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Potential biomarkers of dichlorvos induced neuronal injury in rats

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The present study was designed to develop suitable biochemical markers of chronic dichlorvos exposure using rat as the animal model. Animals were exposed to dichlorvos (6 mg kg^{-1} (body weight) day^{-1}) for 8 weeks and the activities of five potential markers were assayed. Acetylcholinesterase, assayed as an index of cholinergic function, was found to decrease in both haemolysate and brain tissue. Cytochrome oxidase, used as a marker of impaired energy metabolism, was also seen to decrease in platelets and brains of dichlorvos-treated animals. However, acid phosphatase, a lysosomal marker of tissue injury, was increased in both serum and brains of experimental animals. Chronic dichlorvos exposure also led to a decrease in the activity of glucose-6-phosphate dehydrogenase, which was assayed in brain as an index of oxidative stress. Dichlorvos administration did not affect 2', 3'-cyclic nucleotide phosphohydrolase. The present study therefore, indicates that apart from acetylcholinesterase, which is probably a non-specific marker of dichlorvos neurotoxicity, the levels of cytochrome oxidase, acid phosphatase and glucose-6-phosphate dehydrogenase may serve as useful determinants of dichlorvos-induced neuronal injury.

Keywords: dichlorvos, organophosphates, brain.

Introduction

The worldwide production and use of pesticides continues to rise unabated, with an almost 10-fold increase in the past three decades. Among the synthetic organic pesticides, the organophosphate (OP) compounds currently constitute the most widely used class of pesticides, for both agricultural and landscape pest control (WHO 1990, Jeyaratnam 1990). They have gained precedence over the organochlorines due to their relatively non-cumulative nature. Because of their ability to interact with biological systems other than their primary targets, these pesticides constitute a consistent and potent health hazard for humans and animals. In fact, the OP pesticides are currently responsible for more poisonings than any other single class of pesticides. Recent statistics show that these pesticides account for nearly 3 million poisonings with over 2 million deaths annually (Rosenstock *et al.* 1991, Sultatos 1994).

Dichlorvos is an OP pesticide which is being used worldwide for the protection of stored products and crops, as well as a general purpose public-health insecticide. Dichlorvos has been reported to be severely neurotoxic, by virtue of its ability to cross the blood-brain barrier (Dambska *et al.* 1984). It has been reported to produce both neurochemical and neurobehavioural alterations, which include deficits in cognitive as well as motor functions (Sarin and Gill in press). Typical symptoms of dichlorvos poisoning include achrymation, salivation, rhinorrhea, and

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bronchoconstriction accompanied by severe cardiac disturbances. Death may occur as a result of complete central nervous system (CNS) depression (Marrs 1993).

Since the brain is a primary target of dichlorvos toxicity (Kobayashi *et al.* 1986), alterations in CNS function should be among the first and foremost to be assessed following such poisoning. A primary obstacle to the investigation of the CNS, however, lies in the inaccessibility of the brain, which prevents the direct collection of specimens. Brain biopsies are difficult to obtain and postmortem samples, by definition, become available only after the death of the patient. It is therefore imperative to develop sensitive and accurate biomarkers of dichlorvos poisoning, so that suitable therapeutic strategies may be developed soon after the onset of symptoms. Accurate markers of neuronal injury, apart from predictive diagnostic capacity, may also play a pivotal role in the hazard identification of potential neurotoxins (O'Callaghan 1988, Evans 1995).

In the present study, we have therefore attempted to identify suitable biochemical markers of dichlorvos-induced neuronal injury, which may prove useful in defining the onset of dichlorvos poisoning, in view of the general population's increasing risk of exposure to this widely used OP pesticide.

Materials and methods

Tris-hydroxy methyl amino methane, 5, 5'-dithiobis-2-nitrobenzoic acid, 2',3'-cAMP, 2'-AMP, and NADPH, were obtained from Sigma Chemical Co., USA. Glucose-6-phosphate and disodium phenyl phosphate were obtained from Sisco Research Lab, Bombay, and 2, 2-dichlorovinyl dimethyl phosphate (dichlorvos) was purchased from Hindustan Ciba–Geigy Ltd., Bombay, India. All other chemicals used were highest grade commercial products available locally.

Animals

Male albino Wistar rats weighing between 140–160 gms were procured from the Institute Animal House. Animals were kept in polypropylene cages in well-ventilated rooms and were provided *ad lib* access to water and standard laboratory chow (Hindustan Lever Ltd., Bombay, India).

Experimental design

Rats were divided into two groups: dichlorvos-treated, where animals received dichlorvos (6 mg kg^{-1} (body weight) day^{-1}, for 8 weeks, subcutaneously, in corn oil), and controls (the animals received only an equal volume of the vehicle).

During the course of the treatment, blood was drawn at fortnightly intervals from the orbital sinus of the rats (approximately 3 hrs after dichlorvos administration), for the estimation of enzymes in peripheral tissues (serum, platelets and haemolysate). At the end of the treatment schedule, the animals were sacrificed (decapitation under light ether anaesthesia) 5–6 hrs after the final dose of dichlorvos. The brains were excised and a 10% (w/v) homogenate of the brain was prepared. This was then centrifuged at 750 *g* for 10 min to remove the cellular debris and subjected immediately to various biochemical investigations. All procedures were carried out at 4°C.

Analytical procedures

Acetylcholinesterase (AChE). Acetylcholinesterase was assayed in the haemolysate and brain by the method of Ellman *et al* (1961). For the preparation of haemolysate, 0.2 ml of blood was drawn from the animals and washed three times with physiological saline (0.9% NaCl), followed by centrifugation at 3000 rpm for 10 min. The washed cells were then suspended in 0.5 ml digitonin (200 mg l^{-1}) and refrigerated for 15 min to lyse the red blood cells. This was followed by another centrifugation for 10 min at 3000 rpm. The supernatant obtained served as the haemolysate. The reaction mixture contained 0.1M phosphate buffer (pH 7.5), 0.66 mM dithiobisnitrobenzoic acid, 0.013% Triton X-100 (only for brain homogenate) and the requisite amount of sample (0.025 ml of haemolysate or 0.6 mg of brain protein). The reaction was started by the addition of 0.83 mM acetylcholine iodide (substrate), and the increase in absorbance was monitored at 412 nm. The results were calcu[illegible]

molar extinction coefficient of the product, 5-mercapto-2-nitrobenzoic acid (13.6×10^3 M^{-1} cm^{-1}) and the results expressed as nmol product formed min^{-1} ml^{-1} serum or nmol product formed min^{-1} mg^{-1} protein of tissue homogenate. Proteins were quantitated by the method of Lowry *et al.* (1951).

Cytochrome Oxidase (CO)

Cytochrome oxidase was assayed in platelets and brain tissue according to the method of Sotocassa *et al.* (1967). The assay mixture contained 0.3 mM reduced cytochrome C in 75 mM phosphate buffer (pH 7.5). The reaction was started by the addition of requisite amount of sample (platelets 800 μg of protein, brain tissue 23 μg of protein) and the activity was determined by following the oxidation of cytochrome C (molar extinction coefficient 29.5 M^{-1} cm^{-1}) spectrophotometrically at 550 nm for 2 min. The results were expressed as nmol cyt. C oxidized min^{-1} mg protein^{-1}.

Acid phosphatase

Acid phosphatase was assayed in the serum and brain by the method of Wotton (1964). The reaction mixture contained 0.2 M citrate buffer (pH 4.9), 10 mM disodium phenyl phosphate and requisite amount of the sample (0.05 ml of haemolysate, 0.4 mg of brain protein). Following an incubation at 37°C for 30 min, the reaction was terminated by addition of 0.5 N NaOH and the phenol liberated was estimated in the presence of alkaline potassium ferricyanide (2.4%). The colour thus developed was expressed as ng phenol liberated min^{-1} ml serum^{-1} or ng phenol min^{-1} mg protein^{-1}.

2', 3'-cyclic nucleotide phosphohydrolase (CNP)

CNP was assayed in brain by the method of Prohaska *et al.* (1973). To a requisite amount of the sample prepared in 0.32 M sucrose, was added 0.2 M Tris-HCl (pH 7.5) and 1% sodium deoxycholate and the protein concentration adjusted to 0.1 mg protein ml^{-1}. To the final reaction volume (0.2 ml), was added the above sample, 7.5 mM 2', 3'-cAMP and 50 mM Tris-maleate buffer (pH 6.2), and the reaction carried out at 30°C for 10 min. The reaction was terminated in a boiling water bath after which the mixture was returned to 30°C, when 0.3 M Tris-HCl containing 21 mM $MgCl_2$ (pH 9.0) was added along with 0.72 U of alkaline phosphatase, resulting in a final pH of 8.5. The inorganic phosphorus thus released was assayed by the method of Martin and Dotty (1949) and the results expressed as μmoles 2'-AMP formed min^{-1} mg protein^{-1}.

Glucose-6-phosphate dehydrogenase (G-6-PD)

Glucose-6-phosphate dehydrogenase was assayed in brain tissue according to the method of Kornberg and Horecker (1955). The assay mixture contained 0.1M triethanolamine buffer (pH 7.6), 31 mM glucose-6-phosphate, 10 mM $NADP^+$ and an appropriate amount of sample (0.6 mg of brain protein) in a volume of 3.0 ml. The increase in absorbance at 340 nm caused by the reduction of $NADP^+$ was monitored and the results were expressed as nmoles $NADP^+$ reduced min^{-1} mg protein^{-1}.

Statistical analysis

All values are expressed as the mean ± SD of 6 animals in each group. Data were analysed using the Student's t-test and values with $p<0.05$ were considered significant.

Results

Estimations in peripheral tissues

Since OPs are anticholinesterase agents, the effect of chronic dichlorvos exposure on AChE activity was studied in the rat haemolysate at different intervals. A progressive and statistically significant decrease in the activity of AChE became evident from the second week onwards (1.4-fold, 28%), which culminated in a nearly 2.3-fold (61%) decrease by the end of 8 weeks of treatment.

As shown in table 2, a consistent and significant decrease (70%) in the activity of CO was also discernible during the treatment from the second week onwards

Table 1. Effect of chronic dichlorvos exposure on the activity of acetylcholinesterase in the haemolysate of rats.

	Acetylcholinesterase (nmol product formed/min/ml)				
	0 day	2 weeks	4 weeks	6 weeks	8 weeks
Control group	197.42±13.48	163.45±10.59	194.98±10.52	194.16±11.43	181.41±29.07
Dichlorvos treated group	191.92±3.54^{NS}	118.74±14.92***	105.47±13.89***	83.81±12.71***	71.53±19.87***

Rats were exposed to 6 mg dichlorvos /kg body weight for 8 weeks and acetylcholinesterase was assayed in the haemolysate as described in the materials and methods.
Values are Mean±SD, $^{***}p<0.001$, significantly different from control group.
NS – not significant.

Table 2. Effect of chronic dichlorvos exposure on the activity of cytochrome oxidase in the platelets of rats.

	Cytochrome oxidase (nmol cyt. c oxidised/min/mg protein)				
	0 day	2 weeks	4 weeks	6 weeks	8 weeks
Control group	4.25±0.62	4.03±0.70	4.05±0.62	4.44±0.43	4.27±0.82
Dichlorvos treated group	3.90±0.25^{NS}	1.56±0.11***	1.22±0.27***	1.02±0.15***	1.13±0.45***

Rats were exposed to 6 mg dichlorvos/kg body weight/day for 8 weeks and the cytochrome oxidase was assayed in the platelets as described in the materials and methods.
Values are Mean±SD, $^{***}p<0.001$, significantly different from the control group.
NS – not significant.

which persisted until the end of scheduled 8 weeks of treatment (73%). Chronic dichlorvos treatment also resulted in a significant increase in the activity of acid phosphatase from 4 weeks onwards (1.6-fold) with an almost 2-fold increase being discernible towards the end of the treatment (table 3).

Estimations in brain tissue

The activities of all the above mentioned enzymes, along with those of CNP and G-6-PD, were also monitored in the brains of dichlorvos-treated animals. It is clear from the data in table 4, that a nearly identical pattern of results was evident in the brain tissues as well. Whereas the activity of AChE and CO was decreased by 66.5% and 29% respectively, that of acid phosphatase was increased by 12%, as compared to the controls.

Studies on the profile of G-6-PD revealed a nearly 44% decrease in its activity in the brains of experimental animals *vis-a-vis* respective controls. The activity of CNP, a myelin marker, however, remained unaffected following dichlorvos exposure (table 5).

Table 3. Effect of chronic dichlorvos exposure on the activity of acid phosphatase in the serum of rats.

	Acid phosphatase (ng phenol liberated/min/ml)				
	0 day	2 weeks	4 weeks	6 weeks	8 weeks
Control group	430.22±6.88	497.01±45.12	424.85±16.82	390.60±27.70	410.93±38.10
Dichlorvos treated group	425.48±13.24^{NS}	579.38±64.37^{NS}	678.95±16.91***	685.61±30.86***	736.32±9.56***

Rats were exposed to 6 mg dichlorvos/kg body weight, for 8 weeks and acid phosphatase was assayed as described in the materials and methods.
Values are Mean±SD, $^{***}p<0.001$, significantly different from the control group.
NS – not significant

Table 4. Effect of chronic dichlorvos exposure on the activity of acetylcholinesterase, acid phosphatase and cytochrome oxidase in rat brain.

	Acetylcholinesterase (nmol pdt formed/ min/mg protein)	Cytochrome oxidase (nmol cyt. C oxidized/ min/mg protein)	Acid phosphatase (ng phenol liberated/ min/mg protein)
Control group	119.48 ±5.85	539.76 ±42.87	171.68 ±9.53
Dichlorvos treated group	39.94 ±2.01***	381.05 ±40.47***	191.79 ±10.04***

Rats were exposed to dichlorvos (6 mg/kg body weight/day) for 8 weeks and the marker enzymes were assayed as described in the material and methods.
Values are Mean±SD of 6 animals in each group.
$^{***}p<0.001$, significantly different from the control group.

Table 5. Effect of chronic dichlorvos exposure on the activity of glucose-6-phosphate dehydrogenase and 2', 3'-cyclic nucleotide phosphohydrolase in rat brain.

	Glucose-6-phosphate dehydrogenase (nmol $NADP^+$ reduced/min/mg pr.)	2',3'-cyclic nucleotide phosphohydrolase (μmol 2'-AMP formed/min/mg protein)
Control group	61.76±3.31	14.20±0.35
Dichlorvos treated group	34.60±2.99***	13.99±0.10^{NS}

Rats were exposed to dichlorvos (6 mg/kg body weight/day) for 8 weeks and glucose-6-phosphate dehydrogenase and 2',3'-cyclic nucleotide phopshohydrolase were assayed as described in the materials and methods.
Values are Mean±SD of 6 animals in each group.
$^{***}p<0.001$, significantly different from the control group.
NS – not significant.

Discussion

Acetylcholinesterase is a crucial neuronal enzyme and serves to regulate the concentration of free acetylcholine, a neurotransmitter required for transmission of impulses across the cholinergic synapse. Besides its presence in the nervous tissue

AChE activity has also been detected in erythrocytes, where it has been proposed as a useful marker of cholinergic toxicity (Kobayashi *et al.* 1986). Our results, indicative of a decrease in the AChE activity both in the haemolysate and the brains of dichlorvos-treated animals, are in conformity with the reports of Plumlee *et al.* (1994) and Bhatnagar *et al.* (1994). Furthermore, Harlin and Dellinger (1993) also reported nearly 33% reduction in the activity of AChE in the haemolysate, and approximately 45% decrease in its activity in the brains of dichlorvos-treated rats. Inhibition of AChE through the stimulation of muscarinic acetylcholine receptors (mAChR) may lead to an accumulation and resultant intensified action of acetycholine, resulting in bronchospasms and cardiac disturbances, which may eventually prove fatal (Whang and Zhao 1995). The decrease in AChE activity may, however, not be regarded as a specific marker of dichlorvos neurotoxicity. In an earlier study from our lab (Julka *et al.* 1995), an altered cholinergic function due to decreased AChE activity and subsequent altered mAChR binding was reported to occur following chronic aluminium exposure. In addition to this, we have also reported marked AChE inhibition in rat brain following chronic lead exposure (Sandhir *et al.* 1994). Heavy metals such as lead and uranium have also been reported to decrease AChE activity in experimental animals (Labrot *et al.* 1996).

Cytochrome oxidase is the terminal enzyme of the electron transport chain and serves to transfer electrons to molecular oxygen to produce water (Nicholis 1984). Since dichlorvos poisons respiration, activity of this enzyme may serve as a useful marker of impaired energy metabolism. Chronic dichlorvos administration elicited inhibition of CO in both platelets and brains of experimental animals. Platelets were used since these have been reported to be similar to presynaptic terminals in several respects (Julka and Gill 1995). A decreased CO activity in this case could be indicative of a disruption in the electron transport chain with resultant decreased tissue utilization of oxygen. This may further increase cerebral energy requirements and lead to severe neuronal damage. This speculation gains credence from reports indicating that defects in the mitochondrial respiratory chain may lead to excitotoxic neurodegeneration (Blass *et al.* 1988).

Acid phosphatase is a hydrolytic enzyme localized in the lysososmes, the activity of which has been reported to serve as an index of cellular injury. Chronic dichlorvos exposure was seen to cause an increase in the activity of this enzyme in both serum and brain tissue. The activation of the lysosomal system along with increased acid phosphatase activity has been observed in a number of states associated with neuronal injury (Beaudet *et al.* 1991), such as experimental allergic encephalomyelitis, Alzheimer's Disease and Parkinsonism (Nixon and Cataldo 1993). Increased acid phosphatase activity in the present study could be due to a dichlorvos-induced disruption of axonal transport (Moretto *et al.* 1987). This may lead to an altered permeability and subsequent break-down of lysosomal membranes, eventually resulting in the observed increase in acid phosphatase activity.

Chronic dichlorvos administration elicited an appreciable decrease in the activity of G-6-PD in rat brain. Glucose-6-phosphate dehydrogenase is a vital enzyme of the oxidative phase of the hexose monophosphate shunt, as it catalyses the production of NADPH through the dehydrogenation of glucose-6-phosphate to 6-phosphogluconolactone. A major function of the NADPH thus generated is to maintain glutathione (GSH) in its reduced state. GSH is an antioxidant, and acts as a substrate for glutathione peroxidase, which catalyses the reduction of toxic

peroxides. Reduced activity of G-6-PD therefore implies reduced NADPH and consequent reduced biosynthesis of GSH, a state reflective of oxidative stress. This is well in line with an earlier report, indicating reduced levels of GSH and resultant oxidative damage in dichlorvos-treated brains (Julka *et al.* 1992).

The enzyme CNP was assayed in brains of dichlorvos-treated animals as an index of demyelination. Dichlorvos exposure did not have any effect on CNP activity, indicating that dichlorvos may not be inducing any myelin damage at the current level of exposure.

Validation of biomarkers of neuronal damage requires that the changes observed in the peripheral tissues should also be reflected in the brains of experimental animals, as these markers are several steps removed from their actual functional sites in the nervous system. It is clear from the above study that peripheral alterations in the proposed markers of dichlorvos neurotoxicity correlate well with those observed in the brains of treated animals. In addition, this investigation also indicates that apart from AChE, levels of CO, acid phosphatase and G-6-PD may also serve as useful indices of dichlorvos-induced neuronal damage. This conclusion is amply evident from the highly significant alterations in the activities of these enzymes in brains of dichlorvos-treated animals. Based on the results of the present study, it may be reasonable to speculate that inclusion of CO, acid phosphatase and G-6-PD in a test battery, along with AChE which is an established marker of OP neurotoxicity, may further aid in predicting the prognosis of neuronal function following dichlorvos exposure.

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