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An in vivo study in mice: mother gestational exposure to organophosphorus pesticide retards division and migration process of neural progenitors in fetal developing brain

Xiao-Ping Chen, * Ting-Ting Wang, Xiu-Zhong Wu, Da-Wei Wang, Yong-Sheng Chao

Department of Biotechnology, College of Biological Engineering, Zhejiang University of Technology, Hangzhou, China

Running Title: *Gestational pesticide disrupts fetal progenitor*

Corresponding:

Xiao-Ping Chen

Department of Biotechnology,

College of Biological Engineering,

Zhejiang University of Technology,

18 Chao-Wang Road, Hangzhou, 310032, China

Tel: +86-571-88320823

Fax: +86-571-8832-867

E-mail: chxp@zjut.edu.cn

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Abstract

Background: Widely utilized pesticides such as chlorpyrifos (CPF) can cause cognitive abnormalities, neurotransmitter disruptions and brain cytoarchitecture deficits in adulthood after exposing in prenatal period, but the mechanism underlying the development and maintenance of such neurotoxicity in embryonic neurogenesis remains largely unclear. Using embryonic neocortex slices, we investigated mitosis population constituents and characteristic interkinetic nuclear migration (INM) to evaluate CPF effects on proliferation process of neural progenitors. *Methods:* Gestational days (GD) 14 and GD 7.5-11.5 ICR dams were exposed to 5 mg/kg of CPF to investigate immediate toxicity and sustained toxicity. Proliferating nuclei were labeled with 50 mg/kg of Brdu at 1, 3, 6 and 9 hours before samples were collected. Mitoses count and Brdu positive nuclei (BPN) location were measured and analyzed in standard sections of embryonic dorsolateral cortex. *Results:* CPF reduced mitoses count in the primary progenitors but not in the secondary progenitors with time sustained. CPF retarded BPN migration with a 6-9μm delay of relative location in the immediate groups and a 3-6μm delay in the sustained ones. CPF had no or little effects on the global mitoses count and BPN count. *Conclusion:* Prenatal CPF exposure disrupts the proliferation process of primary progenitors in the embryonic dorsolateral cortex immediately and with sustained effects, which may contribute to explain the toxicity mechanism in early neurogenesis.

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1. Introduction

One of the most common reasons of embryonic brain malformation is environmental toxin exposure in ordinary life. Organophosphorus compounds are well known toxins which inhibit cholinesterase activity in animals and induce cell apoptosis in developing brain.[1,2] For example, a popular organophosphorus pesticide with trichloro-pyridyl (Fig.1), Chlorpyrifos (CPF), was reported to cause developmental neurotoxicity after prenatal or neonatal exposing. [2,3] During neural tube stage to nursing stage, CPF exposure on the developmental brain can impair the brain function and structure in the adolescent and adult offspring. The reported impairments include cognitive behavior abnormalities, [4,5] neurotransmitter disruptions, [6,7] and brain cytoarchitecture deficits, [8,9] which could last for a long time after ceasing of the pesticide exposure.[10,11]

It was recently reported that organophosphorus pesticides could induce brain morphological deficits which related to function abnormalities. Chen [9] reported that prenatal CPF exposure at 5mg/kg/d caused selective learning disability in adult offspring mice, accompanying with selective cell loss in the corresponding dorsal hippocampus and media prefrontal cortex. Rauh [12] reported that gestational mother with high CPF exposure had low IQ children with thinner frontal lobe, reinforcing human epidemiological survey evidence for the function-structure relationship of neurotoxicity. In these cases, pesticide was exposed only on the mother gestational period, but the function-structure deficits was represented persistently and even lasted until adulthood, thus intrinsic impairments must exist in the embryonic neurogenesis

There were numerous evidences to prove that embryonic neural progenitors were sensitive and direct targets of organophosphorus pesticide toxicity. For example, CPF exposure elicited diverse changes of gene expression profiles involving proliferation, differentiation, cell cycle and apoptosis of embryonic neurogenesis. [13, 14, 15] CPF and chlorpyrifos oxon (CPO) inhibited axonal outgrowth but stimulated dendrite growth in co-cultured immature superior cervical ganglia neurons. [16] CPF treatment inhibited DNA synthesis and cell replication in undifferentiated PC12 cells, droving them into catecholaminergic phenotype but not cholinergic phenotype. [17] However, the toxicological pathway from neural progenitor impairments into brain morphology and function abnormalities remains largely unclear.

To explain the delicate toxicity pathological mechanism, we have addressed a hypothesis that pesticide-induced developmental neurotoxicity may originate from the imbalance of proliferation and differentiation of neural progenitors in early neurogenesis.[18] That is, pesticide exposure impairs neural progenitors sensitively and directly, which causes abnormalities in proliferation division and differentiation division of neural progenitors. The

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disordered proliferation pool and differentiation pool disrupt the morphology and cytoarchitecture of developing brain, which finally conduct persistent brain function abnormalities in offspring.

There are still scarcely direct evidences to support this hypothesis, embryonic neurogenesis is a highly complex and dynamics progression which is difficult to study. Though there were already a lot of in vivo and in vitro essay to evaluate developmental neurotoxicity, it is still difficult to assess the division and migration progression in embryonic progenitors[19] To overcome the technique bar, recently we set up a morphological based system to evaluate developmental neurotoxicity. It is distinguishable of diversity of developing progenitors by identifying their docking position in embryonic cerebral wall. [20] There are two major neural progenitors with lively multiplication in embryonic cerebral wall. The pseudostratified ventricular epithelium (PVE) progenitors or apical progenitors (APs) are the very origin progenitors of brain cells, which localize predominantly in the ventricular zone (VZ) and divide only at the surface layer of the ventricle. The secondary proliferative population (SPP) progenitors or basal progenitors (BPs) are the daughter cells of PVE, which localize in the subventricular zone (SVZ) and divide in the subsurface layer of the ventricle. [20, 21] APs perform a characteristic division behavior with nuclei up and down movement along cell cycle overall length, called interkinetic nuclear migration (INM). [22, 23] Thus, by analyzing the mitoses localization and tracing the nuclei migration, we can obtain important messages for progenitor population size and APs proliferation process.

This study was designed to determine the immediate and sustained toxicity of CPF prenatal exposure on embryonic neural progenitors. 5mg/kg/d of CPF were exposed exact at GD14 in immediate group and a few days before GD14 in sustained group. The exposure dosage was defined from our previous study and others work which represented no obvious systemic toxicity. [4, 9] Measurement was performed at GD14, a vital time point that neural progenitors switch their division model from proliferating into differentiating, which is sensitive for determine the disorders of progenitor division process. Neural progenitor population size was determined by analyzing mitoses count in their docking location, and INM velocity was determined by BrdU pulse labeling and calculation of positive nuclei migration speed.

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2. Materials and Methods

2.1 Animals and timing embryos

All the animal studies were carried out in accordance with *the Guide for the Care and Use of Laboratory Animals* issued by the National Institutes of Health. The experimental procedures were conducted with approval by the Institutional Animal Ethics Committee of the Research Center of Laboratory Animal Science in Zhejiang Chinese Medical University (Permit Number are SYXK (Zhe) 2008-0116). ICR mice were purchased from the Experimental Animal Centre in Zhejiang Province, Hangzhou. The mice were housed in breeding cages with free access to food, water and a 12/12 dark-light cycle. For timing embryos, male and female mice were paired in 1:1 ratio, vaginal plugs were checked twice a day at 9:00 am and 21:00 pm. The exact time of vaginal plugs found was recorded as gestational days 0 (GD 0) for dams and embryo day 0 (E0) for fetus.

2.2 Pesticide exposure and Brdu labeling

Gestational dams were randomly classified into fourteen groups, each with eight animals. Chlorpyrifos (99.1% purity) was purchased from Xinnong Chemical Company at Jiande, China and dissolved in dimethyl sulfoxide at a concentration of 5 mg/ml. GD14 dams were received three repeated subcutaneous injections of 5 mg/kg.bw of CPF in 3 hr intervals for immediate effects study, while GD7.5-11.5 dams were received daily injections of 5 mg/kg.bw of CPF for sustained effects study.

Brdu was purchased from Sigma Company, USA and dissolved in pH 7.4 saline solution at a concentration of 5mg/ml. 50 mg/kg.bw of Brdu was intraperitoneal injected to label S-phase nuclei in embryonic neocortex at 0.5, 2.5, and 5.5 hour before the last exposure of CPF in immediate groups (Fig.2A), and 2.5 days after the last CPF exposure in sustained groups (Fig. 2B). The controls were received equal volume of vehicle.

2.3 Histochemistry

Gestational dams were sacrificed at 1, 3, 6, or 9 hour after Brdu injection, embryo brains were isolated and fixed in 4% paraformaldehyde for 24 hour. After dehydrated with gradients alcohol and cleared with xylene, the samples were embedded in paraffin. The embedded brains were cut into continuous 4-μm sections in a precise coronary orientation. The sections were de-crumpled in 45℃ water and put onto galss slices.The slices were dried in 65℃ over night, de-waxed with xylene and re-hydrated with gradients alcohol. The samples were then

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denatured in 2 M HCl for 60 minutes, washed with 0.025% TrintonX-100 PBS, and carried out antigen retrieval with 0.125% trypsin at 37℃ for 20 minutes. 5% BSA incubation at 20℃ for 2 hr was conducted to blocking non-specific antigen. Immunostainning was performed with biotin-labeled mouse anti Brdu polyclonal antibody (1:50, Abcam, no.ab2284) at 4℃ overnight and HRP linked anti-biotin second antibody (1:200, Invitrogen, no.43-4323) 1hr RT, followed by DAB coloration and 5% fast green contrast stained. HE staining was performed with hematoxylin stain and eosin contrast stain. The slices were finally mounted with neutral resin.

2.4 Section position definition

Mitosis count and BrdU positive analysis were performed in a standard level which previous reported to study INM and progenitor division process.[20] Briefly, the embedded brains were adjusted into a precise horizontal orientation to obtain perfect left-right symmetry, then were cut into continuous 4-μm sections (Fig. 3A and 3B). The largest cross section of lateral ventricle in the embryonic brain was chosen for measurement, which is the section according to Schambra's atlas level at E14 horizontal 3(Fig. 3C).[24]

2.5 Enumeration of mitoses

Enumeration of mitoses was performed in GD14 samples at 1hr after BrdU labeling. The chosen slices at the defined level were prepared with HE stain. Measurement was performed in an area of 0.1×0.2 mm^2 at the dorsolateral region of cerebral wall, and both sides of lateral ventricle were determined for mitoses count (Fig. 3C). Under an Olympus IX71 microscope, the measurement area was divided into 10μm height profiles (bins) from ventricle margin to abventricular area with a mesh micrometer (Fig. 4C). Mitotic figures in bin I-X were distinguished and represented as total mitosis number and percent of mitotic cells in each bin. PVE population size was assumed by ventricular mitosis number (bin I), while SPP population size was assumed by abventricular mitosis number (bin V-IX). There were no mitotic figures in bin II-IV due to the surface division characteristics of PVE cells, so it is easy to distinguish ventricular mitoses and abventricular mitoses. Mitoses counting were carried out in a clockwise way around the cerebral wall to avoid repeated counting. Cells in prophase, metaphase, anaphase with obvious mitotic figures were enumerated, while cells in telophase were eliminated due to its indistinguishable from two independent mitotic cells (Fig. 4D). The final results are the summation of both side of mitoses count.

2.6 Enumeration of Brdu-positive nuclei

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Measurements were performed in BrdU labeling samples at 1, 3, 6 and 9hr time points. The slices were prepared with BrdU immunohistochemical stain. In an area of 0.1×0.2 mm^2 in dorsolateral ventricle, Brdu-positive nuclei were counted and represented in 10μm height profiles (bins) from the ventricle margin. Global Brdu positive nuclei count was defined as the combined number of Brdu positive nuclei in bin I-X, while percent of positive nuclei was defined as the percent of Brdu-positive nuclei at each bin in global Brdu positive nuclei. To quantitative determine the mean migration distance of positive nuclei, we calculated the relative location of Brdu labeled nuclei (D) with accumulated percent of positive nuclei at each bin (X) multiplied the distance of this bin (Y) in formula $D=\sum XY$. That is, if the percent of positive nuclei at bin I (X_1) = 0 %, X_2 = 0.38 %, X_3 =2.34 %…X_{15} = 0 %, while the distance of bin I (Y_1) =10μm, Y_2 =20μm, Y_3 =30μm…Y_{15} =150μm, thus the relative location of BrdU labeled nuclei (D) = $X_1 Y_1 + X_2 Y_2 + X_3 Y_3 \ldots + X_{15} Y_{15}$.

2.7 Data analysis

Data are presented as means plus standard error (Mean ± SEM). SPSS 17.0 software was used to analysis treatment and control groups with a multivariate analysis of variance. Significance was conducted and expressed as $P<0.05$ and $P<0.01$. To reduce any individual differences in groups, all the results were performed in a double-blind way. The gender features of GD14 fetuses were inconspicuous (Fig.3A), and there was nearly no research to demonstrate any sex differences in embryonic neocortical development during the neurogenic phase, so we did not consider sex as a major modifier and did not perform sex difference analysis. Eight slices in each group was taken from eight different embryos with individual dams.

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3. Results

3.1 CPF effects on progenitor mitoses counts

There are different progenitor populations in embryonic neocortex. [25] To determine the effects of CPF exposure on apical progenitors or basal progenitors, we conducted mitoses count analysis. According previous study, PVE was defined as the apical progenitor population which resides in VZ and divides in ventricle surface of cerebral wall with typical INM behavior, while SPP was defined as the basal progenitor population which predominates in SVZ and represents abventricular mitosis without typical INM behavior. [20] Thus PVE and SPP sizes can be evaluated by calculating the mitoses count in ventricle surface or abventricular zone of GD14 cerebral wall. Under an Olympus IX71 microscope, we observed GD14 cerebral wall represented cellular stratifications, which including VZ, SVZ, cortical plate (CP) and intermediate zone (IZ) (Fig. 4A and 4B). Mitoses count was performed in cells with obvious mitotic figures in prophase, metaphase, anaphase, cells in telophase were eliminated due to its indistinguishable from two independent mitotic cells (Fig. 4D). Since mitotic cells were only found in VZ/SVZ but not in CP/IZ, we termed VZ/SVZ as proliferation pool and CP/IZ as differentiation pool (Fig. 4A and 4B).

The global mitoses count in the measurement area was compared between CPF and control groups. Combined the mitotic cells in all bins, we observed an average 20-24 mitoses at counted regions in control groups. There were no significant difference of global mitoses count with CPF treatment both in immediate groups and sustained groups (Fig. 5B and 5D, $P>0.05$). We then separated the global mitoses count as PVE count and SPP count. The mitoses distribution curve in GD14 cerebral wall was bimodal, with approximately 80 % of mitotic cells concentrated in bin I (PVE) and the remaining broadly distributed in bin V-X (SPP). No mitotic cells were observed in bin II-IV (Fig. 5A and 5C). Combined mitoses count in bin V-X, we found that both CPF immediate treatment and CPF sustained treatment showed no significant effects on SPP counts (Fig.5A, $P>0.05$; Fig.5C, $P>0.05$). For PVE counts, though CPF immediate treatment showed no significant effects on mitoses count (Fig. 5A, $P>0.05$), CPF sustained treatment represented a significant difference from the control (Fig. 5C, $P<0.05$). Thus, here we found a population selective toxicity in embryonic progenitors, which is time sustained.

3.2 CPF immediate effects on INM

Division procession of PVE cells can be traced by determining the INM speed. To label the cycling cells, BrdU was injected to mark S-phase nuclei, and the marked nuclei were counted

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in their localization at defined intervals.[26, 27, 28] For CPF immediate groups, Brdu pulse labeling was performed during the CPF exposure period. We found that control groups revealed a bell curve of Brdu positive nuclei in GD14 cerebral wall at 1hr time point, with approximately 85% of positive nuclei concentrated in the upper and middle VZ (50-90μm). The positive nuclei migrated downwards and scattered into a wide range in the middle and inner VZ (10-80μm) at 3hr time point, then continuously migrated downwards and concentrated in the inner VZ (10-40μm) at 6 hr time point. CPF immediate treatment showed no significant effects on the nuclei distribution curve at beginning (1hr time point), but represented retarding effects in the following time. That is, CPF caused more positive nuclei staying in the middle VZ instead of in the middle and inner VZ at 3 hr time point, and kept more positive nuclei staying in the middle and inner VZ instead of in the inner VZ at 6 hr time point (Fig. 6A).

The nuclear migration velocity was quantitative determined by calculating the relative location of positive nuclei. Percent of positive nuclei multiplying its distance location was accumulated into a global point, which was represented as a relative location in Fig. 6B. Compared with the relative location of positive nuclei at 1, 3, and 6 hour in control groups, we found that CPF immediate exposure induced no significant changes at 1hr time point, but caused an average 8.8 ± 2.00μm lagging at 3hr time point and 6.8 ± 1.17μm lagging at 6hr time point (Fig. 6B).

We finally combined the BrdU labeling nuclei count in all bins and compared the global labeling nuclei count. There was a gradually increasing trend of labeling nuclei counts at 1, 3, 6 hr time points, which represented a S-phase ongoing process after Brdu labeling. Compared the global nuclei count between control and CPF immediate groups, we did not find any statistics difference in all time points (Fig. 6C, $P>0.05$). That is, though CPF immediate exposure arrested the nuclei migration velocity from abventricular zone downwards to ventricle surface, there were no changes in total BrdU labeling cell count.

3.3 CPF sustained effects on INM

To explore whether CPF pretreatment also leave long staying toxicity on embryonic progenitors, we performed daily CPF exposure during GD7.5-11.5 and labeled BrdU 2.5 days after secession of the pesticide treatment. CPF toxicity was measured on GD14 neocortex at 1, 3, 6, and 9 hour after BrdU labeling. It was previous reported that the BrdU labeling nuclei represented a down movement at beginning, then turn upwards in the following time. [20, 28] In control groups, we observed the labeling nuclei moved downwards until attached the apical

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surface of cerebral wall during 1 to 6 hr time points, following by the labeling nuclei staying in apical surface to wait for the turn upwards migration at 6 to 9 hr time points. Comparison of CPF pretreatment and control groups, the labeling curves in CPF groups were lagging to the controls, though the toxicity was not as strong as that in the immediate groups. CPF pretreatment detained more labeling nuclei in upper VZ (80-100μm) at 1-3 hr time points, and retarded the labeling nuclei migrating into inner VZ (10μm) at 6 hr time point. CPF effects at 9 hr time point were nearly undistinguishable, because at this time the downwards migration and the upwards migration combined to make a mixing result (Fig. 7A).

Then we did quantitative analysis of the relative location of positive nuclei. We found that CPF pretreatment induced 3.5 ± 1.81μm lagging of relative location of positive nuclei at 1hr time point, though still no significant difference in statistics (Fig. 7B, $P=0.055$). We found that CPF pretreatment induced 6.3 ± 1.61μm lagging of relative location at 3 hr time point, which has very significant difference versus the control in statistics (Fig 7B, $P<0.01$). However, we did not observed significant difference of relative location at 6 hr time point (Fig 7B, $P>0.05$) and 9 hr time point (Fig 7B, $P>0.05$). Considering there was a reverse movement from downwards migration to upwards migration during 6 hr to 9 hr [20,27], we regards the results as a mixing trajectory of both opposite migrations, and CPF effects on downwards migration retarding was neutralized by the effects on upwards migration retarding.

We then performed global labeling nuclei analysis. There were a gradually increasing trend from 1 to 6 hr time points both in CPF pretreatment and control groups. Except a temporary decrease in CPF sustained group at 1 time point (Fig. 7C, $P<0.01$), CPF pretreatment did not induce obvious changes in labeling nuclei counts at other time points (Fig. 7C, $P>0.05$).

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4. Discussion

The occurrence and development of pesticide neurotoxicity involves a series of neurogenesis mechanisms, including proliferation,[29,30] differentiation [31,32,33] and migration [34,35] of neural progenitors. Division process in origin progenitors is a vital event for all of these activities, which acts as a switch button and balancer.[36] In the present study, we performed an in vivo essay to determining the pesticide effects on the early division process of neural progenitors. BrdU pulse labeling and positive nuclei location measurement were used to determine the division timing progression. In results, we obtained three essential findings. First, CPF exposure induced a population selective mitosis inhibition in sustained effects, which targeting on the primary progenitors (PVE) but not on the secondary progenitors (SPP). Second, CPF exposure arrested the division process of PVE cells both in immediate and sustained effects, showing lagging locations of BrdU labeling nuclei. Finally, CPF exposure did not change the total amount of mitoses cells and S-phase cells in embryonic progenitors. Thus, CPF toxicity at 5mg/kg/d dosage is a faint effect that only disrupts functional activities of neural progenitors but not affects the global cycling cell amount.

4.1 CPF exposure and progenitor population sizes

As two principal classes of proliferation cells in E14 neocortex, PVE comprises of most of the proliferation cells while SPP comprises of the remaining.[20] The population balance in normal embryonic neocortex was disrupted with CPF pretreatment in our study. By measuring the mitoses count separately in ventricular surface and abventricular zone, we compared the pesticide effects on PVE size and SPP size. We found that CPF pretreatment reduced PVE population size but not SPP population size in sustained effects, while CPF immediate exposure had no effects both in PVE size and SPP size. The selective toxicity may be caused by developmental stage difference between these two types of progenitors, for PVE cells being undifferentiated progenitors and SPP cells being progenitors started to differentiation.[20, 37] It was previous reported that CPF exposure impeded DNA synthesis in undifferentiated PC12 cells but not in differentiated cells, [38] and prenatal CPF exposure disturbed neurotransmitter metabolism more in the early formed region than that in the later formed one.[39] There were also reported that CPF and chlorpyrifos oxon (CPO) treatment disorganized the spindle structure and inhibited cell proliferation in vitro. [40, 41] Thus the population selective toxicity may bring about from the selective proliferation toxicity in the embryonic neocortex.

There were evidences that CPF treatment directly impaired spindle organization and de-polymerized microtubules in brain slices. For example, CPO treatment elicited propidium

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iodide to incorporate into progenitor nuclei and disrupted spindle organization in the hippocampus slices. [42] CPF and CPO treatment de-polymerized microtubules by covalently modified tyrosine sites in the purified bovine tubulin and in the pesticides pretreated brain. [43, 44] CPF co-incubation with E9.5 embryo elicited abnormalities in mitotic figures in E11.5 neuroepithelium.[41] This cytoskeleton de-assembly mechanism may identify the M-phase targeting toxicity in pesticide-induced neurotoxicity. However, mitoses count is a slow reaction index that only presents results after more than one cycle length. In our experiment, as we used a subtoxic dosage of CPF exposure with mini toxicity in neural progenitors, there were no obvious changes of mitoses count in CPF immediate groups. In CPF sustained groups, the mini toxicity finally reduced mitoses count after a few days action.

4.2 INM immediate arrest and sustained arrest

It is a characteristic behavior of APs that performs a nuclei up-down migration in VZ along overall cell cycle length. S-phase nuclei usually take place in the upper VZ, followed by a basal-apical migration in G2-phase. The nuclei stay and divide in ventricle surface in M-phase, then perform an apical-basal migration in G1-phase. [22] In our study, S-phase nuclei were labeled with a BrdU single injection. That is, the labeled nuclei represented in upper VZ at beginning, then migrated basal to apical along the cell cycle running. Thus we can trace the nuclei migration speed by measuring the labeled nuclei location, and take the nuclei location as a hallmark of progenitor division progression.

Our results showed that CPF exposure arrested basal to apical migration of INM both in immediate and sustained groups, the different treatment may involve distinctive mechanisms. Immediate effect is an acute toxicity occurring in the CPF exposure period, the rapid reaction feature strongly suggests an inactive mechanism of movement machinery of INM. It is well known that INM is driven by molecular motor when sliding along the microtubule tracks. [22, 45, 46] As above described, organophosphorus pesticide treatment can destroyed microtubule organization in vivo and in vitro. Furthermore, in vitro studies showed that CPF and CPO inhibited microtubulin mobility by directly binding on a molecular motor kinesin. [47] On the other hand, CPF exposure increased DCF-DA, DHE fluorescence, superoxide generation and caspase activity in glia progenitors, decreased activities of antioxidant enzymes in isolated retina cells, indicating oxidative stress and apoptosis mechanism involving the process. [48, 49] Thus, the acute toxicities may identify the immediate mechanisms of pesticides-induced INM retarding.

CPF sustained toxicity must involve other mechanisms, in which INM retarding effects occur

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with a few days interval after termination of pesticide exposure. Our results showed that the INM retarding toxicities in sustained groups were weak and partly recoverability. However, CPF pretreatment significant reduced the mitosis count at PVE and the total BrdU positive nuclei at 1 hr, implying intrinsic changes must leave in the neural progenitors.

There were still not many clues to trace the pathological pathway of these intrinsic toxicities in embryonic neurogenesis. There were previous evidences showed that prenatal or neonatal pesticides exposure could induce cognitive abnormalities, neurotransmitter disorders and cytoarchitecture deficits in adult brain regions [50, 51, 9] There was also study showed that CPF treatment could change the developmental fate of PC12 cells from cholinergic phenotype into catecholamine phenotype. [17] Furthermore, results from pesticide treated PC12 cells showed defined toxicity styles of gene expression profiles and transcriptional responses in defined exposure periods, suggesting gene mechanism involved in the intrinsic toxicity. [38] In our recently study, we reported a switched cleavage plane orientation of neural progenitors as the early indication of changed developmental fate in pesticide treated progenitors. [28] It is possible that CPF sustained toxicity come from the disruption of proliferation and differentiation regulation in progenitors, which result as alternated progenitor fates and deficits of embryonic neurogenesis, though the integrated mechanism remains largely replenished.

There was another vital factor which may affect the final results. In our experiment, CPF was exposed at GD14 in immediate groups and at GD7.5-11.5 in sustained groups, the poisoning stage difference may cause distinctive toxicity reactions. It is generally considered that GD14 is a stage that neural progenitors with proliferation and migration simultaneously, while GD7.5-11.5 is a stage that neural progenitors only with proliferation. In the present study, we found that both CPF exposure at GD14 and at GD7.5-11.5 disrupted progenitor division at GD14. We will explore whether and how the developmental stages affect the final neural toxicity in future.

4.3 CPF classic toxicity and developmental neurotoxicity

It is well known that acetylcholine esterase (AChE) inhibition is the golden standard to evaluate organophosphorus pesticides toxicity. Until now, there was no other toxicity endpoint more sensitive than AChE inhibition. Most of OPs toxicities are based on the AChE inhibition. [3] However, in developing brain, there were toxicities working on no-AChE mechanism at dosage around the toxicity threshold. Developmental toxicities have been observed in AChE gene knock out mice, with AChE activity drugs, and in early neurogenesis

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stage before AChE formation. [4, 52] The no-AChE toxicity mechanism of developmental neurotoxicity need to explore in a deep going way.

The CPF exposure dosage (5mg/kg/d) we used in this study is a subtoxic dosage which could cause developmental neurotoxicity but whitout obvious systemic tocicities. According to previous study, 5mg/kg/d of mother CPF exposure induced a blood chlorpyrifos concentration of 46ng/g in fetuses, which is much higher than the toxicity dosage showing offspring brain structure/cognitive changes in human epidemiological survey. [12, 53,54] However, the dosage is near the CPF NOAEL dose and the toxicological endpoint of AchE inhibition (0.15mg/kg/d for dermal. 0.1mg/kg/d for inhibition, and 0.03 mg/kg/d for oral exposure). [52] Considering the FQPA safety factor, our results are valuable.

This study supplies a novel approaching for developmental neurotoxicity mechanism. Using a morphological based essay, we measured mitoses count and INM velocity in CPF treated embryonic progenitors. Our results represented in vivo evidences for organophosphorus pesticide induced progenitor proliferation disturbance. In future, we plan to perform further experiments to explore the pesticide effects on progenitor differentiation and migration in the developing neocortex. We hope to explain finally the intrinsic toxicity path in pesticide induced developmental neurotoxicity.

Conflict of Interest

There are no conflicts of interest to declare.

Acknowledgments

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Legend to figures

Fig. 1 Chemical structure of chlorpyrifos

From http://www.chemblink.com/products/2921-88-2.htm

Fig. 2 Schedule of Brdu and pesticide treatments

(A) Chlorpyrifos immediate exposure groups. Three repeated injections of chlorpyrifos (5mg/kg) were given to GD14 neocortex at 3hr intervals. Brdu (50mg/kg) was injected during pesticide exposure period, and animals were sacrificed at 1, 3 or 6hr after Brdu labeling.

(B) Chlorpyrifos sustained exposure groups. 5mg/kg of chlorpyrifos was injected daily during GD7.5-11.5. On GD14, 50mg/kg of Brdu was given with animals sacrificed at 1, 3, 6 or 9hr after Brdu labeling. CPF: chlorpyrifos.

Fig. 3 Embryo slices and section position definition

(A) GD14 embryo, left: size measurement, 1.0×0.6 cm; right: embryo in horizontal direction.

(B) Schematic diagram of embryo cutting into horizontal continuous slices. The slice level in thick line is the chosen section for measurement.

(C) The largest cross section of lateral ventricle in GD14 neocortex, which was chosen to measure mitoses count and BrdU labeling nuclei count. Measurement was performed in an area of 0.1×0.2 mm^2 at the dorsolateral region of cerebral wall (in black box), both side of lateral ventricle was used.

Fig. 4 Stratification and mitoses location in GD14 cerebral wall

(A) GD14 cerebral wall in sustained groups. Left: control; right: chlorpyrifos treatment. Black arrows show mitotic cells at ventricle margin (PVE) and abventricular zones (SPP). Scale bar: 15μm.

(B) GD14 cerebral wall in immediate groups. Left: control; right: chlorpyrifos treatment. Black arrows show mitotic cells at ventricle margin (PVE) and abventricular zones (SPP). Scale bar: 15μm.

(C) High magnification image of mitotic cells at ventricle margin (PVE) and abventricular

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zones (SPP).

(D) GD14 cerebral wall was divided into 10μm height profiles (bins) from ventricle margin to abventricular area with a mesh micrometer. The measurement area arranged from bin I to bin X.

(E) Cell figures in interphase, prophase, metaphase, anaphase and telophase. Only cells in prophase, metaphase and anaphase were enumerated as mitosis count. Cells in telophase were eliminated due to its indistinguishable from two independent mitotic cells.

Fig. 5 CPF effects on progenitor population sizes

(A) CPF immediate effects on PVE and SPP sizes. GD14 dams were received three repeated injections of CPF (5 mg/kg/) at 3 hr intervals, the samples were determined at 0.5 hr after the last CPF injection. The pseudostratified ventricular epithelium (PVE) size was assumed with ventricular mitoses number, while the secondary proliferative population (SPP) size was assumed with abventricular mitoses number. Mitoses counts were recorded and expressed in bins. *P<0.05, **P<0.01. CPF: chlorpyrifos.

(B) Global analysis of mitosis count in immediate groups. Total mitosis number was calculated as combined value of all measurement bins. CPF: chlorpyrifos.

(C) CPF sustained effects on PVE and SPP sizes. GD7.5-11.5 dams were received daily injections of CPF (5 mg/kg/), the embryo samples were determined at GD14. The pseudostratified ventricular epithelium (PVE) size was assumed with ventricular mitoses number, while the secondary proliferative population (SPP) size was assumed with abventricular mitoses number. Mitoses counts were recorded and expressed in bins. *P<0.05, **P<0.01. CPF: chlorpyrifos.

(D) Global analysis of mitosis count in sustained groups. Total mitosis number was calculated as combined value of all measurement bins. CPF: chlorpyrifos.

Fig. 6 CPF immediate effects on INM of GD14 PVE cells

(A) Distribution curves of Brdu positive nuclei at 1, 3 and 6hr after labeling. Brdu was injected during chlorpyrifos exposure period. Nuclei count was represented as percent of positive nuclei in each bin. CPF: chlorpyrifos. Scale bar: 5 μm.

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(B) Migration progression of relative location of Brdu positive nuclei in chlorpyrifos immediate groups and control groups. The relative locations were calculated with formula: D = ∑XY. ** p<0.01

(C) Global number of Brdu positive nuclei at 1, 3 and 6hr time points in chlorpyrifos immediate groups and control groups.

Fig. 7 CPF sustained effects on INM of GD14 PVE cells

(A) Distribution curves of Brdu positive nuclei at 1, 3, 6 and 9hr after labeling. Brdu was injected 2.5 day after termination of chlorpyrifos exposure. Nuclei count was represented as percent of positive nuclei in each bin. CPF: chlorpyrifos. Scale bar: 5 μm.

(B) Migration progression of relative location of Brdu positive nuclei in chlorpyrifos sustained groups and control groups. The relative locations were calculated with formula: D = ∑XY. ** p<0.01

(C) Global number of Brdu positive nuclei at 1, 3, 6 and 9hr time points in chlorpyrifos sustained groups and control groups. ** p<0.01

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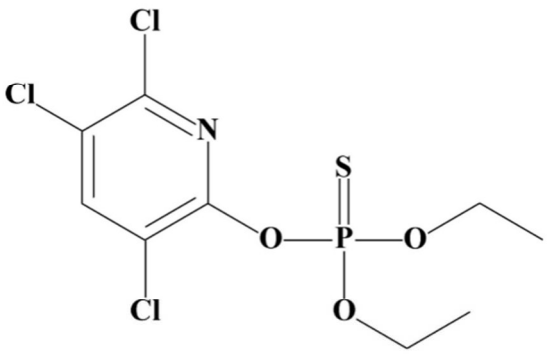

Fig.1 Chemical structure of chlorpyrifos

Figure 1

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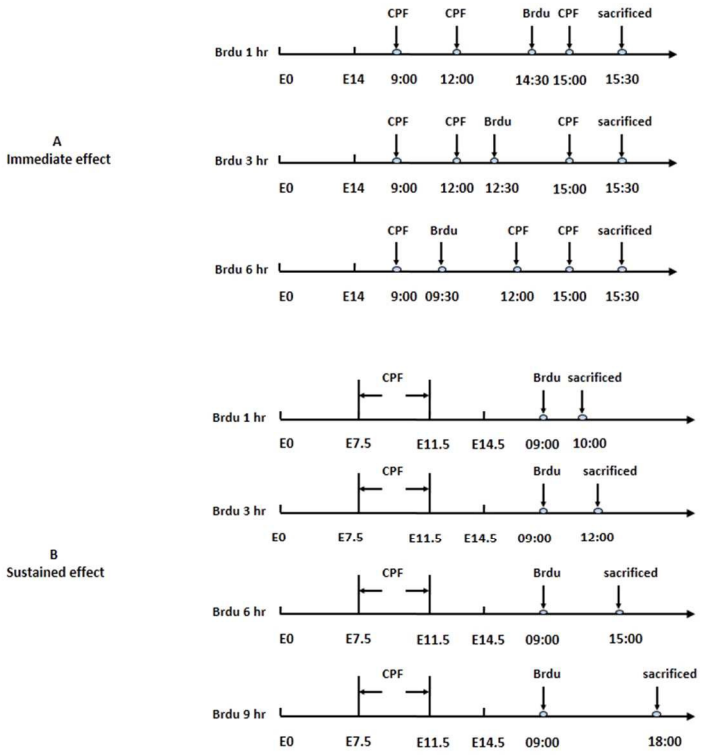

Figure 2

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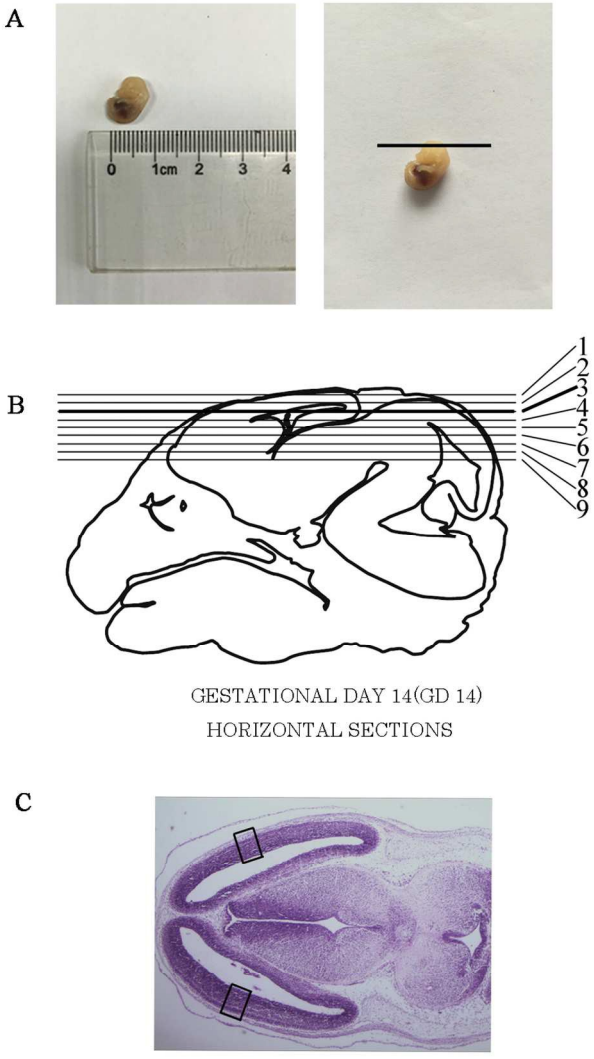

Figure 3

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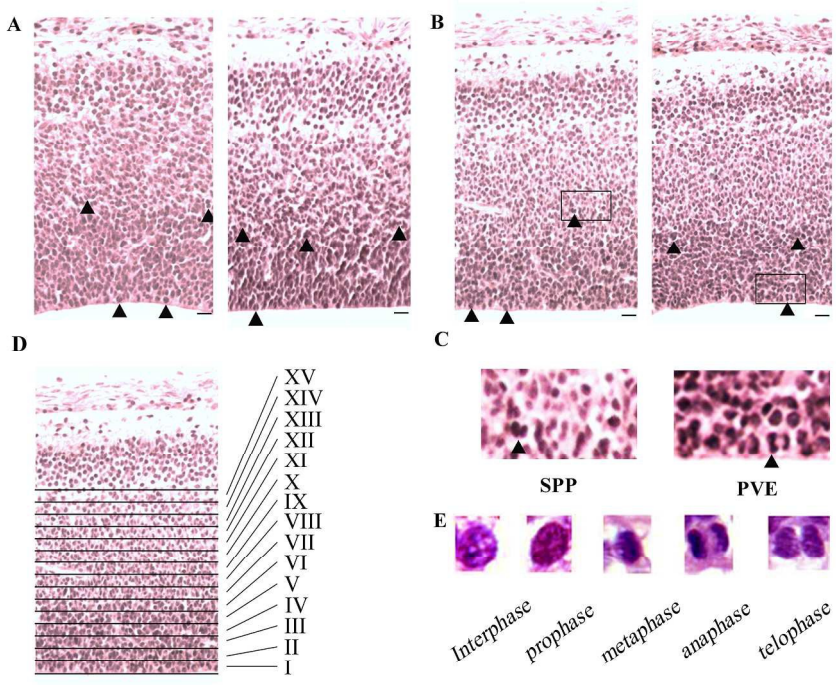

Figure 4

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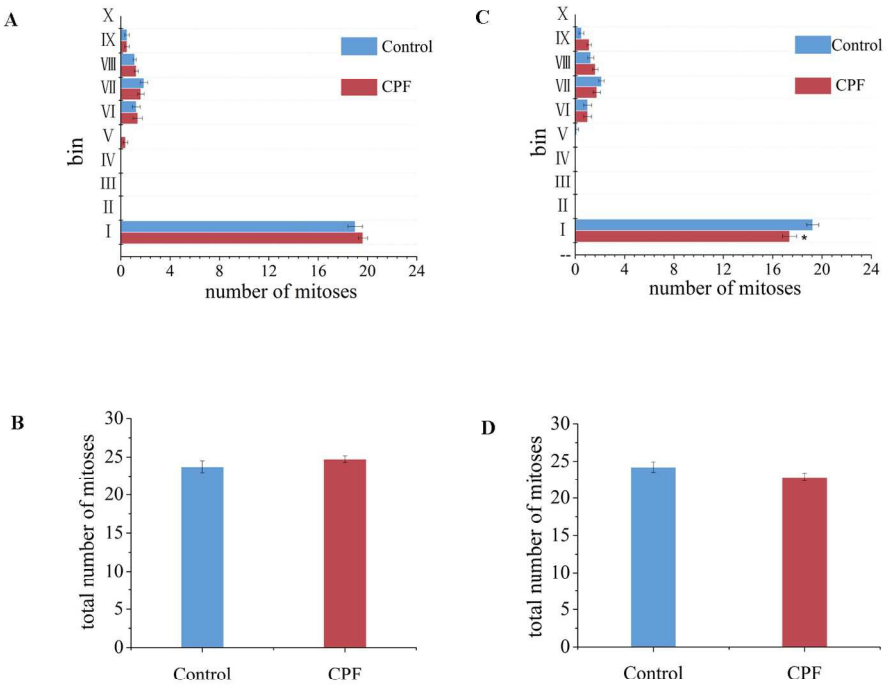

Figure 5

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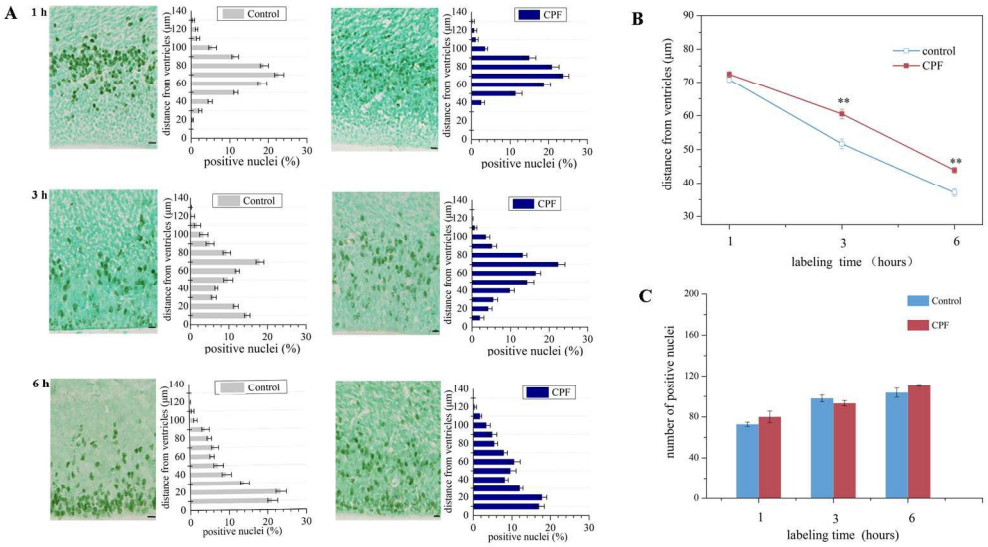

Figure 6

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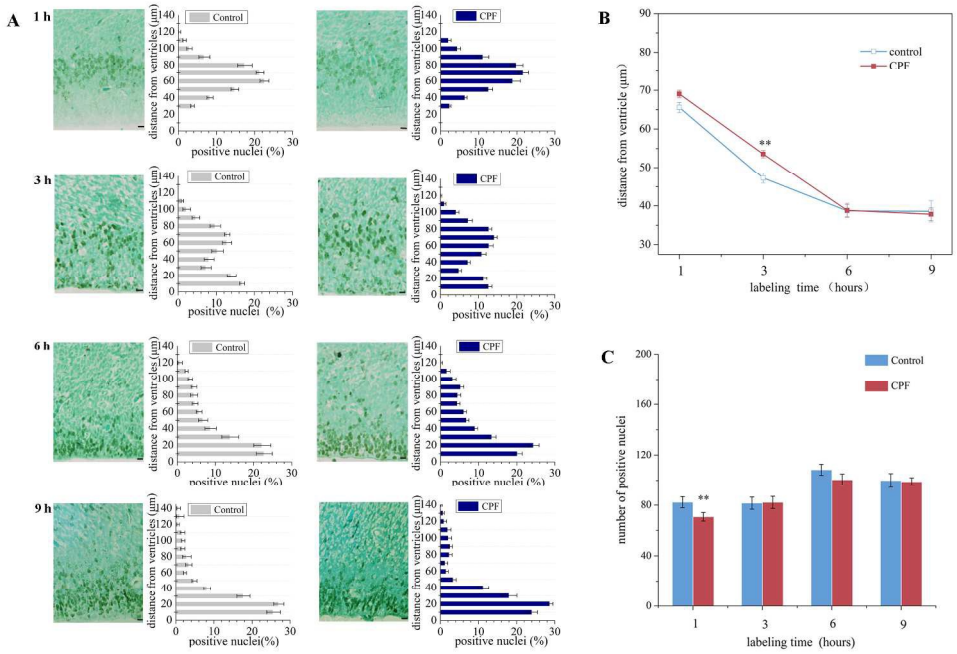

Figure 7

937x681mm (72 x 72 DPI)

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