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EVALUATION OF NEUROTOXICITY OF REPEATED DERMAL APPLICATION OF CHLORPYRIFOS ON HIPPOCAMPUS OF ADULT MICE

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Mitra NK, How HS, Nadarajah VD: Evaluation of neurotoxicity of repeated dermal application of chlorpyrifos on hippocampus of adult mice. *Ann Agric Environ Med* 2008, **15**, 211–216.

Abstract: Dermal absorption of chlorpyrifos, an organophosphate insecticide is important because of its use in agriculture and control of household pests. The objectives of this study are to investigate firstly, the biochemical changes in the blood and secondly, histomorphometric changes in the hippocampus of adult mice following dermal application of chlorpyrifos in sub-toxic doses. Male Swiss albino mice (60 days) were segregated into one control and two treated groups (n=10). Chlorpyrifos, diluted with xylene, was applied in doses of 1/2 of LD_{50} (E1) and 1/5 of LD_{50} (E2) over the tail of mice of the two treated groups, 6 hours daily for 3 weeks. AChE levels in the serum and brain were estimated using a spectrophotometric method (Amplex Red reagent). Coronal serial sections were stained with 0.2% thionin in acetate buffer and pyramidal neurons of Cornu Ammonis of hippocampus were counted at 400× magnification using Image Pro Express software. At the end of 3 weeks, body weights were reduced significantly in E1 group. Serum AChE concentrations were reduced by 97% in E1 and 74% in E2 groups compared to controls. The neurons of CA 3 and CA 1 in the hippocampus showed evidences of morphological damage in both treated groups. Furthermore, the neuronal count was significantly reduced in CA 3 layer of hippocampus in E1 group.

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Key words: dermal toxicity, chlorpyrifos, repeated exposure, cholinesterase inhibition, hippocampus, histomorphometry.

INTRODUCTION

Chlorpyrifos (O, O-diethyl-3, 5, 6-trichloro-2-pyridyl phosphorothioate), an organophosphorus (OP) pesticide was first registered in the United States in 1965. Currently, there are over 850 registered chlorpyrifos products in United States. Despite recent restrictions on home use in certain countries, it remains a popular pesticide throughout the world. In 2000, the National Center for Food and Agriculture Policy, Washington DC, estimated that up to 3 million pounds (1.4 million kg) of chlorpyrifos was being used in the home-and-garden market each year. Chlorpyrifos is used to destroy cockroaches, fleas, spiders and flies indoors, as well as mosquitoes, ticks and bees outdoors [12]. It is absorbed via the gastrointestinal tract and to a lesser extent through intact skin and by inhalation. It is moderately toxic following acute oral, dermal and inhalation exposure routes and is categorized as a Class II toxin by the US Environmental Protection Agency [25].

Most occupational exposures to pesticides are through inhalation or dermal exposure [24]. A study in Oregon found that the neurobehavioural performance of Hispanic immigrant farm workers to be lower than that observed in a non-agricultural Hispanic immigrant population, and a positive correlation was found between urinary organophosphate metabolite levels and poor performance on some neurobehavioural tests [18]. Savage *et al.* [21], in an epidemiological study followed 100 patients for an average

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of nine years after being admitted to hospital with organophosphate poisoning, and compared them with matched controls. Significant deficits in several cognitive tests of memory and abstraction were found, but no differences in Electroencephalography findings were observed [21].

Individuals with a history of exposure to low sub-clinical levels of chlorpyrifos reported defects in concentration, word-finding and short-term-memory [10]. Hippocampal formation of the brain is responsible for consolidating explicit memories, such as the conscious recollection of facts and formation of spatial memories. Studies of lesions in humans, primates and rodents have suggested that the primary role of the hippocampus is to act as temporary memory buffer which is required for the consolidation of long-term memory [8].

Many animal studies have documented significant neurological changes in the developing foetus and postnatal rats following subcutaneous and oral routes of administration of chlorpyrifos [7, 14, 16, 20]. Postnatal chlorpyrifos exposure in rats by subcutaneous injection showed evidence of cell damage and loss, as well as interference with the development of cholinergic pathways [5, 22, 23].

The speed of absorption of chlorpyrifos depends on the solvent used and is usually slower than the uptake by other routes. A study carried out on human volunteers by Meuling *et al.*, in 2004 reported that following single dermal application of chlorpyrifos diluted in ethanol for 4 h, 4.3% of the applied dose was absorbed, and chlorpyrifos was retained by the skin with mean elimination half-life of 41 h [13]. An acute dermal toxicity study on rats using chlorpyrifos soluble in xylene found that acute dermal LD_{50} for male rats is 202 mg/kg [6]; however, acute dermal LD_{50} for mouse was not found in literature search.

Single dermal application of chlorpyrifos in a sub-clinical dose of 30 mg/kg (15% of dermal LD_{50}) on pregnant Sprague-Dawley rats inhibited maternal and foetal brain AChE activity within 24 h of exposure [2]. Very few reports have been found in literature regarding quantitative estimation of neuronal damage in the areas of the central nervous system, affected by repeated sub-toxic exposure of the skin to chlorpyrifos in adult animals. This study evaluates the effect of repeated dermal application of chlorpyrifos in sub-toxic doses in adult Swiss albino mice on serum and brain acetylcholinesterase levels. This was followed by quantitative estimation of neuronal loss, if any, in the cornu ammonis pyramidal neurons of hippocampus (in view of reported neuro-behavioural and short-term-memory changes following repeated exposure to chlorpyrifos in human).

MATERIALS AND METHODS

Chlorpyrifos. Commercial preparation of chlorpyrifos (O, O-diethyl-3, 5, 6-trichloro-2-pyridyl phosphorothioate), Zespest (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) and 1/5 LD_{50} (40.4 mg/kg body weight chlorpyrifos in 1 ml) doses.

Animals. Male Swiss albino white mice (species: Balb/c), 60 days old (20–25 g) were used in this study. All animals were in good condition without any macroscopic changes in the skin and tail. The mice were fed with standard pellet feed and water *ad libitum*. They were housed in plastic cages (5 in a cage) and exposed to a natural 12 h light and dark sequence.

Dermal application of chlorpyrifos. The animals were divided into 3 groups (n=10). The chorpyrifos solution in xylene was applied on the tail skin of 2 experimental groups, in the dose regimen of 1/2 LD_{50} (E1 group) and 1/5 LD_{50} (E2 group). The applications were performed for 3 weeks, excepting Sundays (total application of 18 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 6 h daily. After removing the bandages, the remaining solution over the tail was swabbed off with wet gauze. The mice of the control group were exposed to dermal application of 1 ml of xylene simultaneously and in a similar way.

Body weights of all the animals were measured twice a week. After 3 weeks, the animals were anaesthetized with intraperitoneal administration of pentobarbitone. Blood samples were collected by cardiac puncture and brains were collected for acetylcholinesterase assay and histomorphometric studies.

Estimation of serum acetylcholinestarase (AChE). 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 pooled 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 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 min 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 can be estimated in pooled serum samples of Control, E1 and E2 groups.

Estimation of brain acetylcholinestarase (AChE). The portion of the brain containing the cerebral hemisphere along with cerebellum was removed from half of the animals in each group. After collection, the brain samples were weighed and stored in PBS solution at -80°C temperature until further use. The brain samples were homogenized in PBS solution at 4°C and used for AChE estimation. A procedure for determining the protein concentration of each brain homogenate sample was carried out using Bio-Rad Lab's Quickstart™ Bradford dye reagent, with bovine serum albumin (Sigma-Aldrich) as a standard. This procedure allowed estimation of AChE concentration, in terms of U/mg protein in the sample. This calculation was crucial as the amount of brain samples used to make the homogenate may vary between each animal.

Estimation of AChE concentration in brain homogenate samples was performed using the same materials, equipments and protocol used in estimation of serum AChE. However, the dilutions of the brain homogenate samples and positive controls were performed using PBS solution instead of reaction buffer. The negative control was also the PBS solution.

Histological and histomorphometric studies. Perfusion of brains was carried out by using 10% formal saline and areas of forebrain between optic chiasma and infundibulum showing hippocampus and iso-cortex were dissected. Eight 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 and CA-3 areas of hippocampus were performed (Fig. 3). Every 10^{th} section (5 slides in each animal) containing hippocampal area was chosen from each animal. Using a brightfield compound Nikon microscope, YS100 (attached to a Nikon camera), the slides were examined and photographed under 400× objective. For each slide, two areas of CA1, one area of CA2, two areas of CA3 were randomly selected. Using Image-Pro Express software, a 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 [1]; M= Section thickness in micron (8 micron); L = Mean nuclear diameter of respective area; A = Crude neuronal count per sq.mm of section.

Statistical analysis. The absolute neuronal count (per mm^2) was subjected to statistical analysis using SPSS 11.5. One Way ANOVA was performed on the counts of each area (CA1, CA2 and CA3) to determine if there is any statistically significant differences in absolute neuronal count between the treatment groups in that area.

RESULTS

Body weight. Mice receiving dermal application of 1/2 LD_{50} chlorpyrifos [E1] showed continued reduction in mean body weight, both in the middle (10% reduction) and at the end of the experiment (17.5% reduction). On the contrary, the mice exposed to dermal application of 1/5 LD_{50} of chlorpyrifos [E2] showed slight increase in mean body weight (7.9% in the middle, and 3% at the end of the experiment). The control group, receiving dermal application of xylene showed continued increase in mean body weight (11% in the middle and 19.5% at the end of the experiment).

Changes in serum acetylcholinesterase (AChE). Mean fluorescence readings of the serum samples after 3 weeks of exposure to chlorpyrifos showed a trend that AChE levels were reduced in groups E1 and E2, compared to control.

The percentage of inhibition (Fig. 1) of calculated AChE concentration in the pooled serum of group E2 was 74.64% compared to the serum samples in control group. The group E1 receiving 1/2 LD_{50} of chlorpyrifos produced higher percentages of inhibition (97.06%).

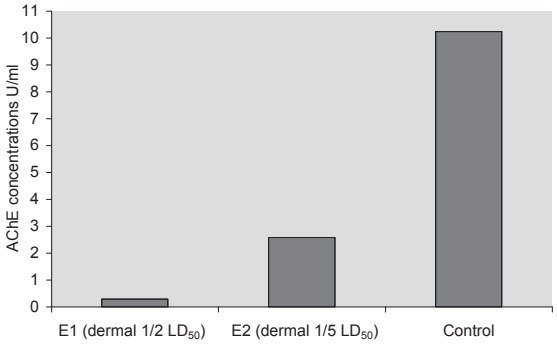

Figure 1. Bar charts showing mean AChE concentration in pooled serum samples (from 10 mice) of each group at the end of 3 weeks, derived from 40× dilution of the samples.

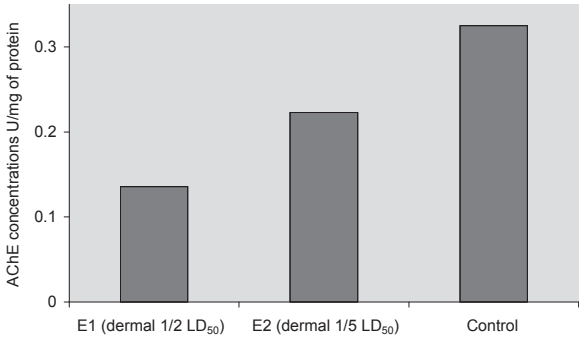

Figure 2. Bar charts showing mean AChE concentration per mg of protein in brain samples of each group derived from 40× dilution of the samples.

Changes in brain acetylcholinesterase (AChE). The mean brain AChE concentration was inhibited by 31.28% in group E2 (Fig. 2) receiving dermal application of 1/5 LD_{50} of chlorpyrifos, compared to control group. Group E1 receiving ½ LD_{50} of chlorpyrifos showed 58.15% inhibition.

Changes in histological and histomorphometric studies. Under qualitative observations, the CA3, CA2 and CA1 hippocampal pyramidal neurons in E1 group receiving dermal application of 1/2 LD_{50} of chlorpyrifos showed evidences of neuronal damage in the form of pyknosis of nuclei and vacuolation of neuropil around the damaged neurons. The pyknosis of nuclei was more commonly seen in CA3 (Fig. 5) and CA1 regions compared to the CA2 region. The hippocampal pyramidal neurons in E2 group receiving dermal application of 1/5 LD_{50} of chlorpyrifos showed dissolution of nissl granules and discontinuity of the nuclear membrane (Fig. 6). Under quantitative histomorphometric study, the density of pyramidal neurons, as evidenced by the absolute neuronal count per sq.mm area of section in control group of mice, was found to be more in CA1 region (6285.9/sq.mm) compared to that in CA3 (5726.9/sq.mm) and CA2 regions (5603.9/sq.mm). The mean absolute neuronal count was reduced in both the mice groups E1 and E2 compared to the mean count of the control group in CA3, CA2 and CA1 regions (Tab. 1). One way ANOVA analysis found statistically significant difference in mean absolute neuronal count between the three groups (control, E1 and E2) only in CA3 region of hippocampus. In *post-hoc* statistical test (Bonferroni), the mean absolute neuronal count per sq.mm area in E1 group of mice receiving dermal application of 1/2 LD_{50} of chlorpyrifos was reduced significantly ($p<0.05$) compared to the count in control group. The reduction in count in E2 group having dermal application of 1/5 LD_{50} of chlorpyrifos was not statistically significant.

Table 1. Results of neuronal count per sq.mm of sections in regions of hippocampus after dermal application 1/2 LD_{50} and 1/5 LD_{50} of chlorpyrifos.

Examined groups (n=10)		CA3 region	CA2 region	CA1 region
Control	Mean	5726.9	5603.9	6285.9
	SD	1282.5	1352.3	1345.6
E1	Mean	5114.6*	5098.2	6040.5
	SD	1281.2	1285	1177.6
E2	Mean	5388.2	5472.7	5742.8
	SD	845.8	809.8	1296.3
One way ANOVA		* ≤ 0.05	ns	ns

ns – not significant; * – mean difference from control group significant (≤0.05) post-hoc Bonferroni test

DISCUSSION

Carr *et al.* [3], observed significant body weight reduction in post-natal rats subjected to higher dose of oral

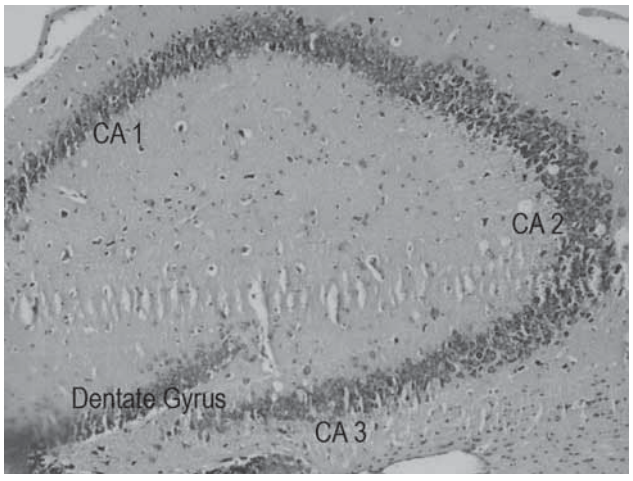

Figure 3. Coronal section of mice hippocampus showing pyramidal neurons of cornuammonis (CA3, CA2 & CA1 regions). Nissl, × 100, 8 μ.

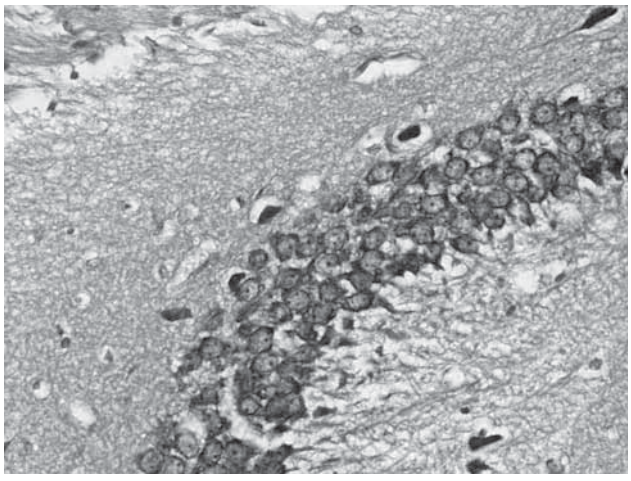

Figure 4. CA3 pyramidal neurons of hippocampus in a mouse of control group with dermal application of xylene for 3 weeks. Central nucleolus with perinuclear rim of nissl granules. Neurons are arranged in 4 to 5 layers and are closely packed. Nissl, ×400, 8.

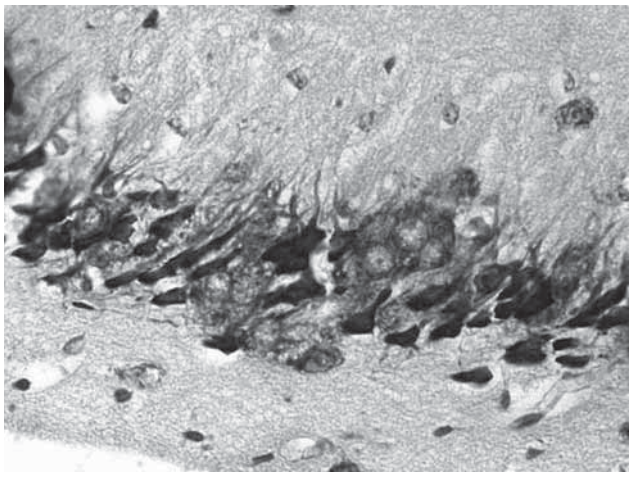

Figure 5. CA3 pyramidal neurons of hippocampus in a mouse exposed to dermal application of chlorpyrifos (101 mg/kg) for 3 weeks. Pyknosis of neurons were prominently seen. Non-pyknosed cells lost normal shape due to loss of perinuclear rim of nissl granules. Nissl, ×400, 8 µ.

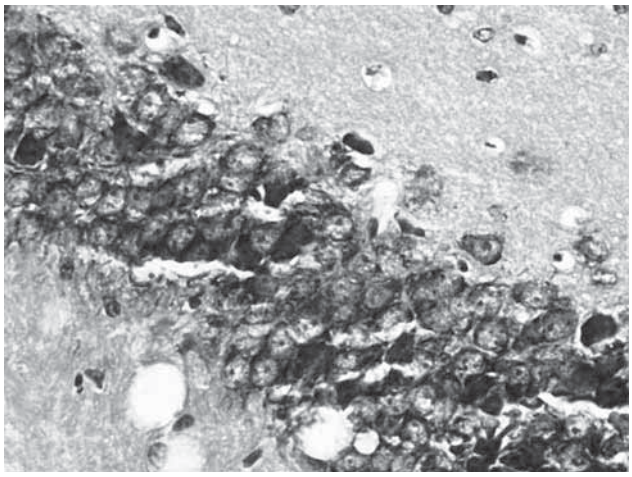

Figure 6. CA3 pyramidal neurons of hippocampus in a mouse exposed to dermal application of chlorpyrifos (40.4 mg/kg) for 3 weeks. Dissolution of peri-nuclear nissl granules and loss of nuclear membrane gave fuzzy appearance to most of the neurons. Few cells showed pyknosis. Nissl, ×400, 8 µ.

chlorpyrifos treatment when the dosage was increased progressively from 3–12 mg/kg bw/day from day 1 to day 21 [3]. But no difference in body weight was found among lower dosage of 3 mg/kg bw/day from day 1 to day 21. This study found that mice receiving dermal application at 1/2 LD_{50} of chlorpyrifos showed continued reduction in mean body weight. Mice receiving dermal application of 1/5 LD_{50} of chlorpyrifos showed increase in body weight, but the percent of increase was less than that in the control group of mice receiving dermal application of xylene.

Latuszyńska *et al.* [11], used two different dosages of dermal chlorpyrifos application (1/70 and 1/14 dermal LD_{50}) in combination with cypermethrin (0.5 and 2.7 mg/cm^2) which resulted in high percentages of plasma AChE inhibition after one week (81% and 92%, respectively) and four weeks (91% and 95%, respectively) [11]. This study observed 74.64% and 97% reduction in serum AChE in mice groups treated with dermal application of 1/5 LD_{50} of chlorpyrifos and 1/2 LD_{50} of chlorpyrifos for 3 weeks.

Pregnant rats treated with a single dermal application of 15% LD_{50} of chlorpyrifos inhibited maternal and foetal brain AChE activity within 24 hours of exposure by 48% and 67% of control activity, respectively [2]. Moser [15], observed that single oral doses of 30 mg/kg b.w. of chlorpyrifos produced 60% inhibition in brain and 80% inhibition in whole blood AChE [15]. Both these studies showed that brain AChE inhibition percentages were relatively less compared to plasma AChE inhibition. This study found 58% and 31% inhibition in mean brain AChE in mice groups treated with dermal application of 1/2 LD_{50} of chlorpyrifos and 1/5 LD_{50} of chlorpyrifos for 3 weeks, respectively. These values were significantly lower than the serum AChE inhibition.

The inhibition in brain AChE levels observed in this study is in contrast to the general perception that dermal exposure to chlorpyrifos is not as significant or as dangerous as other routes of exposure. In fact, chlorpyrifos and its metabolites have been suggested to establish a reservoir and accumulate in skin after dermal exposure, resulting in longer exposure duration and more adverse long-term effects [13].

Icenogle *et al.* [9], observed that learning and memory (assessed using the 16-arm radial maze) were adversely affected in offsprings, when chlorpyrifos was administered in the sub-toxic dose of 1 or 5 mg/kg/day to pregnant rats on gestational days 9 to 12 [9]. Qiao *et al.* [16], found that following a low dose (1 or 2 mg/kg/day subcutaneously) exposure of chlorpyrifos during gestational days 17–20, the cell size of the foetus was enhanced in the brainstem and reduced in the forebrain [16]. These effects were statistically significant overall by ANOVA as well as in *post hoc* tests. In a later study, Qiao *et al.* [17], summarized that in combination with previous results, the current findings indicate that the developing brain, especially the hippocampus, was adversely affected by chlorpyrifos, regardless of whether exposure occurred early or late in brain development.

Roy TS *et al.*, 2005 found significant reduction in the number of neurons in CA1 and CA3 regions of hippocampus of juvenile rats (postnatal days 15 and 20) following subcutaneous administration of 5 mg/kg of chlorpyrifos on postnatal days 11 to 14 [19]. Latuszyńska *et al.* [11], administered a mixture of chlorpyrifos (27.8 mg/cm^2) and cypermethrin (2.7 mg/cm^2) dermally over the tail of rats for 4 weeks, and observed pyknosis of neuronal cells in stratum granulosum of area dentatae and CA1 region of hippocampus [11]. This study found pyknosis of nuclei more commonly seen in CA3 and CA1 regions of hippocampus following dermal application of chlorpyrifos in a dose of 101 mg/kg body weight (1/2 dermal LD_{50}) for 3 weeks. It was also found that a dose of 1/2 dermal LD_{50} was sufficiently potent to cause statistically significant (One way ANOVA followed by *post hoc* test) reduction in mean absolute neuronal count per sq.mm of section in CA3 area of hippocampus after 3 weeks of application.

Although dissolution of nissl granules and discontinuity of nuclear membrane were observed in the pyramidal neurons of hippocampus in the group of mice receiving dermal application of chlorpyrifos in a dose of 40.4 mg/kg body weight (1/5 dermal LD_{50}), no significant difference in mean absolute neuronal count was found in pyramidal neurons of hippocampus. A review by Clegg *et al.*, 1999 suggested that clinical signs of cholinergic toxicity may not be produced if inhibition of brain cholinesterase is below 70% [4]. Cognitive or behavioural defects may not be observed until substantial brain cholinesterase inhibition occurs. They also suggested that there were no indications that chlorpyrifos caused delayed neurotoxicity at dose levels below twice the oral LD_{50}.

Afferent input to the hippocampus from entorhinal cortex projects to the dentate gyrus, from where it passes to CA3 neurons. Dendrites of CA3 neurons project to CA1 neurons. Efferent pathway passes from CA1, then subiculum, alveus and fimbria and ultimately forms fornix. Hence, CA3 region of cornu-ammonis is occupying a central position and plays an important role in the process of consolidation of short-term memory. The reported deficits in several cognitive tests of memory, abstraction, word-finding and concentration [10, 21] following occupational exposure to chlorpyrifos, may be extrapolated to be caused by reduction in neuronal count in CA3 pyramidal neurons of hippocampus, as evidenced by this study which found statistically significant reduction in neuronal count in CA3 region of adult mice following repeated dermal exposure to chlorpyrifos.

CONCLUSIONS

Dermal applications of sub-toxic doses of chlorpyrifos on Swiss albino mice can cause reduction in serum and brain AChE concentration. The neurotoxic effect on the areas of the brain responsible for cognitive functions is further evidenced by qualitative changes indicating neuronal damage and quantitative changes indicating neuronal loss in cornu ammonis neurons of hippocampus.

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