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A comparison of neurotoxicity in cerebellum produced by dermal application of chlorpyrifos in young and adult mice

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Abstract Chlorpyrifos (CPF), an organophosphate pesticide inhibits acetylcholinesterase (AChE) and causes neuromuscular incoordination among children and elderly. The objectives of the present study were to compare the neurotoxic effects of dermal application of CPF on the cerebellum in the parameters of glial fibrillary acidic protein (GFAP) expression in young and adult mice and to correlate with the changes in acetylcholinesterase levels. Male Balb/c mice, 150 days old (adult) and 18 days old (young) were dermally applied with ½ LD_{50} of CPF over the tails for 14 days. Serum AChE concentration was estimated and GFAP immunostaining was performed on sagittal paraffin sections through the vermis of cerebellum. Although reduced in both age-groups exposed to CPF, percentage of reduction in serum AChE was more in adult compared to the young. Under GFAP immunostaining, brown colour fibres and glial cells were observed in cerebellar cortex and medulla in both the experimental groups. The mean GFAP-positive glial cell count in cerebellar medulla per mm^2 of section was significantly ($p < 0.05$) increased in adult mice exposed to CPF when compared with age-matched control. In conclusion, this study confirmed that dermal exposure of CPF was able to exert neurotoxic effect in both young and adult mice. However, the quantitative results revealed that adult mice showed more GFAP expression in cerebellum when compared with the young, when exposed to CPF.

Keywords Chlorpyrifos · Dermal toxicity · Cerebellum · GFAP

Introduction

Chlorpyrifos (CPF) is a broad spectrum organophosphate (OP) pesticide widely used in Malaysia. It inhibits the enzyme acetylcholinesterase (AChE) by phosphorylating the active site of the enzyme, which results in accumulation of acetylcholine leading to disruption of proper transmission of nerve impulses (Vale et al. 2003). OP poisoning is the commonest occupational illness reported in farmers, especially during preparation of the spray solutions, loading of spray tanks and application of the pesticides (Rathinam et al. 2005). An Indonesian study among west Sumatran female pesticide sprayers showed that neurological signs like staggering gait, muscle fasciculation and tremors emerged among the pesticide sprayers (Murphy et al. 2004). This study also showed that 160 out of 161 sprayers provided history of skin contact over the hands with pesticides during measuring and mixing. Gloves were rarely worn by the sprayers. Analysis of data in Malaysian National Poison Centre between 1996 and 2000 showed increased rate of exposure to pesticides among poisoned children (Rahman et al. 2001). Eskenazi et al. (1999) in a review article highlighted the potential problem of widespread low level exposure to pesticides in children residing in agricultural areas. Dermal absorption might be the major pathway of exposure in the children crawling over dusty surfaces.

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Glial fibrillary acidic protein (GFAP) is the principal intermediate filament (IF) protein found predominantly in mature astrocytes of the central nervous system (CNS). GFAP is believed to be crucial in regulating astrocyte motility. Astrocytes become reactive following variable injury to the CNS. The cells respond in a distinctive manner termed reactive gliosis rapidly increasing the expression of GFAP (Pekny et al. 1999). The blood–brain barrier (BBB) was found to be structurally and functionally impaired in GFAP-deficient mice, suggesting that GFAP expression was critical for the normal integrity of the BBB (Liedtke et al. 1996).

Previous study by the author reported that the 150-day old mice showed higher reduction in neuronal count in hippocampal areas compared to 18 days old mice when ½ LD_{50} of CPF was applied dermally over the tails for 14 days (Mitra et al. 2010). Increased gait abnormalities and tremors were observed when CPF exposure occurred in the early postnatal period (Eskenazi et al. 1999). Cerebellar lesions give rise to ataxia or loss of coordination (Voogd 2003). Previous study also noted that animals that were exposed in utero to intraperitoneal administration of CPF revealed decreased balance in rotarod and reduced open-field motor activity in post-natal examination (Muto et al. 1992). Subcutaneous administration of CPF in post-natal day 1–4 showed reduced coordination skill in complex motor behaviour in male rat pups compared to the females. However, administration of CPF on postnatal day 11–14 had a much less notable effect in both sexes (Dam et al. 2000). To date, the comparison on the changes in the morphology of neurons and GFAP expression by glial cells in the cerebellum between young and adult animals following dermal application of sub-toxic dose of CPF has not been reported.

Thus, the objectives of the present study were to compare the neurotoxic effects of dermal application of CPF on the cerebellum in the parameters of GFAP expression in young and adult mice and to correlate with the changes in the AChE levels.

Materials and methods

Chlorpyrifos

A commercial formulation of chlorpyrifos (*O*, *O*-diethyl-3, 5, 6-trichloro-2-pyridyl phosphorothioate), from a manufacturer of pesticides in Kuala Lumpur, Malaysia, was used in this study. The formulation contained 38.7% *w/w* of chlorpyrifos (CPF) in organic solvent xylene. This commercial form of CPF was further diluted with xylene to prepare solutions containing 1/2 dermal LD_{50} (101 mg/kg body weight chlorpyrifos in 1 mL) dosage.

Animals

Male Balb/c albino mice, 150 days old (25–30 g) and 18 days old (11–15 g) were used in this study. The animals with normal skin and tail were purchased from Institute of Medical Research (Laboratory Animal Resource unit of MRRC), NIH, Ministry of Health, Malaysia. Standard pellet feed and water was provided ad libitum to the mice. The mice were exposed to natural 12-hourly light and dark cycle. Animal experiments were conducted according to the principles stated in the guide-book of Laboratory Animal Care and Use Committee (ACUC) of the university. The mice were divided into four groups ($n = 6$). 150 days old mice (Group A) and 18 days old (Group Y) mice were further grouped as control (group Ac and Yc) and experimental CPF treated (group Ae and Ye). Xylene was applied over the tails of the control groups of mice whereas chlorpyrifos in 1/2 dermal LD_{50} dosage was applied over the tails of the experimental groups of mice.

Dermal exposure to chlorpyrifos

The tails were secured and CPF solution in xylene was applied over the skin of the tail of the mice. The applications were done for 4 h daily for 2 weeks. A piece of surgical gauze soaked with the solution was wrapped around the tail, followed by one layer of plaster, one layer of aluminium foil and another layer of plaster. Amount of solution used each time was 1 mL. The layers of barriers were applied to prevent evaporation of the CPF solution. After removing the bandages, the remaining solution over the tail was swabbed off with wet gauze.

Serum acetylcholinesterase (AChE)

Blood samples were collected after 2 weeks of application, by cardiac puncture with the mice kept under ether anaesthesia. Centrifugation at 13,000 rpm for 5 min at 4°C was done to collect the serum. Serum AChE concentration was estimated using Amplex Red acetylcholinesterase assay kit from Molecular probes Inc, USA (Invitrogen detection technologies). This kit was employed to estimate AChE concentration using a fluorescence microplate reader. The serum samples were treated with Amplex Red reagent (10-acetyl-3, 7-dihydroxyphenoxazine), a sensitive fluorogenic probe for hydrogen peroxide. The stock solutions were prepared as per manufacturer's protocol. The estimation of AChE was conducted using serum sample of the mice in different dilutions. 100 µL of the diluted samples and controls were pipetted into separate wells of the Nunc F96 black plate. Each individual sample had triplicate wells. A working solution of 400 µM Amplex Red reagent containing 2 U/ml horse radish peroxidase (HRP), 0.2 U/ml choline oxidase and 100 µM

acetylcholine (ACh) was prepared from the stock solutions given in the kit. On adding 100 μL of the working solution prepared earlier to each well containing the samples and controls, the reactions started. Incubation in dark for 30 min at room temperature was carried out. Using a Tecan microplate reader (excitation at 560 nm and emission at 590 nm) fluorescence emitted by the individual samples was measured. Background fluorescence was corrected, at each point, by subtracting the values derived from the negative control.

A linear regression curve was obtained by plotting Log_{10} of the mean fluorescence readings against Log_{10} of the AChE concentration of the positive controls. The regression analysis indicated that the AChE activity in a sample increased exponentially with increasing concentration of a sample. The Log_{10} of the mean fluorescence readings were substituted into the equation of the linear regression line obtained from the positive controls using Minitab and Microsoft Excel. In this way, the Log_{10} of AChE concentration of the experimental samples can be calculated. AChE concentration was estimated in serum samples of individual animals of Group Ac, Yc, Ae and Ye.

Histological and immunohistochemistry studies

Perfusion of brains was carried out in six animals in each group by using 10% formal saline. The cerebellum which was separated from the forebrain, was cut sagittaly through the middle vermis. Following processing of the tissues with paraffin, serial sagittal sections (4 μ) were cut with a Leica microtome. Left half of the cerebellum was used for staining with 0.2% thionin to observe the changes in the neuronal nuclei and Nissl substance. Immunostaining was done with the sections from the right half of the cerebellum using Dako RealTM EnVision System together with the polyclonal rabbit anti- GFAP to estimate GFAP expression in the cerebellum under the light microscope. Following deparaffinisation, the sections were washed with buffer solution followed by peroxidase blocking. The sections were then incubated for 20 min with anti-GFAP antibody followed by application of peroxidase-labelled polymer to enhance the binding of GFAP and antibody. The sections were treated with DAB (3, 3′-diaminobenzidine) and substrate-chromogen. Following haematoxylin counterstaining, the sections were dehydrated, cleared with xylene and mounted with DPX mounting media. Five stained slides (every third section) containing cerebellum sections were chosen from each animal and screened using the Nikon Eclipse 80i microscope (attached with Nikon camera) with NIS Element version 3.2 software under 400× magnification. Two random areas of the cerebellum medullary layer were observed for histological changes and the brown-colour-stained glial cells were counted under square graticules (150 μm × 150 μm). The evidence for neuronal damage such as loss of nucleolus, pyknosis of neurons and vacuolation of neuropil was observed.

Statistical analysis

The mean serum AChE levels of the control and experimental groups in young and adult groups were subjected to Mann–Whitney *U* test. The mean count of GFAP-positive cells for the random areas of medullary layer were calculated and subjected to statistical analysis by using SPSS 11.5. Mean counts of GFAP-positive cells in the control and experimental groups were subjected to one way ANOVA followed by Post Hoc Bonferroni test.

Results

Changes in serum AChE level

Changes in mean serum AChE levels in control animals of both age groups are shown in Fig. 1. Young control group of mice had 23% lower serum AChE than 150-day-old control group of mice. Dermal exposure of ½ LD_{50} of CPF for 14 days caused reduction in serum AChE levels significantly (Mann–Whitney *U* test, $p < 0.05$) in both young and adult groups of mice (Fig. 1). Serum AChE level was reduced by 69% in group Ye when compared with the control group Yc, whereas in group Ae the similar reduction was 82%.

Histological changes in the neurons of cerebellum

Under Nissl stain, in both the control groups (Yc and Ac), the Purkinje cell layer showed large-sized neurons with

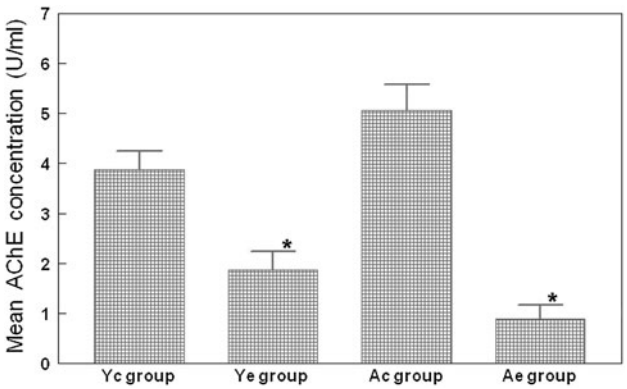

Fig. 1 Bar Chart showing changes in mean (±SD) serum AChE levels in young (18-day-old) and adult (150-day-old) mice groups. Mean AChE concentration per ml of serum at the end of 14 days derived from 40× dilution of the samples. *Yc* Young mice control group, *Ye* Young mice CPF exposed group, *Ac* adult mice control group, *Ae* adult mice CPF-exposed group. *Significance levels compared to age-matched control ($p < 0.05$ in Mann–Whitney *U* test)

prominent nuclei and perinuclear rim of Nissl bodies. The neonatal mice group exposed to CPF (group Ye) showed evidence of pyknosis of neurons whereas the adult mice exposed to CPF (group Ae) showed dissolution of Nissl granules (Fig. 2).

Changes in the GFAP expression

Under GFAP immunostaining, the brown coloured cells and processes were observed prominently in the molecular layer of cerebellar cortex in group (Ye) when compared with the control group (Yc). The cerebellum of the control group in adult mice (group Ac) showed that the lobules were fully developed compared to Group Yc. In group Ae (adult mice exposed to dermal CPF), there were more intense brown coloured stain in the white matter of cerebellar medulla compared to that of group Ye (young mice exposed to dermal CPF) (Fig. 3).

Quantitative analysis

The mean count of GFAP-positive cells per mm^2 of the section was calculated and compared between the control and experimental mice in both young and adult group. The mean count in each group was subjected to one way ANOVA test. On comparison of the control groups, the young mice (group Yc) showed a mean count of 290.12/mm^2 GFAP-positive cells, which was higher than the count in group Ac of 270.37/mm^2. However, on comparison of the experimental groups, the adult mice (group Ae) showed a higher density of GFAP-positive cells of 411.85/mm^2, compared to the density in group Ye of young mice of

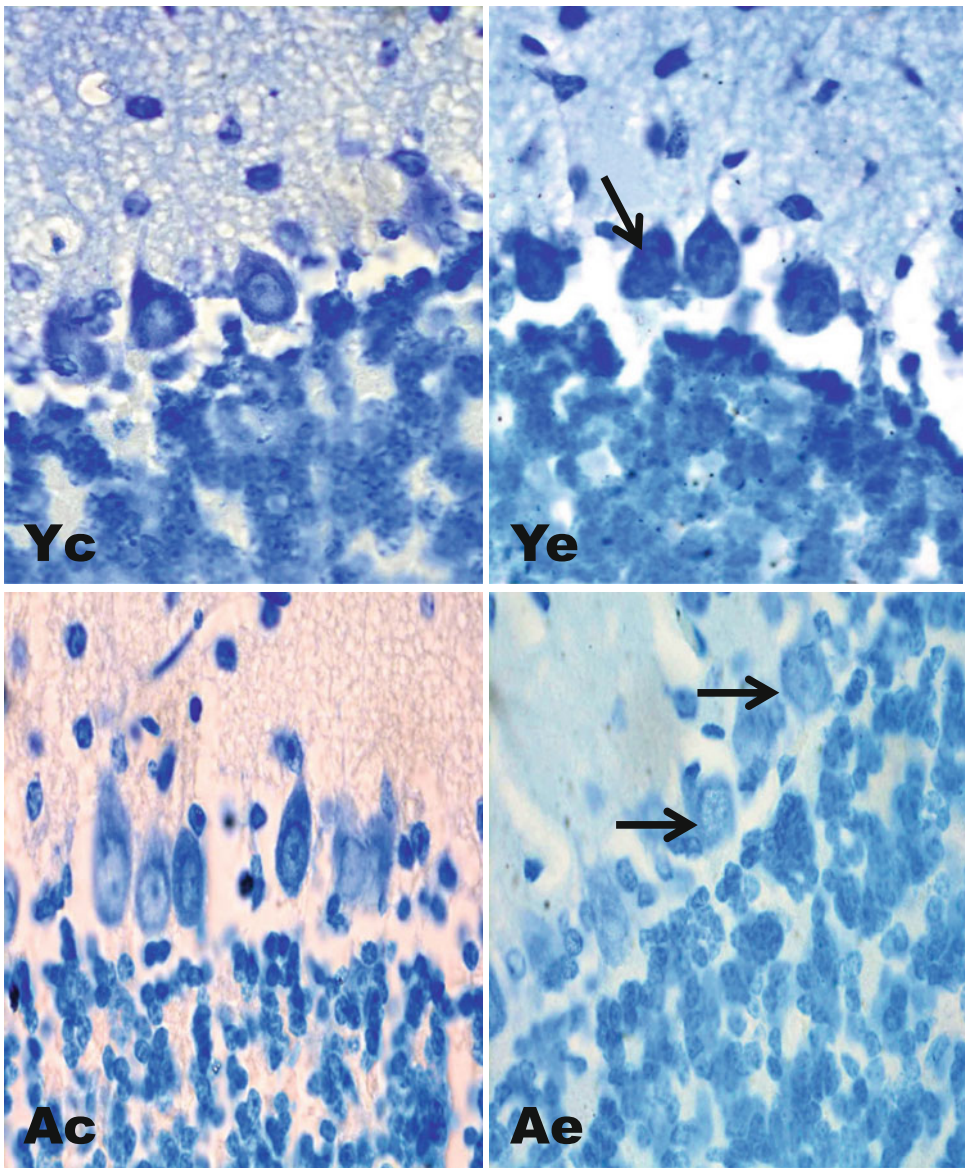

Fig. 2 Photomicrograph showing the changes in Purkinje neurons of cerebellum in different groups of mice under histological staining. Pyknosis was observed (*arrow*) in young mice group (*Ye*) exposed to dermal application of CPF. Dissolution of Nissl granules was observed in adult mice exposed to dermal application of CPF (*Ae*). *Yc* Young mice control group, *Ac* adult mice control group (Nissl stain, Thionin, 400×)

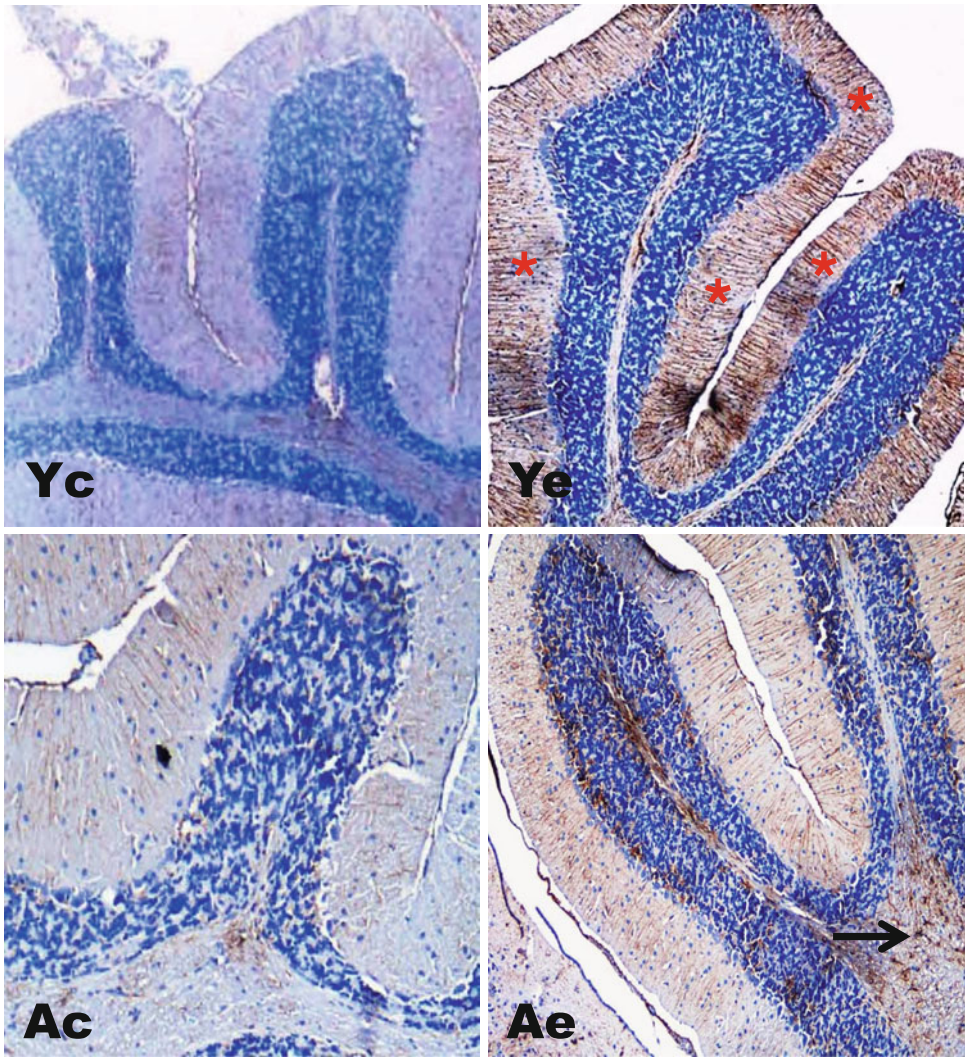

Fig. 3 Photomicrograph showing the changes in GFAP expression in cerebellar cortex and medulla in different groups of mice under immunohistochemical staining. Compared to the control (*Yc*) group, the young mice group exposed to dermal application of CPF (*Ye*) showed higher expression of GFAP (*) in the form of brown colour glial fibres in cerebellar cortex. Compared to the control group (*Ac*), the adult mice group exposed to dermal application of CPF (*Ae*) showed GFAP stained glial cells in cerebellar medulla (*arrow*) in addition to glial fibres in cerebellar cortex (GFAP stain 100×)

330.86/mm^2 (Fig. 4). Thus, following dermal exposure to ½ LD_{50} of CPF for 14 days, the young mice (group Ye) showed an increase in GFAP expression with increased mean cell count of 14% when compared with the control group (group Yc). While for the adult CPF-treated group (group Ae), the increase in the mean cell count was 52% higher compared to the control group (group Ac). The comparison of increase in GFAP-positive cells in cerebellar medulla between young and adult CPF-treated mice groups showed that the percentage of increase in mean cell count in adult CPF-treated group was almost fourfold higher than the corresponding increase in young CPF-treated group. There was statistically significant ($p < 0.05$) difference between the mean GFAP counts of group Ae and Ye in post hoc Bonferroni test. The post hoc Bonferroni test also showed significant difference ($p < 0.05$) in mean GFAP-positive cell count in adult CPF-treated group compared to the adult control group (Fig. 4). Thus, GFAP-positive cells were more significantly expressed in adult

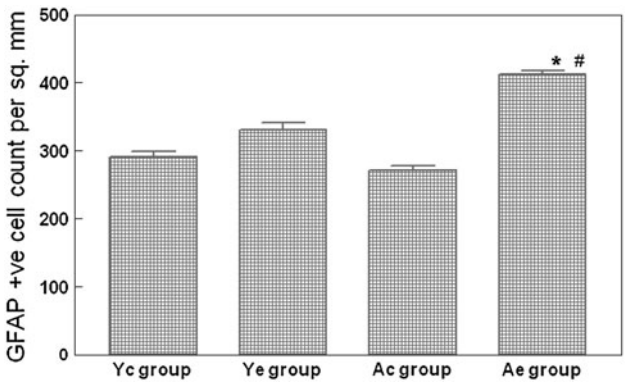

Fig. 4 Bar chart showing mean (±SD) count of GFAP-positive cells per sq. mm of sections in cerebellar medulla of young (18-day-old) and adult (150-day-old) mice groups. *Yc* Young mice control group, *Ye* young mice CPF exposed group, *Ac* adult mice control group, *Ae* adult mice CPF-exposed group. One-way ANOVA [$F(3,65) = 5.631$; $p < 0.05$], $^{*}p < 0.05$ compared to Ac (adult control); $^{\#}p < 0.05$ compared to Ye (young CPF)

mice group (group Ae) exposed to dermal ½ LD_{50} of CPF for 14 days when compared with the age-matched control.

Discussion

Chlorpyrifos (CPF) is bioactivated to the neurotoxic metabolite, CPF-oxon and elicits toxicity through inhibition of AChE. The CYP450 metabolic capacity to metabolize CPF to both 3,5,6-trichloro-2-pyridinol (TCP), and CPF-oxon was adequate in rats as young as post-natal day 5. Furthermore, TCP concentration was seen to be three-fold higher in post-natal day 17 rats (Timchalk et al. 2006). Another study on comparison of in vivo AChE inhibition in neonatal and adult rats revealed that maximal brain AChE inhibition was similar in both age groups (Pope et al. 1991). Moreover, age related differences are much less pronounced or even nonexistent in case of repeated exposures to chlorpyrifos. We studied the CPF toxicity in young (18 days old) and adult (120 days old) mice and both the age groups have the ability to metabolise CPF.

Serum AChE level was significantly inhibited in both age groups of mice exposed to CPF. Group Ae (150 days old) had higher suppression of serum AChE than Group Ye (18 days old). The evidence of suppression of AChE following dermal application of CPF is described in a few studies. Latuszynska et al. (2003) found similar (79%) suppression of serum AChE levels in 3-month-old rats following dermal application of mixture of CPF and cypermethrin. Mitra et al. (2008) observed inhibition of serum AChE by 97% on dermal application of ½ LD_{50} of CPF for 3 weeks in adult mice. Howard et al. (2007) compared the effect of acute oral CPF exposure at $1 \times LD_{10}$ on neonatal and adult mice and noted similar suppression of cardiac cholinesterase activity in both neonate and adult with earlier reductions in muscarinic receptor binding in adults.

Browne et al. (2006) in an enzymatic and electrophysiological study of individuals (between 4 and 90 years) exposed to environmental OP found that serum AChE activity was significantly lower in exposed individuals compared to controls (41%) whereas both paraoxonase (PON) and arylesterase activity were significantly higher in the exposed subjects (447 and 441% of the control, respectively). Likewise, this study also noted that with dermal application of CPF, serum AChE level was reduced by 82% in adult and 69% in neonatal mice.

There is extensive literature showing that the developing brain might be far more sensitive than the mature one to low-dose effects of organophosphates (Johnson et al. 2009; Stapleton and Chan 2009). Young animals were reported more susceptible than adults to the acute toxicity of CPF (Dam et al. 2000). This is related to lower expression levels of PON in newborns whereby PON is responsible to inhibit and metabolise the AChE inhibitor. Foetal brain AChE activity was found to be 10% lower than the maternal one (Lassiter et al. 1999). However, our findings revealed otherwise. Young mice showed less and no significant increment in mean count of GFAP-positive cells compared to adult after exposure to CPF. One of the possible explanations for this would be due to faster recovery rate of AChE in young mice (Eaton et al. 2008). The rate of AChE synthesis was higher in young animals than in adults, leading to a faster recovery of AChE activity, which caused less neuronal damage. Besides that, exposure at lower, subchronic, repeated-dose (less than 1 mg/kg/day) of CPF, detoxication pathways in young animals are adequate to protect against cholinesterase inhibition. To corroborate, previous work (Mitra et al. 2010) reported that there was 800-fold increase in PON expression in young experimental mice after application of CPF for 14 days. Thus, it may be possible that due to upregulation of PON enzymes and an increased rate of AChE synthesis following repeated dose application of CPF, reduced GFAP expression was seen in young experimental group compared to the adult.

Young children are at higher risk of OP intoxication as they explore OP contaminated surfaces with the hand to mouth behaviour (Commission on life sciences 1993). Latuszynska et al. (2003) reported that dermal application of Nurelle D 550 EC (500 g of CPF and 50 g of cypermethrin) on 3-month-old rats showed histopathological changes such as pyknosis of Purkinje cells in cerebellum after 4 weeks of the experiment. Another study on prolonged exposure to a combination of stress and low doses of the pesticides (Pyridostigmine bromide, DEET, and Permethrin) in adult rats showed loss of Purkinje cells with locomotor and sensorimotor performance deficit (Abdel-Rahman et al. 2004). Damage or loss of Purkinje neurons could affect information integration and signal transmission in cerebellum. These changes would lead to incoordination in motor movement and reduction in motor learning (Hilber and Caston 2001). Among the regions of the central nervous system, the brain stem and forebrain are suggested to be more severely affected compared to the cerebellum due to rich cholinergic projections (Campbell et al. 1997). However Crumpton et al. (2000) observed changes in transcription factor expression both in forebrain and cerebellum following subtoxic doses of CPF in neonatal rats.

Glial fibrillary acidic protein has been recognised as a special marker for astrocytes (Gomes et al. 1999). It has been reported that the number and size of GFAP-immunoreactive astrocytes in the CNS increases following certain types of brain injury or in various pathological conditions (Davis et al. 2002). A few studies suggested that GFAP is a sensitive and early biomarker of neurotoxicity and the up-regulation of it precedes anatomically perceptible damages in the brain (O'Callaghan and Jensen. 1992; Ho et al. 2007). In this study, GFAP immunostaining in the

cerebellar cortex showed expression of brown stained GFAP-positive fibres in both young and adult mice exposed to dermal application of ½ LD_{50} of CPF. Cerebellar medulla showed more numbers of GFAP-positive cells in CPF exposed adult and young mice groups. This observation suggested that there is an increase in GFAP expression following dermal exposure to CPF. Abou-Donia et al. (2006) observed the effect of gestational exposure to combined CPF (1.0 mg/kg/day, dermal) and nicotine (3.3 mg/kg/day, subcutaneous) in postnatal day 30 rats. Similar to this study, reduction in Purkinje neurons and increased GFAP expression in white matter and granular cell layer of the cerebellum were observed. In addition, significant motor deficits in beam walk score and inclined plane performance and forepaw grip time were detected.

Activation of astrocytes may contribute to glial scar formation, which prevents regeneration and growth of axons. This may cause disturbance in neurotransmitter transmission associated with pathogenesis of chronic neurodegenerative diseases like Parkinson's disease (Wakabayashi et al. 2000).

In terms of quantitative evaluations, the young control mice displayed higher mean count of GFAP-positive cells when compared with adult control mice. In the rat brain, GFAP is fully expressed by astrocytes on postnatal days 10–32 and reach the peak of gliogenesis at postnatal days 11–14 (Roy et al. 2004). In this experiment, the young mice (18 days old) were undergoing brain growth spurt and increased mitotic activity of glial cells. Hence, more glial cells were differentiated into astrocytes and this leads to more GFAP-positive cells being expressed in young control group compared to the adult group.

Present study showed that sub-toxic dermal application of CPF was able to exert neurotoxic effects in both age groups, with significant increase in GFAP expression and count of GFAP-positive glial cells in the cerebellar medulla of adult mice. The findings are similar to that of a study by Veronesi et al. (1990) whereby repeated fenthion (organothiophosphate insecticide) exposures resulted in more extensive neuropathological changes in 12-month-old rats when compared with similar exposures in younger (2 months old) rats.

Conclusion

The relatively higher suppression of serum AChE level and more statistically significant increase in counts of GFAP-positive cells in cerebellar medulla observed in aged mice indicated their sensitivity towards neurotoxic effects of CPF. In conclusion, this study confirmed that the dermal exposure of CPF was able to exert neurotoxic effect in both young and adult groups. However, qualitative immunohistochemical observation along with quantitative count of GFAP-positive glial cells revealed that adult group (150-day-old mice) showed more GFAP expression in cerebellum compared to young group (18 days old) when exposed dermally to CPF (sub-toxic dose ½ LD_{50}).

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Conflict of interest The authors declare that there is no conflict of interest.

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