang et al., 2009) in animals. It is now widely used in agricultural pest control and household pest cleanup. The pyrethroids can be classified into type I and type II, and cypermethrin (CP) and deltamethrin (DM) are common pyrethroid II pesticides used worldwide in agriculture, home pest control and disease vector control. In addition, they are considered as an insecticide closely related to human health and food safety (Jin et al., 2015; Singh et al., 2012).

CP and DM accumulate in soil, and traces of them may appear in vegetables, tea, fruits and other foods. CP and DM also have stomach toxicity and brain toxicity (Ncir et al., 2017; Singh et al., 2012). Although long-term exposure to low dose of CP is not enough to cause obvious symptoms of poisoning, the potential damage for reproduction cannot be ignored because of accumulation (Muangpha et al., 2015). CP and DM cause morphometric and structural changes in the genital organs by reducing the number of follicular cells, oocytes and corpora lutea through dose-dependent effects (Marettova et al., 2017; Petr et al., 2013). The previous studies showed that relative toxic potency of six individual pyrethroids for cortical neurons was followed by beta-cyfluthrin, lambda-cyhalothrin, deltamethrin, cypermethrin, bifenthrin and permethrin by disrupting voltage-gated sodium channels and altering cell excitability (Chen et al., 2017; Johnstone et al., 2017; Mohana Krishnan and Prakhya, 2016). In addition, pyrethroids were correlated with carboxylesterase metabolism in liver (Anand et al., 2006), and next generation sequence was used to identify differentially expressed genes for precise molecular mechanisms (Mamidala et al., 2012; Zimmer et al., 2017). Although DM and CP are widely used in human activities, the mechanism of them on cortical neurogenesis remain unclear, so we want to investigate the effect of DM and CP on neuronal progenitor proliferation, cell maturation, neuronal differentiation, apoptosis and neuronal migration in mammalian.

Our results demonstrated that CP/DM exposure inhibited the proliferation of neural precursor cells and neural stem cells, and promoted cell apoptosis in vivo and in vitro. The cell fate decision of newborn neurons was affected by CP and DM, respectively. These findings may be helpful for understanding the neurotoxicity mechanisms of pyrethroids.
2. Materials and methods

2.1. ICR mice

The mice were used and all procedures were performed according to the institutional guidelines for animal experiments. The day of vaginal plug detection was considered as gestation day 0.5 (E0.5), and the day of birth was designated as postnatal day 0 (P0). Schematic structure and the procedure in this study showed in Fig. 1, especially, purpose of the part A was to determine for cell proliferation and part B was to determine neuronal migration and apoptosis. All the animal experiments were performed according to the guidelines for the care and use of laboratory animals of Huaihe Hospital of Henan University.

2.2. Drug treatment

To assess the effect of CP/DM on cell proliferation in the VZ (Ventricular zone) and SVZ (subventricular zone) of cerebral cortex, mice were randomized into CP/DM and control groups (6 mice per group). Mice in each group then received intragastric administration of either CP/DM (1.2 mg/kg) or an equivalent volume vehicle (corn oil) from E10.5 to E15.5, and the dose was selected after observing toxicity signs with no death refer to previous studies (Cao et al., 2015; Ogaly et al., 2015). All animals were intraperitoneal (i.p) injected with 5-Bromo-2-Deoxyuridine (BrdU, 50 mg/kg) at E15.5 (Fig. 1), and sacrificed at 2 h after injection. All samples from each group were sacrificed and collected for cell cycle exit analysis.

To determine whether or not the CP/DM can affect neuronal migration at this dosage, the mice were randomized into control and CP/DM groups. Mice received intragastric administration of either vehicle or CP/DM from E10.5 to E16.5. Timed pregnant mice at E15.5 received a single intraperitoneal injection of BrdU (50 mg/kg) and mice were sacrificed at E18.5 or P1 after BrdU injection.

2.3. Tissue preparation

Postnatal mice were deeply anesthetized with sodium pentobarbital and perfused intracardially with 4% paraformaldehyde (PFA) in a 0.1 M phosphate buffer at a pH of 7.2-7.4. Brains were extracted and sections were sliced for 50 mm coronal sections. All brains were fixed overnight in 4% PFA at 4 °C for at least 24 h, embedded with O.C.T. (Sakura Finetek) on dry ice and ethanol slush.

2.4. Chemicals and antibodies

Deltamethrin (DM, CAS: 52918-63-5) and Cypermethrin (CP, CAS: 52315-07-8) were purchased from J&K chemical, China. BrdU (CAS: 59-14-3, Sigma) and Propidium Iodide (CAS: P4170, Sigma) were
purchased from Sigma Aldrich (USA). F12K culture, Opti-MEM culture and Fetal bovine serum (FBS) were purchased from Gibco (USA); mouse monoclonal anti-Ki67 (1:1000, 556003) was purchased from BD Pharmingen (USA); mouse monoclonal anti-NeuN (1:500) was purchased from Merck Millipore (MAB377; USA); Rabbit monoclonal anti-Pax6 (1:1200) was purchased from MBL (PD022; USA); rabbit monoclonal anti-Tbr1 (ab31940) and rabbit monoclonal anti-Tbr2 (ab23345) were purchased from Abcam (USA); goat anti-mouse-Alexa Fluor 488 (1:300); goat anti-Rabbit-Alexa Fluor 568 (1:300) and goat anti-mouse-Alexa Fluor 568 (1:300) was purchased from Invitrogen (USA).

2.5. Immunohistochemistry

Preparation of coronal slices of cerebral cortex and immunohistochemistry were performed as described previously (An et al., 2014). Briefly, mice brains were removed and fixed in 4% paraformaldehyde (PFA), then brains were cut at coronal cryostat sections. Sections were processed for immunostaining by a free-floating protocol. The sections were incubated with primary antibody diluted in solution I (1% BSA in 0.1M PB containing 0.1% Triton X-100) overnight at 4°C; after washing in 0.1M PB for 30 min, the sections were incubated with...
secondary antibody diluted in solution II (0.1M PB containing 0.1% NaN3) for 3 h at Room Temperature; finally, propidium iodide (PI) was used for nucleus staining.

Hematoxylin and Eosin (HE) strain was used to check changes of pathological structure in this study. Briefly, the brains were immersed in 4% paraformaldehyde for 6 h and transferred to 70% ethanol. Individual samples were placed in processing cassettes, dehydrated through a serial alcohol gradient, and embedded in paraffin wax blocks. Before staining, the tissue sections were dewaxed in xylene and rehydrated through decreasing concentrations of ethanol. After staining, samples were dehydrated through increasing concentrations of ethanol and xylene.

2.6. Cell cycle analysis

Cell cycle analysis of cerebral cortex and immunohistochemistry were performed as described previously (Naveau et al., 2014). The thymidine analogue BrdU labels cells in S-phase of the cell cycle at the time of injection. pHH3 also allows mitotic cells distinguished from apoptosis. Ki67 is absent in quiescent cells (G0) but is present in active phases of the cell cycle (G1, S and G2).

2.7. Cell lines

We treated PC12 cells in vitro with 1 μM DM and 100 μM CP, respectively (Huang et al., 2016; Ihara et al., 2017; Wu et al., 2003). The PC12 cells were cultured in DMEM (Gibco, USA) containing 10% (v/v) inactivated calf serum 5% and inactivated horse serum (Hyclone, USA), respectively.
100 U/mL penicillin, 100 μg/mL streptomycin at 37 °C in a humidified 5% CO2 atmosphere. The culture medium was changed every 2–3 days and apoptosis of the PC12 cells was assessed with the Annexin V-FITC/PI cell apoptosis detection kit according to manufacturer’s instructions. Using flow cytometry, data for early phase (LR) and late phase (UR) were analyzed.

2.8. Tunel staining and western blot

DNA fragmentation in apoptotic cells can be detected by terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling (TUNEL). The TUNEL assay relies on the presence of nicks in the DNA which can be identified by TdT, an enzyme that catalyzes the addition of dUTPs that are secondarily labeled with a marker. Briefly, after fixation and permeabilization, the slices were fixed with 4% paraformaldehyde for 20–30 min at RT and then treated with PBS 2–3 times. TUNEL was performed with One Step TUNEL Apoptosis Assay Kit according to manufacturer’s instructions (Sangon Biotech, China).

Mice brain protein lysate were used to investigate apoptosis relative protein, Bax and Bcl-xl. Cellular protein was extracted in lysis buffer (50 mM Tris-HCl, 0.5% Triton X-100, 2 mM EDTA, and 150 mM NaCl; pH 7.3) with 1 mM phenylmethanesulfonyl fluoride. Western blot for...
Bax (Millipore, USA), Bcl-xl (Millipore, USA) and GAPDH (Santa Cruz, USA) was performed as previously described (An et al., 2014).

2.9. Statistical analysis

All data are presented as mean ± SEM. Two-tailed Student’s t-tests were used for statistical comparisons between two groups, and one-way ANOVA followed by post hoc Newman-Keuls test was used for comparisons between more than two groups. Tests were carried out using GraphPad Prism 5.0 software. Statistically significant differences were considered if \( p < 0.05 \) (**), \( p < 0.01 \) (###) or \( p < 0.001 \) (****/#####).

2.10. Equipment

All immunofluorescence staining images presented were acquired using Olympus FV1000 confocal microscope system (Japan). Confocal images were reconstructed using the FV10-ASW software and cropped, adjusted and optimized in Photoshop CS5. BD FACSVerse flow cytometer (USA) and Leica CM1950 slicer (Germany) were used for the study.

3. Results

3.1. Alteration of histomorphology in CP/DM-treated dams and cubs

In this study, Schematic diagram of the experimental protocol was showed as Fig. 1. Experimental schedule to investigate the effect of CP/DM on cell proliferation of newly generated cells in the cerebral cortex and neuronal migration. Firstly, the number and the weight of live dams and offspring were counted in E16.5 and P1, then we could get the ratio (b/a and B/A) of body (g.b at E16.5 and g.B at P1) and brain (g.a at E16.5 and g.A at P1), respectively. The main purpose of this part was to determine whether CP/DM influence the development of offspring during cerebral cortex development. There was significant difference in live body weight of dams between control mice and mice treated with CP (**\( p < .01 \)) or DM (##\( p < .01 \)) in E16.5 but not P1.

In addition, number of live fetuses per dam did show significant difference between the control group and group treated with CP (***\( p < .001 \)) and DM (#\( p < .05 \)) at E16.5. Similar results occurred in CP (\( p < .05 \)) and DM (#\( p < .05 \)) at P1, respectively (Fig. 2A). However, no significant difference in body/brain ratio was found between control mice and mice treated with CP/DM at E16.5 and P1. Moreover, HE stains analysis did not show any significant difference between treated groups and the control group (Fig. 2B). The histopathological findings suggest that CP/DM did not cause the obvious changes of pathological structure during cortical development, but our results indicated that the pregnant mice had toxic symptoms (not shown).

3.2. CP/DM decreased neuronal progenitor proliferation and promoted cell cycle exit

To determine whether neurons treated with CP/DM are able to effect cell proliferation of neurogenesis in subventricular zone (SVZ), confocal analysis was performed as Fig. 3. Ki67 is a cellular marker for proliferation and increases cell progression in S phase of the cell cycle. Here, the effect of CP/DM on progenitor proliferation in the developing cerebral cortex was studied using Ki67 and pH3H after BrdU injection 2h. The average number was remarkable difference (**\( p < .001 \), Fig. 3G) in BrdU positive cells between control group (Control, 148.09, n = 14) and treated groups (CP, 106.58, n = 14; DM, 108.76, n = 14). In addition, the average number of the Ki67 positive cells in VZ/SVZ was difference (CP, **\( p < .01 \) and DM, *\( p < .05 \)) between control group (Control, 41.14, n = 14) and treated groups (CP, 32.23, **\( p < .01 \), n = 12 and DM, 34.78, *\( p < .05 \), n = 12, Fig. 3H). Interestingly, most of the pH3H labeled cells were not affected in treated groups with CP/DM (ns, not significant between control group and treated groups, Fig. 3I).

Since cell proliferation was decreased by exposure to CP/DM, we evaluated cell cycle exit index. The ratio of BrdU positive and Ki67-
percentage of Pax6 was obvious higher than control (Fig. 5B1–B3 and C), which indicated CP/DM delayed neuronal proliferation of stem cell and precursor cell (Control, 15.96%; CP, 21.26%; DM, 21.35%; \( *p < .05 \)). In addition, the marker Tbr1 and Tbr2 were used to analysis cell differentiation, and the relative percentage of Tbr2 (Fig. 5D1–D3 and E) was significate decreased comprising to control group (Control, 32.07%; CP, 19.75%; DM, 19.12%; \( **p < .01 \)). Meanwhile, consistent with Tbr2, the relative percentage of Tbr1 (Fig. 5F1–F3 and G) was decreased comprising to control group (Control, 30.98%; CP, 21.16%; DM, 21.76%; \( *p < .05 \)). These results indicated CP/DM delayed maturation and neuronal differentiation of stem cell and progenitor cell. To examine the phenotype of mature cells, double immunohistochemical staining for BrdU and NeuN was carried out (Fig. 5H1–H3 and I). In control mice, about 90.91% of BrdU positive cells in the cortical plate were co-labeled with NeuN (a maker of mature neuron), in contrast, only 81.15% of BrdU-positive cells were NeuN-positive in CP group and 82.47% of BrdU-positive cells were NeuN-positive in DM group. Interestingly, the percentage of BrdU and NeuN double positive cells was lower in the CP/DM mice than in control (Fig. 5I), indicating that pyrethroids indeed delayed maturation of neurons in mammalian, and pyrethroids impaired the fate decision of newly generated cells during cortical neurogenesis.

3.4. Both CP and DM induced apoptosis in vivo and in vitro

The PC12 cell lines (a common nerve cell line) were treated with CP/DM to examine the effect of apoptosis. Annexin V is one of the most sensitive indexes for detecting early apoptosis, while PI can stain apoptotic nuclei in the middle and late stages. As Fig. 6 showed, the PC12 apoptosis rate in the CP/DM treatment group increased no significant compared to that of the control group during early phase (CP, Q1-UR = 15.11%; DM, Q1-UR = 14.45% and Control, Q1-UR = 14.18%). However, during late phase, the apoptosis rate of the CP and DM treatment group increased and the difference between the treatment group (CP, Q1-UR = 16.22% in Fig. 6B and DM, Q1-UR = 16.32% in Fig. 6C) and control group (Q1-UR = 3.46% in Fig. 6A) was extremely significant (\( p < .01 \), Fig. 6D). Western blot analysis showed that CP/DM increased the expression of Bax (\( p < .05 \)) but reduced the expression of Bcl-xl (\( p < .05 \)) in control (n = 6). In addition, CP/DM-induced apoptosis was detected using TUNEL. Arrows indicated the apoptosis cells in control group, CP group and DM group. Analysis of apoptosis cells in vivo as Fig. 6H showed (cells/mm\(^2\), \( *p < .01 \)), these results showed that apoptosis was affected by prenatal exposure to CP/DM in vivo and in vitro.

3.5. Neuronal migration was not affected by CP and DM

During neuronal migration, newly generated postmitotic neurons have the ability to enter into cortical plate (CP) from ventricular zone (VZ) to the intermediate zone (IZ), converting into a multipolar morphology. To determine whether neurons treated with CP/DM are able to affect neuronal migration, confocal analysis was performed at E18.5 by using BrdU maker. In each group we compared the percentage of neurons in CP, IZ and VZ/SVZ in the neocortex, individually. Cell number of migration was quantified in the VZ/SVZ (n = 15), IZ (n = 15) and CP (n = 15) at three days after BrdU injection (E15.5) by PI/BrdU double positive makers. Compared with control group (VZ/SVZ, 52.60%; IZ, 32.89% and CP, 21.16%), we did not observe any effect of exposure to cypermethrin (VZ/SVZ, 51.86%; IZ, 33.89% and CP, 14.04%) and deltamethrin (VZ/SVZ, 52.30%; IZ, 33.68% and CP, 13.94%) groups, which indicated that neuronal migration was not affected by prenatal exposure to CP/DM (Fig. 7).
4. Discussion

The maximum residue limits for different pyrethroids have been made in China, although both United Nations Food and Agriculture Organization and the World Health Organization made strict limits in vegetables and fruits (Pang et al., 2000). Pyrethroid exposure remains an important public health problem, especially for chronic toxicity. Previous studies showed that exposure to pyrethroids can induce various neurotoxic symptoms upon central nervous system, such as numbness, seizure and memory impairment (Kannarkat et al., 2015; Ray and Fry, 2006). Although some neurotoxic cases after acute or chronic intoxication of pyrethroids have been reported (Christen et al., 2017), there are no reports that alteration of neural stem cell and cortical development after exposure to pyrethroids, for example, cypermethrin and deltamethrin. Herein, we investigated how CP and DM affect neurogenesis in neural stem cell as well as the effects of them on apoptosis and differentiation.

Pyrethroids have also slowly neurotoxicity on motor neuron disorder (MND) via prolongation of the kinetics of voltage-gated sodium channels, for instance, clinical neurophysiologic studies indicated that both upper and lower motor neuron signs in bulbar, cervical and lumbosacral regions were involved chronic pyrethroid intoxication, resulting to ALS (Doi et al., 2006), tongue spasms and MND (Ahdab et al., 2011). In the present study, body weights of dams and offspring were impaired after exposure to CP/DM, our results proved that CP/DM induced the decreased number and the weight of live dams. CP/DM could reduce the survival rate of pups significantly while pathological change was not obvious with mild toxic symptoms.

Neurogenesis in the cerebral cortex is involved neuronal progenitor division, proliferation and cell cycle exit (Naveau et al., 2014). Previous studies found that DM exposure induced apoptosis in hippocampal precursor, neurodegeneration and cognitive dysfunction (Hossain et al., 2015). Similarly, DM also impaired granule cell and Purkinje cell migration, inhibition of neurite outgrowth and caused motor coordination deficits (Kumar et al., 2013). Therefore, we want to verify whether neurogenesis dysfunction caused by CP and DM. BrdU and Ki67 double labeling were used to identify the cell cycle exit index and the exit index was performed as previous study (Naveau et al., 2014). As an excellent marker of by cell cycle and proliferation, BrdU can incorporate into cells during cell division. From early prophase through metaphase, anaphase and telophase, pHH3 also allows mitotic cells distinguished from apoptosis. Ki67 is absent in quiescent cells (G0) but is present in active phases of the cell cycle (G1, S and G2). Thus, our results indicated that CP/DM elevated the cell cycle exit index on stem cells and progenitor cells in the developing cerebral cortex, indicating neurogenesis dysfunction.

Neurogenesis in mammalian brain occurs throughout life-cycle in mice, and has been clearly demonstrated in two different regions in brain: the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and the subventricular zone (SVZ) in the anterior lateral ventricles (Li et al., 2017). In the present study, maturely neuronal marker NeuN was used for identify neuron differentiation (Guselnikova and Kozhevskiy, 2015). The relative percentage of BrdU+/NeuN+ mature cells were altered in CP, these results presumably reflected the abnormal maturation of the CP. Furthermore, we also found that the number of neurons in Tbr1+ and Tbr2+ decreased in VZ/SVZ while the number of PAX6+ and BrdU+ in SVZ/VZ increased. It has been reported that newborn Pax6 neurons were preferentially generated in radial glia cell in ventricular surface, (Englund et al., 2005). The Tbr2 was expressed by a subpopulation of neuronal progenitors in the VZ/SVZ and Tbr1 was detected in postmitotic projection neurons (Heven et al., 2001). Results demonstrated that progenitor cells in the neo-cortex were more sensitive in response to the neurotoxicity induced by CP/DM from Pax6, Tbr2 to Tbr1. The reduction of radial glia, IPC and postmitotic neurons observed in this study indicates that CP/DM exposure could lead to cerebral cortex dysfunction. In a word, these results showed that CP/DM delayed progenitor cell maturation and neuronal differentiation.

Annexin V can be used to detect early apoptosis by binding to phosphatidylserine and PI can penetrate late-apoptotic cells but not the early apoptotic cells. The combination of the Annexin V and PI can separate different apoptotic periods. Previous studies on aquatic animals showed that CP was harmful for the organs with inflammation, DNA damage and apoptosis. Several candidate genes involved apoptosis were changed significantly, for instance, caspase 3, Bax, Bcl, p53 and inducible nitric oxide synthetase (iNOS) (Arslan et al., 2017). DM-induced hepatoportal toxicity was confirmed with studies on expression changes of bel-2 (Maaelej et al., 2017), and the toxicity was identified through the oxidative status and inflammation. Agreeing to previous results, the apoptosis rate of the CP treatment group increased during late phase rather than early phase in PC12 cells, the findings are consistent with DM treated cells. These results on CP and DM indicated that pyrethroids induced cell apoptosis with neurotoxicology during late-phase. Furthermore, the results of western blot and TUNEL to detect the apoptosis. Our results showed that the expression of Bax was significantly increased after treated with CP/DM, while the relative expression level of Bcl-xl was decreased. Also, the results of TUNEL and WB suggested that the treatment of CP/DM induced cell apoptosis. These results were in line with previous researches in vitro (Wu et al., 2003; Huang et al., 2016).

The mammalian cerebral cortex is a six-layered structure. The newly generated postmitotic neurons enter into cortical plate from VZ to final destinations (MZ) during development (An et al., 2014). We observed that neuronal migration treated by CP or DM was not altered. This phenomenon might be the mechanism through which pyrethroids decrease neuronal proliferation, maturation and differentiation. Furthermore, more techniques, like RNA sequencing, will be our next steps to identify causative genes and possible mechanisms.

5. Conclusion

Our results demonstrated that CP and DM exposure inhibited neurogenesis of neural precursor cells and promoted apoptosis. These findings may be helpful for understanding neurotoxicity of pyrethroids, and prevent its toxicity on infants in mammalian.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by Natural Science Foundation of Education Department of Henan Province (number: 16A320003) and Project of Henan University Science Research (number: 2015YBZR010).

References


Petr, J., et al., 2013. Pyrethroids cypermethrin, deltamethrin and fenvalerate have different effects on in vitro maturation of pig oocytes at different stages of growth. Animal 7, 134–142.


