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Biochemical and Behavioral Deficits in Adult Rat Following Chronic Dichlorvos Exposure

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SARIN, S. AND K. D. GILL. *Biochemical and behavioral deficits in adult rat following chronic dichlorvos exposure.* PHARMACOL BIOCHEM BEHAV **59**(4) 1081–1086, 1998.—The present study was carried out to assess the biochemical and behavioral sequelae of chronic dichlorvos (6 mg/kg b.wt/day for 8 weeks) exposure in rats. Dichlorvos administration significantly decreased the activities of neuropathy target esterase and other carboxylesterase viz., paraoxon resistant and mipafox and paraoxon resistant esterases. The acetylcholinesterase activity was also appreciably decreased following dichlorvos exposure. The alterations in biochemical parameters were also reflected in the behavioral patterns of dichlorvos-treated animals. Dichlorvos administration caused a marked decrease in both the ambulatory and stereotypic components of spontaneous locomotor activity of rats. The muscle strength and coordination of the dichlorvos-treated animals was also significantly impaired. Besides, a marked deterioration in the memory function assessed in terms of the conditioned avoidance response was discernible at the end of the treatment schedule in the experimental animals. © 1998 Elsevier Science Inc.

Organophosphate Dichlorvos Rat Brain Behavior Esterases

THE world-wide production and use of organophosphorus (OP) pesticides continues to rise with an approximate 10-fold increase in the past 3 decades (26). This particular class of pesticides has come under severe criticism because of toxic effects on biological systems other than their primary targets.

Organophosphates, including dichlorvos, have been reported to exert their primary pharmacological and toxicological effects through the inhibition of acetylcholinesterase (17), required for the transmission of impulse across the cholinergic synapse. The typical symptoms of OP poisoning are largely due to excessive cholinergic effects and include bronchospasms, hypersecretion from cholinergic innervated glands along with cardiac disturbances caused by vagotonus and anoxia (4). In addition to the acute neurological effects, the organophosphates are also associated with a delayed neurological syndrome, the organphosphate-induced delayed neuropathy (OPIDN). This effect is characterized by a delay of 10–14 days following an acute exposure or may alternatively develop as a result of long-term exposure to organophosphates (24). It occurs without the accompaniment of cholinergic symptoms, and usually develops after the cessation of the cholinergic crisis (14). OPIDN is characterized primarily by a partial paralysis of the hind limbs followed by complete locomotor ataxia (19). Histopathological lesions of delayed neuropathy include axonal degeneration, proliferation of the smooth endoplasmic reticulum, and profound gliosis along with a near complete disorganization of the neuronal cytoskeleton (2). Although the precise biochemical mechanism behind the pathogenesis of OPIDN is still obscure, it is largely attributed to the phosphorylation and resultant inhibition of a novel neuronal esterase, the neuropathy target esterase (NTE) (33).

Whereas the neuropathological lesions associated with OP poisoning are well characterized, there is considerable lack of information on their behavioral effects, especially after chronic exposure. The importance of neurobehavioral studies lies in the fact that behavior is considered as a functional end product of the various sensory, motor, and integrative processes occurring in the nervous system. This particular aspect, therefore, requires intense investigation, more so because behavioral changes are now being regarded as a standard indicators of toxicity in humans and animals, chronically exposed to low concentrations of potential neurotoxicants (31).

Further, in view of the almost ubiquitous nature of the central, including spinal and supraspinal cholinergic pathways and synaptic networks, the anticholinesterase agents such as the OP pesticides may have the widest range of central ac-

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tions, including mental health effects. The present study has, therefore, been designed to evaluate the behavioral and biochemical sequelae of chronic dichlorvos exposure and assess their efficacy as possible markers of dichlorvos neurotoxicity. Dichlorvos is an OP pesticide, which is a direct acting cholinesterase inhibitor, also known to cause delayed neuropathy in both humans and animals (20,30).

METHODS

Subjects

The experimental design was prepared in strict accordance with the guidelines of the Institute's ethics committee. Male albino rats (Wistar strain) in the weight range of 140–160 g procured from the Institute Animal House, were used throughout the study. Prior to and during the study, the rats were individually housed in polypropylene cages in a well-ventilated room with a 12 L:12 D cycle. The animals were provided with standard laboratory chow (Hindustan Lever Ltd., Bombay) and ad lib access to water.

Experimental Design

For the purpose of carrying out the proposed studies, the rats were divided into the following two groups. The dichlorvos-treated group: rats in this group received dichlorvos (2,2-dichlorovinyl dimethylphosphate) manufactured by Hindustan Ciba-Geigy Ltd., Bombay, at a dose of 6 mg/kg b.wt./day, subcutaneously with corn oil as the vehicle for 8 weeks. The control group: in this group, the animals received an equal volume of corn oil.

Apparatus

Motor function tests: open-field behavior. Spontaneous locomotor activity was assessed using a microprocessor-based animal activity meter (Columbia Instruments Inc., USA), which monitors both ambulatory and streotype components of the spontaneous locomotor activity. These include the distance travelled by the animal, time spent in rest, ambulation, and the sterotype behavior of the animal along with the number of sterotypic and vertical movements with the help of an Auto-Track software (version 3.1). The instrument consists of an acrylic activity chamber ($17' \times 17'$) with an array of 15 infrared (IR) beams placed at different angles, an interface, and an Apple IIe computer. The interruption of an IR beam on account of the movement made by the animal placed in the chamber generates an electric impulse that is processed by the computer.

Rotarod treadmill test. This test was carried out to evaluate the muscle strength and coordination of the experimental animals (6). The apparatus consists of a metallic rod (5 cm in diameter), turning at the rate of 8 revolutions per minute. The rod is dividied into four lengths by circular sections, so that four animals can be tested simultaneously.

Memory function tests

Active avoidance test: shuttlebox apparatus. In this case, the shuttlebox ($24' \times 24' \times 15'$) consisted of two equal-sized adjacent chambers connected through a passage of sliding manual door. Both the chambers are uniformly lit, with an electric buzzer fitted in one chamber that serves as the source of the conditioned stimulus (CS). The apparatus is also provided with a milliammeter to monitor the intensity of foot shock delivered through the floor.

Pole-climbing apparatus. This apparatus consisted of a Perspex chamber ($12' \times 12' \times 12'$) and a grid floor made of stainless steel rods. The chamber is provided with a buzzer that serves as the source of CS and a milliammeter to monitor the intensity of the foot shock. The chamber is also fitted with a lid to which is attached a wooden pole (3/4th of an inch in diameter). This pole serves as the safety area for the animals.

Aggressive Behavior

Aggressive behavior was tested in an aggressometer (Techno India, Lucknow). The apparatus consisted of a Persepex chamber ($8' \times 8' \times 7'$), which has a grid floor of stainless steel rods for the purpose of delivering the foot shock.

Motor function tests: spontaneous locomotor activity. Test Procedure—each rat was placed individually in the acrylic test chamber. Following habituation of the animal for 15 min, the different components of the locomoter activity were monitored in an automatic manner on the basis of the interruptions in IR beam, made by the movement of the animal. The electric pulses thus generated were then processed by the attached computer with the help of the autotrack V software. Each animal was monitored for locomoter activity for a period of 5 min. At the time of changing the animals, the chamber was thoroughly cleaned and swiped with a swab of 50% ethanol to remove any possible odour cues.

Rotarod tread mill test. Test procedure—this test was carried out to assess the muscle strength and coordination of the experimental animals. For this purpose, the animals were initially trained (15 trials) to maintain themselves on the rotating rod for a period of more than 3 min. Subsequently, i.e., after a period of 24 h, the animals were again screened for their ability to remain on the rotating rod for three successive trials of 3 min. each. In the event of their inability to do so the test was considered positive, i.e., motor coordination was said to have been produced.

Memory function tests. These were basically the step-through inhibitory avoidance tests, in which following an auditory warning or illumination signal, the satisfactorily trained and conditioned animals jump to a shock-free chamber to avoid the foot shock delivered through the grid at the floor of the cage. Memory functions were assessed in the following manner.

Shuttlebox apparatus. Test procedure—this test was carried out according to the method of Piala et al. (25). Untrained animals from control and experimental groups were individually placed in one chamber and habituated for 5 min. After this, the animals received a conditioned stimulus in the form of a buzzer for 10 s, followed by a buzzer plus foot shock (0.1 mA, 40 V) for 10 s. The animals were subjected to 20 trials/day for 3 days, keeping the test time constant. The intertrial interval was of 1 min. Trials on day 1 were a part of the training drill. Total number of avoidances out of 20 trials and the number of trials for first avoidance were recorded for each animal. Animal was considered to have made avoidance when it jumped to a shock free chamber to avoid the foot shock.

Pole-climbing test. Test procedure—this test was also performed to assess the memory function, and is another measure of the active avoidance response of experimental animals (5). For the purpose of conducting the test, the animals were placed one at a time in the Persepex test chamber and habituated for 5 min. After this the animal was given a conditioned stimulus (CS), i.e., the buzzer for 10 s, followed by a buzzer and foot shock (0.1 mA, 40 V) for 10 s. The intertrial interval was of 1 min. The animals were subjected to 15 trials/day for 3 days, with the first day being a part of the training drill. The

animal was considered to have made the avoidance when it climbed up the pole to avoid the foot shock at the sound of the buzzer (CS). Failure to respond to CS was taken to be a measure of the cognitive deficit.

Aggressive Behavior

Aggressive behavior was tested as per the method desribed by Tedeschi et al. (28). Preliminary screening was done to exclude the nonresponding animals. The rat pairs (one at a time) were placed in the test chamber, acclimatized for 3 min, and a foot shock of 100 V, 2 mA, 5 Hz was administered for 10 s. Following this, the number of fighting episodes in 1 min were counted. Only those pairs of rats that exhibited at least one fighting episode in 1 min were included in the study. The fighting episode was considered positive when the rats converged abruptly to close quarters, stood face to face with each other, and struck each other with their forelimbs. The return of the rats to quadrupedal posture was considered as the demarcation between the fighting episodes.

Biochemical procedures. For the purpose of carrying out the biochemical investigations, the animals were sacrificed at the end of the treatment schedule (8 weeks) by decapitation following cervical dislocation. The brains were excised and dissected into various regions, i.e., cerebral cortex, cerebellum, and brain stem, as per the guidelines of Glowinski and Iverson (11).

Assay of neuropathy target esterase and other esterases. Neuropathy target esterase (NTE) was assayed according to the method of Johnson (13), wherein the hydrolysis of phenyl valerate yields phenol that couples with 4-amino antipyrine in the presence of alkaline potassium ferricyanide to yield a red coloured complex (4N (1,4-benzoquonoeimine)-antipyrine), the intensity of which was measured at 510 nm. The assay was carried out under two conditions: i) incubation of brain homogenate (10% w/v) in the presence of Paraoxon (40 μM) and 50 mM Tris-EDTA buffer (pH 8.0) to yield the paraoxon resistant carboxylesterase; and 2) incubation of homogenate (10% w/v) in the presence of paraoxon (40 μM) and mipafox (50 μm) to yield the paraoxon and mipafox resistant carboxylesterase.

The activity of NTE was assayed as the difference in the activities of paraoxon resistant and paraoxon and mipafox resistant carboxylesterases, i.e., under conditions 1) and 2), and the results were expressed in terms of nmol of phenyl valerate hydrolyzed/min/mg protein.

Acetylcholinesterase assay. Acetylcholinesterase was assayed by the method of Ellman et al. (7), wherein the hydrolysis of acetylthiocholine to thiocholine and acetate is measured. Thiocholine reacts with 5,5′-dithiobis nitrobenzoic acid to form 5-mercapto-2-nitrobenzoic acid, a yellow-colored compound, the intensity of which was measured spectrophotometrically at 412 nm.

Protein estimation. Proteins were quantitated according to the method of Lowry et al. (22).

Statistical Analysis

Data was analyzed using Student's *t*-test, and the values with $p < 0.05$ were considered significant.

RESULTS

Motor Function Tests

Spontaneous locomotor activity. Dichlorvos exposure (6 mg/kg b.wt./day) for 8 weeks caused a significant decrease in all the components of the spontaneous locomotor activity, as evident from the data presented in Table 1. In this context, there was an appreciable decrease in the amount of distance traveled accompanied by a concomittant increase in the resting time. The dichlorvos-treated animals exhibited a marked decrease in the number of stereotypic movements as well as the stereotypic and ambulatory time. The number of horizontal movements were also decreased significantly in the treated animals compared to the animals in the control group. The above observations were further confirmed by the movement pattern of the animals. It was evident from the movement pattern that animals in the control group exhibited a significantly increased extent of activity, exploring the entire chamber and rapidly running along the walls and across the center, making complete circuits of the apparatus. In contrast, the dichlorvos-treated animals exhibited a certain degree of relutance towards exploration; a majority of movements being confined along the walls of the chamber with most of the animals failing to even complete a single circuit of the apparatus.

Rotarod performance. The Rotarod test (Table 2) revealed a marked impairment in the muscle strength and coordination of dichlorvos treated animals. There was a significant reduction in the retention time in case of experimental animals compared to the control animals. None of the tested animals exposed to dichlorvos could maintain themselves on the rotating rod for the full quota of 180 s.

Memory function tests: shuttlebox apparatus. This test was of the step-through inhibitory avoidance type (Table 3). Initially, rats in both the groups made errors, with the animals jumping into the shock-free chamber only after receiving the foot shock. But as the training progressed, there was a marked improvement in the performance of control animals, who re-

TABLE 1

EFFECT OF CHRONIC DICHLORVOS (6 mg/kg b.wt.) EXPOSURE ON THE OPEN-FIELD BEHAVIOR OF RATS

Group	Distance Travelled	Resting Time (s)	Ambulatory Time (s)	Stereotype Time (s)	No. of Stereotype Movements	Horizontal Counts	Vertical Counts
Control	419.13 ± 113.02	189.16 ± 24.81	34.26 ± 8.91	75.68 ± 23.15	301.83 ± 33.25	365.83 ± 100.91	5.00 ± 3.09
Dichlorvos treated	181.13 ± 49.95*	263.4 ± 9.35*	12.53 ± 4.69*	23.93 ± 7.33*	102.83 ± 32.78*	130.83 ± 38.22†	4.33^{NS} ± 2.87

Values are Mean ± SD of six animals in each group.
*$p < 0.001$, †$p < 0.01$, significantly different from the control group.
^{NS}Not Significant.

TABLE 2

EFFECT OF CHRONIC DICHLORVOS (6 mg/kg b.wt.) EXPOSURE ON THE ROTA ROD PERFORMANCE OF RATS

Group	Retention Time (s)
Control	108.94 ± 13.24
Treated	25.33 ± 15.08*

Values are Mean ± SD of six animals in each group.

*$p < 0.001$, significantly different from control group.

TABLE 4

EFFECT OF CHRONIC DICHLORVOS (6 mg/kg b.wt.) EXPOSURE ON THE ACTIVE AVOIDANCE RESPONSE (POLE-CLIMBING APPARATUS) OF RATS

Group	Number of Trials for First Avoidance	Mean Percent Avoidance
Control	1.33 ± 0.41	92.21 ± 5.02
Treated	4.91 ± 1.77*	33.37 ± 7.30*

Values are Mean ± SD of six animals in each group, on day 3 of the experimental schedule.

*$p < 0.001$, significantly different from the control group.

quired only a minimum number (one to two trials) of trials to make the first avoidance (by the second and third day of training). The dichlorvos-treated animals, on other hand, continued to perform poorly and needed a minimum of five to seven trials to make the first avoidance, even at the fourth day of training. Thus, it is clearly evident from the data that there was an overall significant increase in the number of trials required to make the first avoidance by the dichlorvos-treated rats compared to the control rats.

Further, there was also a significant decrease in mean number of avoidances/trial in the dichlorvos-treated group compared to the control group (Table 3).

Pole-climbing apparatus. This test also revealed an almost similar pattern, with the dichlorvos-treated animals exhibiting a marked decline in the mean percent avoidance compared to the control animals (Table 4). Further, the control animals needed a significantly less number of trials to make the first avoidance compared to the treated animals.

Aggressive Behavior

Animals in the dichlorvos-treated group exhibited marked aggression, evidenced by the significantly higher number of fighting episodes observed in them compared to the animals in the control group (Table 5).

Biochemistry

Neuropathy target esterase and other esterases. Dichlorvos treatment (6 mg/kg b.wt./day) for 8 weeks brought about a marked decrease in the activity of neuropathy target esterase in all the brain regions investigated (Table 6). The order of decrease being, brain stem (49%), cerebral cortex (40%), cerebellum (38%).

A similar decrease was observed in the paraoxon resistant component of the total phenylvalerate hydrolase activity. In this case, also, the decrease was maximal in the brain stem (41%) followed by cerebral cortex (35%) and cerebellum (27%). The mipafox and paraoxon-resistant esterase was also significantly inhibited in all the regions of the dichlorvos-treated rat brain viz., cerebral cortex (28%), cerebellum (21%), and brain stem (27%) (Table 6).

Acetycholinesterase. The acetylcholinesterase activity was found to be markedly inhibited in all the regions of rat brain following chronic dichlorvos treatment (Table 7). The decrease was maximal in the brain stem (86%), followed by cerebral cortex (66%) and cerebellum (55%).

DISCUSSION

Dichlorvos is a direct-acting inhibitor of the enzyme acetylcholinesterase, a mechanism through which it exerts its primary toxic effects following acute and chronic exposure. However, in addition to its acute effects, dichlorvos has also been associated with a neurodegenerative disorder, the organophosphate-induced delayed neuropathy that has been attributed to the phosphorylation and resultant inhibition of neuropathy target esterase (NTE), a membrane-bound enzyme present in the neural tissue (23). OPIDN is defined as the delayed onset of prolonged locomotor ataxia resulting from a single or repeated exposure to an organophosphate compound (1,15,27).

TABLE 3

EFFECT OF CHRONIC DICHLORVOS (6 mg/kg b.wt.) EXPOSURE ON THE ACTIVE AVOIDANCE RESPONSE (SHUTTLE BOX APPARATUS) OF RATS

Group	Number of Trials for First Avoidance	Number of Avoidances
Control	1.83 ± 1.16	13.16 ± 1.12
Treated	5.41 ± 2.01*	4.66 ± 1.86†

Values are Mean ± SD of six animals in each group, on day 3 of the experimental schedule.

*$p < 0.01$, †$p < 0.001$, significantly different from control group.

TABLE 5

EFFECT OF CHRONIC DICHLORVOS (6 mg/kg b.wt./day) ON THE AGGRESSIVE BEHAVIOR IN RATS

Group	Number of Fighting Episodes/min
Control	3.20 ± 1.92
Treated	15.60 ± 3.04*

Values are Mean ± SD of six animals in each group.

*$p < 0.001$, signficantly different from control group.

TABLE 6

EFFECT OF CHRONIC DICHLORVOS (6 mg/kg b.wt.) EXPOSURE ON THE ACTIVITY OF NEUROPATHY TARGET ESTERASE AND OTHER CARBOXYLESTERASES IN DIFFERENT REGIONS OF THE RAT BRAIN

	Neuropathy Target Esterase		Paraoxon Resistant Carboxylesterase		Mipafox and Paraoxon Resistant Carboxylesterase	
Region	Control	Treated	Control	Treated	Control	Treated
Cerebral cortex	4.90 ± 0.61	2.88 ± 0.25‡	9.29 ± 0.78	6.03 ± 0.66‡	4.33 ± 0.28	3.13 ± 0.39†
Cerebellum	3.08 ± 0.42	1.91 ± 0.07‡	7.11 ± 0.60	5.18 ± 0.21‡	4.03 ± 0.44	3.18 ± 0.23†
Brain stem	4.63 ± 0.57	2.35 ± 0.13‡	7.57 ± 0.71	4.47 ± 0.22‡	2.84 ± 0.21	2.08 ± 0.16*

Units: μmol of phenyl valerate hydrolysed/min/mg protein.
Values are Mean ± SD of six animals in each group.
*$p < 0.05$, †$p < 0.01$, ‡$p < 0.001$, significantly different from control group.

The present study revealed a significant decrease in all the components of spontaneous locomoter activity in rats, exposed to dichlorvos (6 mg/kg b.wt./day). Dichlorvos administration also caused a marked impairment in the muscle strength and coordination of experimental rats, as revealed by their performance on the rotarod apparatus. The impairment in the motor functions of rats could be attributed to the dichlorvos-induced phosphorylation and inhibition of NTE, as observed during the course of the present study. This argument is further strengthened in view of the reported appearance of clinical signs of motor deficit as a possible consequence of NTE inhibition following exposure to organophosphates (10).

Besides NTE, dichlorvos treatment also brought about a marked decline in the activity of paraoxon resistant and mipafox and paraoxon-resistant carboxylesterases in different regions of rat brain. The decrease in the activity of these enzymes could also contribute to the poor metabolism and resultant toxic manifestations of dichlorvos on the behavior of experimental animals (16). The reported decrease in the motor conduction velocity following exposure to organophosphates (18) could also be responsible for the observed decrease in the locomotor activity and poor coordination of animals, following dichlorvos treatment.

Cognitive functions are sensitive markers of neurotoxicity (32). The significant alterations in the memory function occurred both in terms of the observed increase in the number of trials needed to make the first avoidance and decrease in the number of avoidances/trial in the dichlorvos-exposed animals. Evidence from both animal and human studies has implicated the central cholinergic system, to be an important component of the neural circuitry of learning, memory, and cognition (3). In view of this, the deficits in cognitive behavior of animals could be attributed to dichlorvos induced inhibition of cholinesterase, as observed during the course of the present study.

Enhanced cholinergic activity consequent to cholinesterase inhibition has been believed to be the cause of various toxic effects of organophosphates (21). It has also been suggested that the behavioral effects as a consequence of chronic OP exposure may result from a decrease in the number of muscarinic cholinergic receptors, especially the M2 receptors, which are located presynaptically and mediate the inhibition of acetycholine release (9). Thus, a decrease in the number of muscarinic receptors may be responsible for reduced transmission of the cholinergic impulse resulting, thereby in the observed neurobehavioral alterations. The argument that cholinergic system is the primary locus of OP action in causing memory deficit, gains further support from a report indicating similar extinction of active avoidance response following administration of a cholinotoxin AF64A (ethylcholine aziridinium ion) a synthetic analogue of choline (8). The altered status of cholinergic function is further substantiated by the marked increase in aggression observed in the animals exposed to dichlorvos. The involvement of cholinergic function herein is further supported by the observations of Hintgen and Aprison (12), who reported anticholinesterase agents to be reliable activators of aggression in several animal models by virtue of their ability to modulate cholinergic function.

The altered neurobehavioral pattern of dichlorvos-treated animals may also have a pathologic basis in view of the reported sustained cell drop out and necrosis in the rat cerebrum, the principal seat of memory and learning, following chronic OP exposure (29).

In summary, the present data suggest that chronic dichlorvos exposure can have severe toxic manifestations on both motor and memory functions of experimental animals, which in turn, could serve as sensitive end points of dichlorvos-induced neuronal dysfunction.

TABLE 7

EFFECT OF CHRONIC DICHLORVOS (6 mg/kg b.wt.) EXPOSURE ON THE ACTIVITY OF ACETYLCHOLINESTERASE IN DIFFERENT REGIONS OF THE RAT BRAIN

Region	Control	Treated
Cerebral cortex	119.48 ± 5.85	39.94 ± 2.01*
Cerebellum	46.99 ± 2.08	21.24 ± 1.42*
Brain stem	125.05 ± 2.75	16.70 ± 1.21*

Units: nmol of acetylthiocholine hydrolyzed/min/mg protein.
Values are Mean ± SD of six animals in each group.
*$p < 0.001$, significantly different from control group.

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