Tropical Biomedicine 27(1): 19–29 (2010)

Evaluation of difference in the neurotoxicity produced by dermal application of chlorpyrifos on the neonatal and adult mice

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Received 25 September 2009; received in revised form 30 November 2009; accepted 1December 2009

Abstract. Dermal exposure to organophosphate pesticide is important because of its popular use. This study planned to compare the changes in serum acetylcholinesterase, paraoxonase and neuronal density of hippocampus and iso-cortex between two age groups of Swiss albino mice (18-day-old and 150-day-old) after dermal application of ½ LD_{50} of chlorpyrifos for 14 days. Statistically significant reduction was observed in serum acetylcholinesterase (Mann-Whitney test, $p<0.05$) and neuronal density (Independent samples t-test, $p<0.05$) in exposed groups compared to the control. The reduction in serum AChE and neuronal density was more pronounced in exposed adult mice than in exposed neonatal mice. The paraoxonase level was insignificant in control neonatal mice, whereas it was 890-fold more in exposed neonatal mice. Upregulated paraoxonase levels may be extrapolated to produce relatively lower reduction of cholinesterase and neuronal density in neonatal mice.

INTRODUCTION

Organophosphate (OP) is the most commonly used synthetic pesticide. Chlorpyrifos (CPF) is one of the most popular OP pesticides that are being used in Malaysia. From 1998 to 2001, the total amount of pesticides containing CPF imported into Malaysia was 1410,940 kg (Kin Chai *et al*., 2005). The organophosphate CPF is a well-known inhibitor of acetylcholinesterase (AChE). OP poisoning is the commonest occupational injury and illness occurring in farmers, especially during preparation of the spray solutions, loading of spray tanks and applying the pesticides (Rathinam *et al*., 2005). Almost 50% of all poisoning related inquiries made in Malaysian National Poison Centre between1996 and 2000 were regarding pesticides. Analysis of data on poisoned children below 12 years showed an increased rate of exposure to pharmaceuticals and pesticides (Rahman *et al*., 2001). Neonates can be biologically more sensitive to the similar amount of toxicant on a body weight basis than adults. The infant physiologic systems usually produce higher blood levels for longer periods for most chemicals. The newborn's metabolic capacity matures rapidly and by 6 months of age, children display a sensitivity which is not more than adults based on the pharmacokinetic competence (Dourson *et al*., 2004). In a study by Thompson *et al*. (2003) urine samples were collected from 211 children and 213 adult relations of farm workers exposed to pesticide. DMTP (dimethylthiophosphate), metabolite of a pesticide was found in 88% of the child samples and 92% of the adult samples.

Three large epidemiological studies, examining 100 adult patients of nine years post-poisoning in a matched case control study, observed significant deficits in several cognitive tests of memory and abstraction (Rosenstock *et al.*, 1991). Rauh *et al.* (2006) found that children with prenatal exposure to CPF scored 3.3 to 6.5 points lower on the Psychomotor Development Index at 3 years of age.

Adverse health effects from exposure to pesticides depend on the intrinsic properties of the chemical, its dosage, and genetically determined variations in the host (Costa *et al.*, 2003). Paraoxonase (PON), an arylesterase is synthesized in liver and is closely associated with high-density lipoprotein in serum. The PON 1 activity is genetically determined and is reported to have marked inter-individual and racial variation (Singh *et al.*, 1999) and is believed to confer protection against OPs by catalytically inactivating oxons before they bind to the cholinesterase (Karanth *et al.*, 2000).

Some studies stated that young animals are more sensitive to OP toxicants compared to adults (Brodeur & DuBois, 1963; Harbison, 1975; Gaines & Linder, 1986). The mechanism(s) for the greater susceptibility of young rats is not well defined. One of the implied reasons was the relativity of PON levels. It was observed that animals with high PON1 activities against the toxic metabolites of a specific OP were more resistant to that OP than other animals with low PON1 activity (Brealey *et al.*, 1980; Furlong *et al.*, 1989).

Differences in the levels of PON among different age groups of same species are not well-established in scientific literature. The differences in the quantitative estimation of neuronal loss in the brain of different age groups following dermal exposure to CPF have also not been studied. The objectives of the present study were to investigate the levels of PON activity in juvenile and adult mice following dermal CPF exposure and also to compare the changes in the AChE levels & quantitative neuronal loss in Hippocampus and iso-cortex.

MATERIALS AND METHODS

Chlorpyrifos

Commercial preparation of chlorpyrifos (O, O-diethyl-3, 5, 6-trichloro-2-pyridyl phosphorothioate), Zespest, produced by Zeenex Agro Science Sdn Bhd, Kuala Lumpur, Malaysia was used in this study. The preparation contained 38.7% W/W of chlorpyrifos in organic solvent xylene. The commercial preparation was further diluted with xylene to prepare solutions containing 1/2 LD_{50} (101 mg/kg body weight chlorpyrifos in 1 ml) doses.

Animals and groupings

Male Swiss albino mice (species: Balb/c), 150 days old (25-32 g) and 18 days old (11-14 g) were used in this study. The animals were in good condition with normal skin and tail and were purchased from Laboratory Animal Resource unit of MRRC of Institute of Medical Research, NIH, Ministry of Health, Malaysia. The mice were fed with standard pellet feed and water *ad libitum*. They were housed in plastic cages (five in a cage) and were exposed to natural 12 hourly light and dark sequence. Animal experiments adhered to the principles stated in the guide-book of Laboratory Animal Care and Use Committee (ACUC) of the institute. The animals 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 group of mice whereas chlorpyrifos in the dose regimen of 1/2 LD_{50} was applied over the tails of experimental groups of mice.

Dermal application of chlorpyrifos

The chlorpyrifos solution in xylene was applied over the tail skin of the mice. The applications were done for 2 weeks (total application of 14 days). An absorptive fabric of surgical gauze was wrapped around the tail, followed by one layer of plaster, one layer of aluminium foil and another layer of plaster. One ml of the solution was applied onto the surgical gauze wrapping. The layers of barriers were applied to prevent the

solution from evaporation. Time of exposure was 4 hours daily. After removing the bandages, the remaining solution over the tail was swabbed off with wet gauze.

Estimation of serum acetylcholinestarase (AChE)

Blood samples were collected on 15th day of experiment by cardiac puncture of the individual mice kept under ether anaesthesia. Subsequently serum samples were pipetted out after refrigerated centrifugation at 13,000 rpm for 5 minutes. Serum AChE concentration was estimated by using Amplex Red acetylcholinesterase assay kit from Molecular probes Inc, USA (Invitrogen detection technologies). This kit provides an ultrasensitive method for continuously monitoring AChE concentration in a fluorescence microplate reader. The serum samples containing AChE were treated with Amplex Red reagent (10-acetyl-3, 7-dihydroxyphenoxazine) a sensitive fluorogenic probe for H_2O_2. After preparing the stock solutions (as per manufacturer's protocol), the AChE estimation was conducted using serum sample of the mice in different dilutions. One hundred 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 HRP, 0.2 U/ml choline oxidase and 100 µM ACh was prepared from the stock solutions given in the kit. The reaction began when 100 µL of the working solution prepared earlier was added to each well containing the samples and controls. The reactions were incubated for 30 minutes at room temperature, protected from light. The fluorescence emitted by the individual samples was measured in the Tecan microplate reader using excitation in the range of 560 nm and emission detection at 590 nm. For each point, background fluorescence was corrected by subtracting the values derived from the negative control.

The Log_{10} of the mean fluorescence readings were then plotted against the Log_{10} of the AChE concentration of the positive controls. A linear regression was obtained, indicating that the AChE activity in a sample increases exponentially with increasing concentration of a sample. Using Minitab and Microsoft Excel, the Log_{10} of the mean fluorescence readings were substituted into the equation of the linear regression line obtained from the positive controls. The Log_{10} of AChE concentration of the experimental samples thus could be calculated. Following this, the AChE concentration of the undiluted experimental samples could be estimated in serum samples of individual animals of Group Ac, Yc, Ae and Ye.

Estimation of serum paraoxonase (PON)

A part of the centrifuged serum sample was used for estimation of PON. Serum PON concentration was estimated by using EnzChek® Paraoxonase Enzyme assay kit from Molecular probes Inc, USA (Invitrogen detection technologies). Based on the hydrolysis of a fluorogenic organophosphate analog, a fluorometric assay was done to detect the oranophosphatase activity of paraoxonase (PON) enzyme present in the serum sample with the excitation/emission maxima of 360/450nm. Serum sample from each mouse under experiment was diluted 50-fold and 10 µL of the diluted sample was used. The diluted serum sample was added to the paraoxonase substrate in the wells of the microplate. Using a Tecan microplate reader the fluorescence generated by the chemical process of the organophosphate hydrolysis by the serum sample was estimated. Standard curve for fluorescence reference standard was generated by using stock solutions of the assay kit. The equation of the line fit to the standard curve generated was used to calculate the amount of fluorescent product (F). The amount of PON enzyme (E) involved in the reaction was determined by the formula (F/30min) X (1U/1nmol per min) = E.

Histological and histomorphometric studies

Perfusion of brains was carried out in six animals in each group by using 10% formal saline and areas of forebrain between optic

chiasma and infundibulum showing hippocampus were dissected out. 8 micron thick coronal serial sections of dissected area processed with paraffin were stained with Nissl stain (0.2% thionin in acetate buffer). Qualitative observations of stratum pyramidalis of CA-1, CA-2 & CA-3 areas of hippocampus and layer V of iso-cortex were done. Every 10^{th} section (5 slides in each animal) containing hippocampal and iso-cortical areas was chosen from each animal. Using brightfield compound Nikon microscope, YS100 (attached with Nikon camera), the slides were examined and photographed under 400X objective. For each slide, two areas of CA1, one area of CA2, two areas of CA3 and two areas of iso-cortex were randomly selected. Using Image-Pro Express software, count of neurons with prominent nucleolus within a measured rectangular area was performed in the selected regions. Random measurements of neuronal cell diameter were also taken for each region. The absolute neuronal density (P) per unit area of section was estimated using the formula P = A. M / L+M [Abercrombie, 1946]; M = Section thickness in micron (8 micron); L = Mean nuclear diameter of respective area; A = Crude neuronal count per sq.cm of section.

Statistical analysis

The absolute neuronal count (per cm^2) was subjected to statistical analysis using SPSS 11.5. Independent samples t-test was performed on the counts of each area (CA1, CA2, CA3 and iso-cortex) to determine if there is any statistically significant difference in absolute neuronal count between the control and CPF exposed groups in neonatal and adult mice. Likewise Mann-Whitney test was performed on the mean serum AChE and PON levels of control and treatment groups in each age-group.

RESULTS

Changes in the body weight

Control neonatal group Yc displayed weight reduction of 4.8% at the end of experiment. Comparatively CPF treated group Ye showed higher weight reduction of 10.76%. In adult mice, control group Ac showed a weight reduction of 7.43% and CPF treated group Ae showed weight reduction of 19.25% respectively. When comparing the percentage of weight reduction between control and experimental groups of each age group, the reduction rate in adult group of mice was almost two-fold higher than that of neonatal group.

Changes in serum AChE and PON levels

Mean serum AChE in control animals of both age groups is shown in Table 1. In neonatal group of mice it was 23% lower than 150 days old group of mice. Dermal application of ½ LD_{50} of CPF for 14 days decreased serum AChE levels significantly (Mann-Whitney U test, $p<0.05$) in both neonatal and adult groups of mice (Table 1). After dermal application of CPF for 14 days, serum AChE level was reduced by 69% in group Ye compared to the control group Yc, whereas in group Ae the similar reduction was 82%. Mean serum PON level in neonatal control group Yc at the end of experiment was insignificant (0.0008 U/ml) whereas the adult control group at the end of experiment showed a mean serum PON level of 0.65 U/ml which is 800-fold more than the neonatal group. After dermal application of ½ LD_{50} of CPF for 14 days, the mean serum PON level in group Ye was found to be 0.72 U/ml (Table 1) which was 890-fold more than the mean PON levels in control group Yc, unexposed to CPF. The mean serum PON level in group Ae exposed to dermal application of CPF was only 9.7% higher compared to the control adult group Ac, unexposed to CPF. Fig. 1 and Fig. 2 showed an inverse relationship between alteration in serum AChE levels and serum PON levels in both neonatal and adult groups of mice. With dermal application of CPF, serum AChE levels were reduced whereas an upregulation in serum PON levels were observed in both age-groups of mice.

Changes in the neurons of hippocampus and iso-cortex

Under nissl stain, the hippocampal neurons of group Yc (neonatal mice) showed

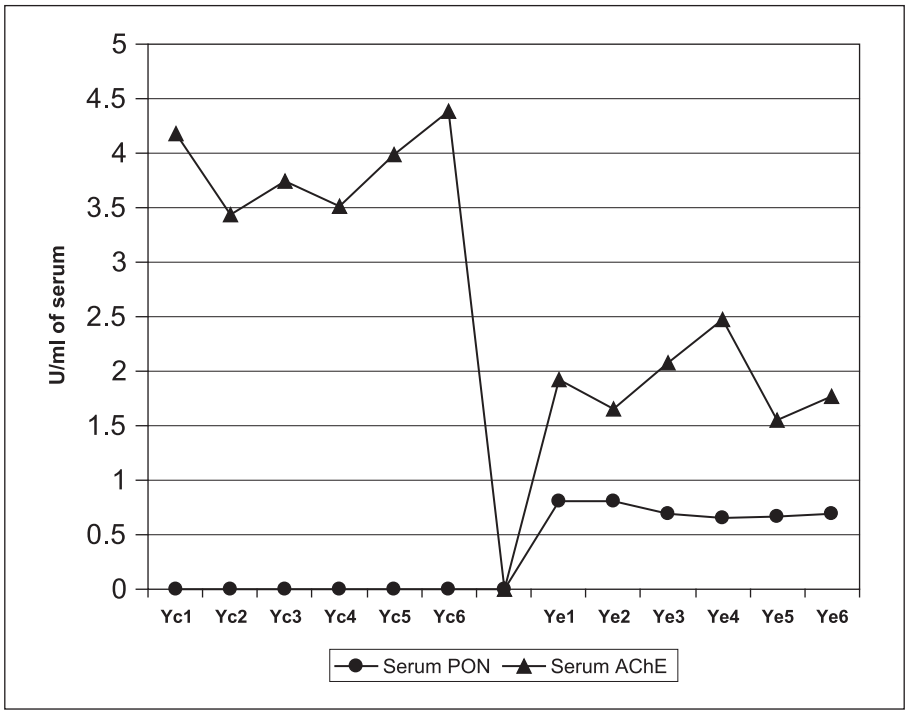

Figure 1. Line chart showing relative distribution of serum PON and serum AChE levels of individual mice in control and experimental CPF treated groups in neonatal mice. Yc = Neonatal control mice; Ye = Neonatal ½ dermal LD_{50} CPF treated mice.

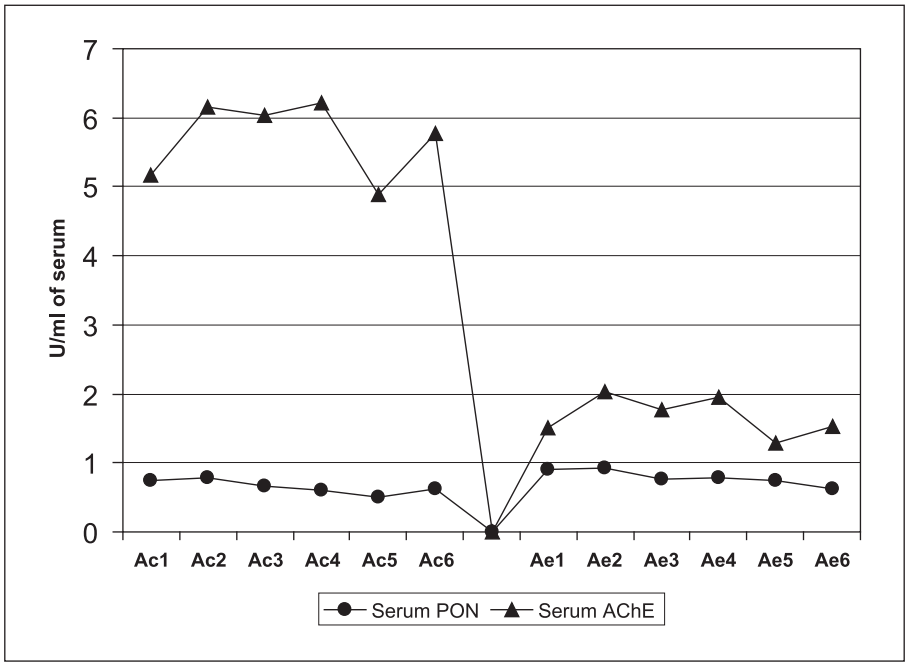

Figure 2. Line chart showing relative distribution of serum PON and serum AChE levels of individual mice in control and experimental CPF treated groups in adult mice Ac = Adult control mice; Ae = Adult ½ dermal LD_{50} CPF treated mice.

Table 1. Serum AChE and PON levels (Mean ± S.D) in control and CPF treated animals of neonatal and adult groups of mice

	Group Yc (Neonatal control)	Group Ye (Neonatal CPF)	Group Ac (Adult control)	Group Ae (Adult CPF)
Serum AChE (U/ml)	3.87 ± 0.37	1.19 ± 0.37#	5.05 ± 0.53	0.89 ± 0.27#
Serum PON (U/ml)	0.0008 ± 0.00002	0.72 ± 0.071#	0.65 ± 0.09	0.79 ± 0.11

Each value is expressed as Mean ± SD for six mice in each group. Mann-Whitney U test was done between control and CPF treated groups in each age-group; # represents $p < 0.05$ compared to the respective age-based control group.

evidence of continued mitosis (double nucleolus, Fig. 3A). Following dermal application of CPF in group Ye, the layers of hippocampal neurons were less dense and few neurons showed evidence of dissolution of nissl granules (Fig. 3B). Fig. 3C showed the hippocampal neurons of group Ac (150 days old mice). The neurons were apparently larger than the neurons in the neonatal group and showed prominent rim of nissl granules. Hippocampal neurons of group Ae, exposed to CPF showed evidence of increased pyknosis of neurons and vacuolation of neuropil around damaged neurons (Fig. 3D). The layers of neuron were found to be less dense also. Compared to group Ye (Fig. 3E), the iso-cortex of group Ae (Fig. 3F) showed evidence of increased pyknosis.

Changes in the neuronal density

All 3 areas of hippocampus in neonatal mice in control group Yc showed higher neuronal density per cm^2 compared to the adult mice control group Ac. However in iso-cortex, the group Ac showed higher neuronal density compared to the group Yc. The higher neuronal density in neonatal mice in hippocampal area is caused by higher brain growth spurt in hippocampal area of neonatal mice. The percentage of reduction in neuronal density in CA1, CA2 and CA3 hippocampal areas in CPF exposed group Ye was 4.9%, 3.5% and 14.9% respectively. Only CA3 area of hippocampus showed significant reduction ($p<0.001$) in neuronal density compared to the group Yc (Table 2). The iso-cortex layer V neurons in group Ye showed 12.9% reduction in neuronal density compared to the control group Yc and it was significant ($p<0.05$). The percentage of reduction in neuronal density in CA1, CA2 and CA3 hippocampal areas in CPF exposed adult group Ae was 20%, 11.3%, 26% respectively, which was higher than reduction seen in neonatal group of mice. Compared to the CPF exposed neonatal group Ye, the CPF exposed adult group Ae showed significant reduction in both CA1 and CA3 areas of the hippocampus. The iso-cortex layer V neurons in group Ye showed 12.9% reduction in neuronal density compared to the control group Yc and it was significant ($p<0.05$). Comparatively, the iso-cortex layer V neurons in group Ae showed 50% reduction in neuronal density (significant $p<0.001$) compared to the control group Ac (Table 2).

DISCUSSION

The weight loss observed in this study is consistent with previous findings following CPF exposure. Weight loss was identified as a major effect of acute CPF intoxication by Sánchez-Amate *et al.* (2001) and Chakraborti *et al.* (1993). Other signs observed in the mice after application of CPF were tremors, excessive movements as well as irritability and aggressiveness. Studies have proved that application of CPF produced neurobehavioural changes in animals. For example behavioural assessment (the plus-maze test of anxiety) on rats following acute CPF exposure had shown anxiogenic effects and significant behavioural alteration in the absence of any sign of cholinergic toxicity (Sánchez-Amate *et al.*, 2001).

The mean serum AChE level of control neonatal mice was found to be 23% lower

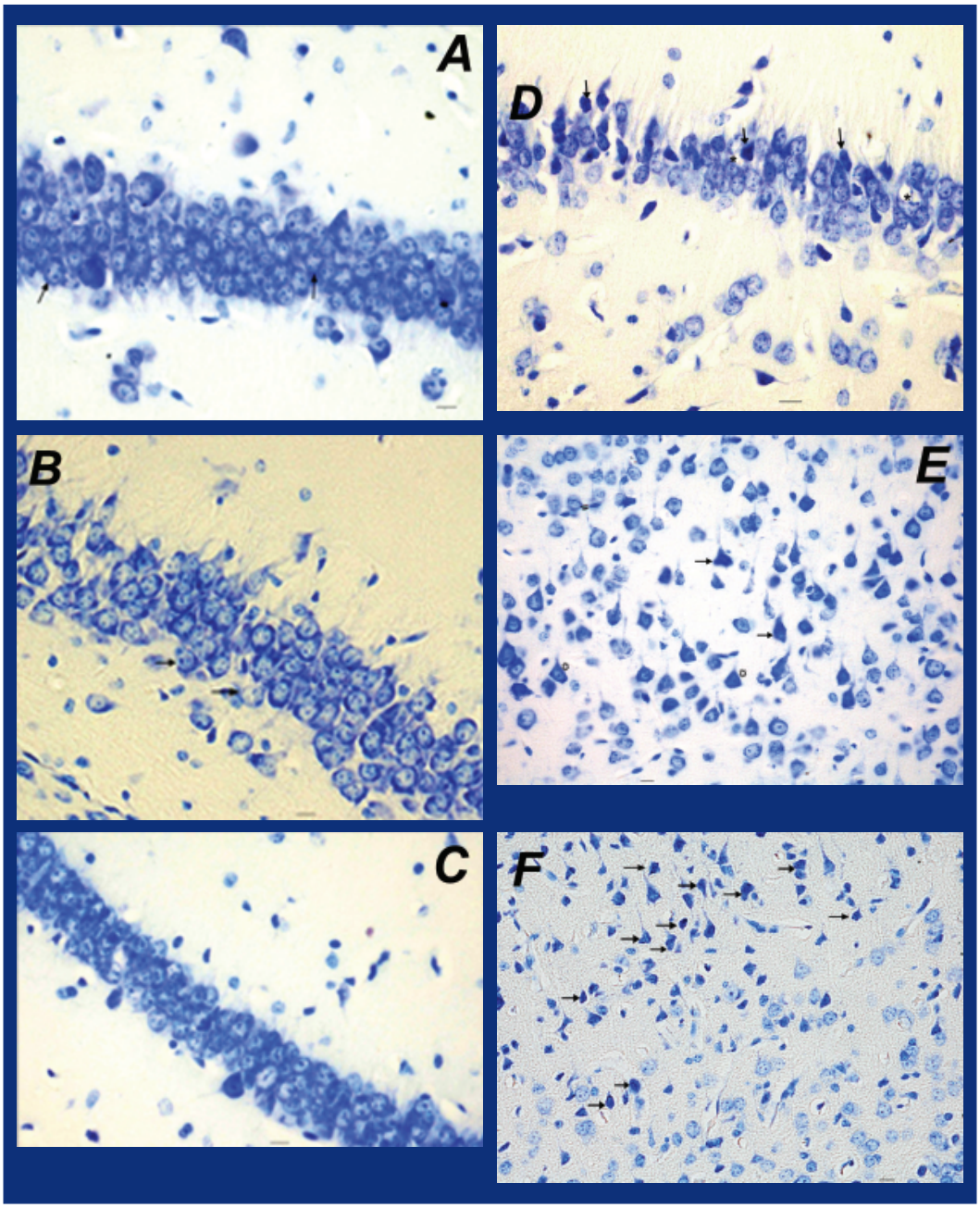

Figure 3. Photomicrograph of hippocampal CA3 neurons and iso-cortex in different groups of mice. A. Group Yc: CA3 neurons showing multiple layers, arrows show neurons with multiple nucleolus and prominent perinuclear nissl granules; B. Group Ye: CA3 neurons showing less density compared to group Yc, arrow shows dissolution of perinuclear nissl; C. Group Ac: CA3 neurons showing multiple layers but less dense than group Yc. D. Group Ae: CA3 neurons showing less density with arrows showing pyknosis of neurons, star showing vacuolation of neuropil; E. Group Ye: Neurons of iso-cortex, arrows showing pyknosis of neurons, star showing pyramidal neurons with prominent nissl; F. Group Ae: Neurons of iso-cortex, arrows showing multiple pyknosis of neurons; (Thionin stain, x400, Bar 8 µ)

Table 2. Neuronal density per sq cm of section in control and CPF treated animals of neonatal and adult groups of mice

	Group Yc (Neonatal control)	Group Ye (Neonatal CPF)	Group Ac (Adult control)	Group Ae (Adult CPF)
CA-1 area	75715.2 ± 12137.2	72006.4 ± 13408.7	72565.4 ± 7643.4	57990.3 ± 8853.9$^{\#}$
CA-2 area	58572.8 ± 11914.7	5651.4 ± 7937.9	51837.1 ± 11193.6	45998.4 ± 9085.9
CA-3 area	47436.3 ± 3052.2	40363.8 ± 5278.3**	29991.5 ± 12824.8	22145.9 ± 8633$^{\$}$
Iso-cortex	8965.2 ± 1915.9	7806.3 ± 1574.1*	10696.5 ± 2605.6	5301.9 ± 727.1$^{\#}$

Each value is expressed as Mean ± SD for six mice in each group. Independent samples t-test was done between control and CPF treated groups in each age-group; $^{\#}$ represents $p< 0.001$ compared to the control group Ac; $^{\$}$ represents $p< 0.05$ compared to the control group Ac; ** represents $p<0.001$ compared to the control Group Yc; * represents $p<0.05$ compared to control group Yc

than that in control adult mice. The mean serum PON level in control neonatal mice was also insignificant compared to that in control adult mice. Foetal brain cholinesterase activity was only 10% of maternal brain AChE activity and maternal carboxyl-esterase activity was 23-fold higher than foetal activity (Lassiter *et al.*, 1999). Serum AChE was significantly inhibited in both age groups of mice exposed to CPF. Group Ae (150-day-old) had higher suppression of serum AchE than Group Ye (18-day-old). The evidence of suppression of AChE following dermal application of CPF is described in just a few studies. Latuszyńska *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 effect of acute oral CPF exposure at 1xLD_{10} on neonate 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.

Costa *et al.* (1999) worked with a mouse model system and have shown that PON plays a major role in the detoxication of OP compounds processed through the P450/ PON1 pathway. It was also found that with injection of PON purified from rabbit serum in the mice, acute toxicity (assessed by the degree of acetylcholinesterase inhibition) of paraoxon and chlorpyrifos oxon is significantly decreased, compared to control animals. This study showed very low PON activity in young age group, supporting the suggestion that PON is developmentally regulated. Furlong *et al.* (2000) noted that newborn mice usually express very low levels of PON and adult levels are reached only after 3 weeks. In this study, following CPF exposure, PON level was highly elevated in 32-day-old mice (after 14 days of experiment), almost reaching the aged mice PON level. One possible suggestion would be that following application of CPF, the body is trying to 'correct' and eliminate the oxons to prevent further damage. As young mice are still in the growing phase of the life cycle, and PON level increases with age reaching adult level after 20 days of age (Furlong *et al.*, 2000), genes of CPF-exposed mice may be up regulated to produce more PON earlier in order to protect the vulnerable young mice from CPF.

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 with controls (41%) whereas both PON and arylesterase activity were significantly higher in the exposed subjects (447% and 441% of the control). Likewise this study also found that with dermal application of CPF, serum AchE level was reduced (82% in adult and 69% in neonatal mice) whereas an increase in serum PON level was observed in both age-groups

of mice (21% in adults and 890-fold in neonates).

As young children eat, drink, and breathe more per unit of body weight than adults, and often explore their environment orally, engaging in extensive hand-to-mouth behavior, hence they are more particularly at risk to OP intoxication, especially after contact with OP contaminated surfaces (Commission on Life Sciences, 1993).

Quantitative study showed that the neuronal density of hippocampus in control neonatal (32 days old) mice was higher than the control adult mice (164 days old). Miller (1995) found that neurons in the rat hippocampal formation are born over a protracted period, from gestational day 15 into adulthood. The phase of rapid growth or growth spurt in the neurons in rodents starts at birth and continues until adult neuronal numbers are obtained (5^{th} post-natal month). Similar to this study Mitra *et al.* (2009) observed pyknosis of neurons and vacuolation of neuropil in hippocampal CA1 and CA3 areas of adult mice with dermal application of both 1/5 LD_{50} and ½ LD_{50} of CPF for 3 weeks. Histomorphometric study revealed reduction in mean absolute neuronal density in all experimental mice, with overall greater reduction seen in aged group, statistically significant in CA3, CA1 and Iso-cortex areas. However in the young mice only changes in CA3 and Iso-cortex areas were statistically significant. Young animals are apparently found to be more sensitive than the adults to the neurotoxicity induced by CPF administered by different routes (Pope *et al.*, 1991; Pope & Chakraborti, 1992; Whitney *et al.*, 1995; Atterberry *et al.*, 1997; Moser & Padilla, 1998). However the relative sensitivity of aged group is not well understood. Chakraborti *et al.* (1993) and Stanton *et al.* (1994) claimed that following CPF exposure, the neurochemical and neurobehavioural changes in neonatal rats are more transient and short-lived compared to the adults. This study proved that neurotoxicity was more severe in the adult mice in the parameters of serum AchE inhibition and neuronal density reduction in hippocampus and iso-cortex compared to the neonatal mice, following dermal application of chlorpyrifos. Veronesi *et al.* (1990) reported that following fenthion exposure, 12 month old rats showed more neuropathological changes compared to 2 month old ones.

It was found that the adult mice were more sensitive towards dermal application of CPF. The relatively higher suppression of serum AChE level and more statistically significant reduction in neuronal counts in CA3, CA1 areas of hippocampus and layer V neurons of Iso-cortex observed in aged mice indicate their sensitivity towards neurotoxic effects of chlorpyrifos. Interestingly, PON levels seemed to have been up-regulated following CPF exposure in neonatal mice which may explain the relatively lower reduction of serum AChE and neuronal density suppression than aged mice. Considering inverse relationship between serum AChE and serum PON levels observed in this study, it may be useful to incorporate these two biomarkers into the health screening programme of community exposed to organophosphate pesticides.

Acknowledgements. The authors would like to thank the Chairman of the Research Committee and the Dean of the Postgraduate Studies and Research Department of the International Medical University for the grant to carry out this research project.

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