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An acetylcholinesterase-independent mechanism for neurobehavioral impairments after chronic low level exposure to dichlorvos in rats

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ABSTRACT

The present study was designed to explore an a[illegible]te mechanism[illegible]actio[illegible] other than acetylcholinesterase inhibition for the chronic, low-level exposu[illegible]o dic[illegible]vos, an organo[illegible]sphate, *in vivo*. Dichlorvos, at dose of 1.0 as well as 6.0 mg/kg b. wt. for 12 weeks to rats sh[illegible]ed impairment in neurobehavioral indices viz. rota rod, passive avoidance and water maze tests. Though hig[illegible] dose of dichlorvos had a detrimental effect on acetylcholinesterase activity, no si[illegible]ficant inhibition was se[illegible] with lower dose of dichlorvos for the same period of exposure i.e. 12 weeks. [illegible]uscarinic acetylcholine receptor binding studies revealed a decrease in the number of binding sites (B_{max}) i[illegible]w as well as hi[illegible] dose groups but the dissociation constant (K_d) value was unaffected with both doses of d[illegible]lorvos. Use of s[illegible]ective ligands against M_1, M_2 and M_3 receptor subtypes indicated that M_2 is the major re[illegible]tor subtype b[illegible]g affected by chronic low-level exposure to dichlorvos. Western blot analysi[illegible] and immunofl[illegible]escence s[illegible]udies also confirmed these biochemical findings. Thus, the present study sugg[illegible]s[illegible] M_2 receptor[illegible] may play a major role in the development of neurobehavioral impairments after ch[illegible]nic exp[illegible]e to dichlorvos.

1. Introduction

Organophosphate (OP) poisoning c[illegible]ntinues to b[illegible]e major cause of morbidity and mortality in third w[illegible]countries (Pe[illegible]nd Cherian, 2000). More than 10,000 cases o[illegible]rgano[illegible]sphate pesti[illegible]de poisoning are annually reported in[illegible]he United St[illegible]s alone (Litovitz et al., 2002). In the developing c[illegible]untries over 3 mi[illegible]n poisonings occur annually, out of which 2[illegible],000 are fatal (Eddleston et al., 2002).

Dichlorvos, an or[illegible]ophosph[illegible]us pesticide, has been used as a crop protectant and as[illegible]ner[illegible] public he[illegible]lth insecticide since 1961. Inhibition of ace[illegible]cholin[illegible]rase (ACh[illegible]s is the major mechanism of action for [illegible] co[illegible]ounds, [illegible]adin[illegible] to increase in the level of acetylcho[illegible]e in the [illegible]naptic cle[illegible]d hence producing both nicotinic and mu[illegible]rinic sy[illegible]toms and signs of intoxication in the peripheral and centr[illegible]ne[illegible]ous syste[illegible] like nausea, vomiting, lacrimation, salivation, br[illegible]cardia, miosis and finally death may occur due to respiratory failu[illegible] (Deer et al., 1993). Humans exposed to low levels of OP agents in indust[illegible]l or agricultural settings have reported difficulty in concentration as well as memory impairment, long after such exposure has ceased (Jett et al., 2001). It has been shown that long-term use of OPs without evidence of acute poisoning appears to produce subtle changes in neuropsychological test performance, like slower reaction time (Fiedler et al., 1997). Agricultural workers tested about 2 years after a pesticide poisoning episode showed significantly lower performance in verbal and visual attention, visual memory, visuomotor speed, sequencing and problem solving (Rosenstock et al., 1991). Neuropsychological and brain-evoked potential deficits were found to remain 6–8 months after the terrorist attack with the chemical weapon, Sarin gas, in the Tokyo Subway (Yakoyama et al., 1998). Altogether, these data point to the neurotoxic potential of OPs, either as pesticides or as nerve agents, as being responsible for the long-term neurobehavioral impairments or deficits.

Several consequences of chronic, low-level exposure to pesticides are not directly attributable to the accumulation of acetylcholine since tissue acetylcholinesterase activity returns to normal levels in about 3 to 4 months (Milby, 1971). Neurobehavioral effects such as impairment on maze performance and locomotion have also been shown to be affected by repeated, low-level exposure to organophosphate pesticides without any signs of acute toxicity (Eskenazi et al., 1999). Sarin and Gill (1998) have also shown significant impairment in muscle strength and co-ordination after chronic exposure to OP pesticides, in addition to a marked deterioration in the memory functions as assessed in terms of conditioned avoidance response.

It has been reported that very low concentrations of organophosphate compounds compete directly with quinuclidinyl benzilate (QNB), a muscarinic receptor antagonist, which binds equally to all receptor subtypes (Eldefrawi et al., 1992). It has also been observed that down regulation of muscarinic receptor subtypes varies according to the organophosphate used for e.g., chlorpyrifos oxon (CPO) preferentially affects second messenger system associated to M_2/M_4 receptors, whereas paraoxon has high affinity for M_2 and M_3 muscarinic subtypes

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(Bomser and Casida, 2001). The large variety of compounds and test systems employed in the past make it difficult to predict the effect of different organophosphates on muscarinic receptors in the central nervous system. Thus, knowing which subtype of muscarinic receptor binds and interacts with which organophosphate, an *in vivo* study will help in the elucidation of the mechanism through which organophosphates act other than inhibiting acetylcholine esterase.

Therefore, the present study was designed to study the effect of chronic low exposure to dichlorvos on neurobehavioral impairments as well as its interaction with muscarinic receptors in rat brain.

2. Materials and methods

2.1. Materials

Dichlorvos was purchased from Hindustan Ciba Geigy Ltd., Mumbai, India. [^{3}H] QNB (specific activity 47 Ci/mmol) was purchased from Amersham, International Plc., UK. Polyclonal antibodies were obtained from Santa Cruz Biotech. USA. Pirenzepine, himbacine, 4-DAMP were obtained from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used in the study were of the highest quality available.

2.2. Animals and their use

Male albino rats (Wistar strain) in the weight range of 140–160 g were housed in polypropylene cages, kept in well-ventilated rooms under hygienic conditions. Animals were provided standard rat pellet diet (Hindustan Lever Ltd., Bombay, India) and water *ad libitum*. Ethical clearance for killing of animals was duly obtained from the Institu[illegible] Animal Ethics committee.

2.3. Experimental design

The animals were divided into two sets (18 a[illegible]mals in e[illegible]ch set) consisting of three groups in each set. Each g[illegible]up cons[illegible] of 6 animals:

Control group Animals received an eq[illegible]olume of corn [illegible](vehicle) as administered to the an[illegible]als o[illegible]chlorvos treat[illegible] group.

Low dose group Animals recei[illegible]d dichlorvos [illegible]issolved in corn oil (1 mg/kg b. wt./da[illegible].c.) for 12 weeks.

High dose group Animal[illegible] eceived [illegible]chlorvos dissolved in corn oil (6 mg/kg b. w[illegible]day, s.c.) [illegible]r 12 weeks.

The animals [illegible]me) w[illegible] exami[illegible]d for motor deficits and passive avoi[illegible]nce tes[illegible] after 6 [illegible] of dichlorvos exposure. At the end of[illegible] weeks o[illegible] dichlorvos [illegible]xposure the same animals were used for ro[illegible]od te[illegible] pas[illegible]idance test and Morris Water Maze test. The ani[illegible] of second set were euthanized with sodium pentathol and s[illegible]rificed by decapitation. The whole brain was isolated and rinse[illegible]in ice-cold physiological saline (0.9% NaCl) and used immediately for various biochemical and immunoblotting experiments.

2.4. Neurobehavioral studies

In order to determine behavioral impairments, both motor and memory function tests were performed in control as well as dichlorvos treated animals. Every animal performed a set of behavioral experiments in a sequence of rota rod, passive avoidance and Morris Water Maze. Since the stress of behavioral tests may affect the biochemical parameters in the animals, these animals were not used for biochemical studies.

2.4.1. Motor function test

This test was carried out to evaluate the muscle strength and coordination in the experimental animals according to the method of Dunham and Meya (1957). The apparatus consisted of a metallic rod (5 cm in diameter), turning at the rate of 8 rpm. As a part of the test procedure, the animals were initially trained for 3 days (3 trials/day) to maintain themselves on the rotating rod for a period of 3 min. Subsequently, 24 h after last training, the animals were put on the rotating rod for a period of 3 min. in the event of their inability to remain on rotating rod, the test was considered as positive i.e. motor incoordination was said to have been produced.

2.4.2. Memory function tests

2.4.2.1. Passive avoidance test. Exper[illegible]nts were perf[illegible]med by the method of Piala et al. (1959) using [illegible] shutt[illegible]box apparatu[illegible] consisting of a dark unlit chamber and an [illegible]luminated [illegible]amber s[illegible]arated by a controllable door. The floor c[illegible]nsisted of a meta[illegible]id [illegible]red to deliver shocks of controlled inten[illegible]ies and d[illegible]ations. On [illegible]e day of the test, each rat was placed int[illegible] the [illegible]umi[illegible]ted compa[illegible]tment and allowed to explore both chamb[illegible]s of the a[illegible]ratus for [illegible]min. On the second day, each rat was plac[illegible]into the illum[illegible]ted c[illegible]mber of the apparatus. As soon as the ra[illegible]nte[illegible] the dark cha[illegible]t, the door was closed, and a foot shock was applie[illegible](0.1 mA, 40 V). After the shock, rat was removed and returned to [illegible]me cage. On day 3, each rat was placed into [illegible] illuminated chambe[illegible]nd the latency to enter into the dark cha[illegible]ber was measured, which served as measure of retention of av[illegible]dance respons[illegible]

2.4.2.[illegible] Morris Wa[illegible] Maze test. This test was carried out by the method [illegible] (1984); in which the rat is trained to escape from [illegible]ter by swimming to a hidden platform. It can find the platform, w[illegible] under the water and serves as a 'rescue' from the stress [illegible]ituation, by using visual extra-maze cues. In navigation tank, the place of the platform is the same on each day but the starting point of the rat varies. This method requires a long-term spatial memory and learning. MWM consisted of a circular water tank (210 cm diameter and 50 cm height), filled with water (30 cm from bottom) maintained at 25 °C. The water was made opaque with nontoxic white color dye. The tank was divided into four equal quadrants with the help of two white threads running at right angle to each other over the rim of pool. A platform (8×8 cm top surface) of 29 cm height was submerged in the center of one of these four quadrants and this quadrant was treated as the target quadrant for whole study period. The position of the platform was kept unaltered throughout the training period. The top surface of the platform was submerged about 1 cm below the surface of the water.

2.4.2.2.1. Acquisition test. A water tank of 210 cm diameter was filled with water up to 20 cm from the top of the tank; a nontoxic white paint was dispersed in the water to make it opaque. A platform with an 8×8 cm top surface was placed in the middle of one quadrant about 24 cm from the side. The top surface of the platform was submerged about 1 cm below the surface of the water. All rats were given four training trials (acquisition) on days 1–4. On each training trial the rat was placed into the water with its nose facing the side of the tank at one of four randomly selected locations corresponding to each quadrant of the maze, and then it was released. The time spent in searching the hidden platform was recorded.

2.4.2.2.2. Retrieval test. On 5th day, the platform was removed, and each rat was placed in the center of the tub facing the same direction and allowed to swim for 90 s. The time spent in the target area (where the platform had been positioned on days 1–4) was recorded. The 4-day acquisition test is considered a measure of spatial learning and the retrieval test (probe trial) is considered a measure of reference memory.

2.5. Biochemical studies

2.5.1. Preparation of synaptic plasma membrane

Synaptic plasma membranes (SPMs) were prepared from the rat brains by discontinuous sucrose density gradient centrifugation by the method of Jones and Matus (1974).

2.5.2. Acetylcholinesterase assay

The acetylcholinesterase activity was assayed in the SPMs according to the method of Ellman et al. (1961). Butyrylcholinesterase was inhibited by the addition of 10 μM ethopropazine to the assay mixture and the change in absorbance was measured at 412 nm.

2.5.3. Muscarinic acetylcholine receptor (mAChR) binding assay

Muscarinic acetylcholine receptor binding studies were carried out in the SPMs according to the method of Yamamura and Snyder (1974). Different concentrations of [^{3}H] QNB (0–2.0 nM) were added to the sample in the presence and absence of 1 μM atropine. The total volume of reaction mixture was adjusted to 1 ml with 50 mM sodium phosphate buffer (pH 7.4) followed by incubation for 60 min at 30 °C with constant shaking. The reaction was terminated with 1 ml ice-cold sodium phosphate buffer and the content were filtered through glass fiber filter (GF/B) presoaked in polyethylene (0.5% w/v) and radioactivity was measured by scintillation counting. The specific binding was obtained by subtracting non-specific binding from the total QNB bound. The results were expressed as fmol QNB bound/mg protein and the values of K_d and B_{max} were obtained from the Scatchard analysis of the data using Graph pad Prism computer software.

2.6. Selective ligand binding studies

Displacement of [^{3}H] QNB by selective muscarinic antagonists, pirenzepeine (5,11-dihydro-11-[(4-methyl-1-piperazinyl) acetyl]-6-pyrido [2,3 b] [1,4] benzodiazepin-6-one) for M_1 receptor subtype himbacine for M_2 receptor subtype and 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide) for M_3 receptor subtype was carried out by the methods described above except the incubation time was increased to 120 min to ensure equilibrium conditions.

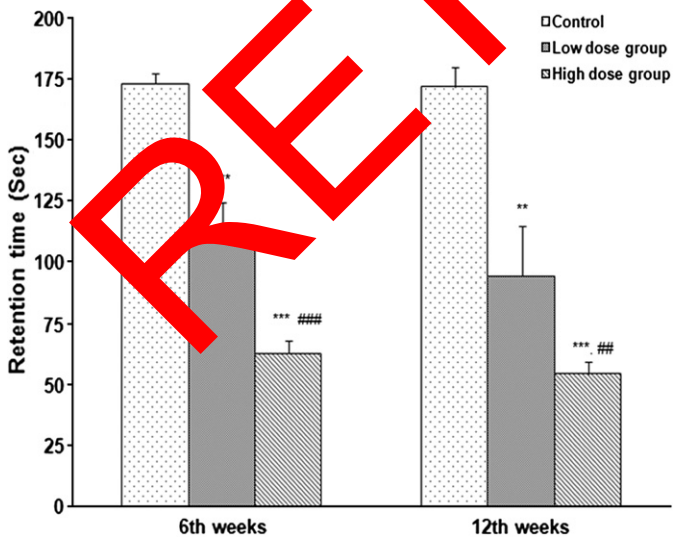

Fig. 1. Effect of chronic dichlorvos exposure on the neuromuscular coordination in rats using rota rod test. The values are mean±SD of 6 animals. ***p<0.001, significantly different from the control group. **p<0.01, significantly different from the control group. ##p<0.05, significantly different from the low dose group. ###p<0.001, significantly different from the low dose group.

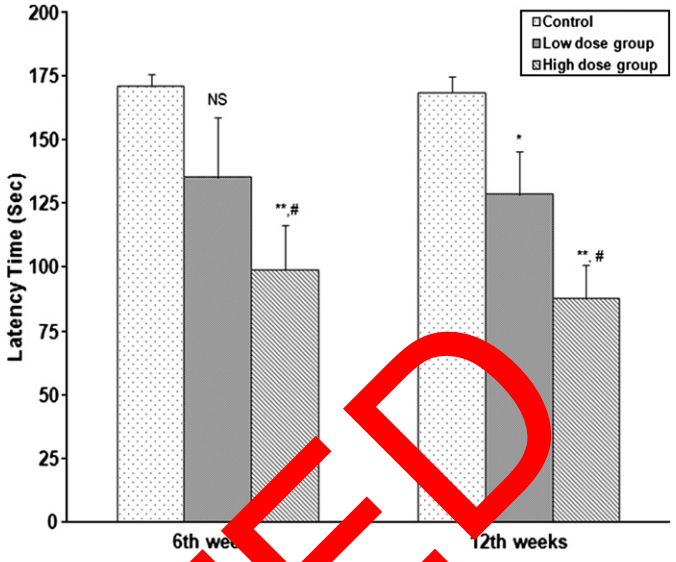

Fig. 2. Effect of chronic dichlorvos exposure on memory function tests viz. passive avoidance test in ... The values are mean±SD of 6 animals. **p<0.01, significantly different from the control group. *p<0.05, ...cantly different from the control group. #p<0.05, significantly different from the low dose group. NSNot significant.

2.7. Western blot analysis of muscarinic receptor subtypes

The membrane protein was prepared as described by Wang et al. (2001) and resolved on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and transferred to nitrocellulose membrane. The protein blots were incubated with primary antibody (respective anti-muscarinic receptors subtype specific polyclonal antibodies from Santa Cruz, USA) at 4 °C overnight, followed by incubation with horseradish peroxidase conjugated anti-goat IgG antibody (Bangalore Genei, India). Immunoreactive protein was visualized by diaminobenzidine (DAB) from Bangalore Genei. The amount of protein was detected by measuring the density of immunodetected band using densitometry analysis.

2.8. Immunofluorescence staining of muscarinic receptors in rat brain

Rat brain was isolated and washed with normal saline and dissected into hippocampus and cerebral cortex. Thin sections of

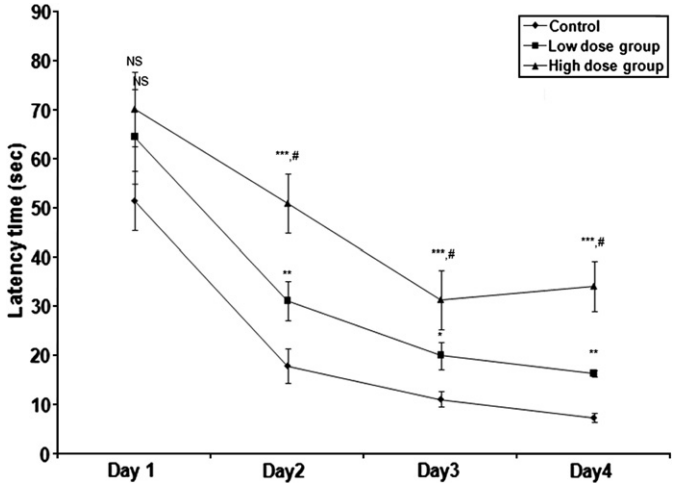

Fig. 3. Effect of chronic dichlorvos exposure (12 weeks) on spatial learning (acquisition test) using Morris Water Maze in rat. The values are mean±SD of 6 animals. ***p<0.001, significantly different from the control group. **p<0.01, significantly different from the control group. *p<0.05, significantly different from the control group. #p<0.05, significantly different from the low dose group. NSNot significant.

Table 1
Effect of chronic dichlorvos exposure on spatial memory (retrieval test) using Morris Water Maze in rat

	Time (s) duration in different quadrants			
	RPQ1	PQ2	LPQ3	OPQ4
Control group	20.8±3.2	52.5±1.8	10.0±2.7	5.3±0.4
Low dose group (1.0 mg/kg b. wt)	28.2±2.3	32.5±2.8	13.0±1.2	14.4±3.4
High dose group (6.0 mg/kg b. wt)	35.2±3.7	30.0±2.8	13.8±2.2	10.5±1.9

The values are mean±SD of 6 animals.
PQ, quadrant in which platform was placed.
RPQ — right to PQ; LPQ — left to PQ and OPQ — opposite to PQ.

brain regions were cut by microtome and rinsed in phosphate buffer saline (PBS) three times for 5 min each. Respective primary antibodies were added and the slides were incubated for 1 h at 37 °C. Following incubation, the slides were washed in PBS three times for 5 min each, then the secondary antibody (FITC labeled) was added and the slides were incubated for 30 min at 37 °C. Again these were washed thrice for 5 min each in PBS. The slides were then mounted with glycerol and kept in the dark. Fluorescence was visualized under a fluorescence microscope and the photographs were taken.

2.9. Protein estimation

Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.10. Statistical analysis

One way ANOVA, with Student–Newman–Keuls test were used for analysis of the data and values with $p<0.05$ were considered statistically significant. All the calculations were carried out using Sigma Stat computer software program.

3. Results

Different doses of dichlorvos resulted in reduction in body weight. The animals in the control group showed an average gain of 146.9 g in body weight, whereas, animals in the low dose group and high dose group showed 30% and 70% reduction in gain in body weight after 12 weeks of treatment respectively (data not shown). This decrease was statistically significant ($p<0.05$) in both treated groups. No changes were observed in the dietary intake.

3.1. Effect of dichlorvos exposure on neuromuscular coordination

The rota rod (Fig. 1) revealed a marked impairment in the muscle strength and coordination in both dichlorvos treated groups. There was significant reduction in the retention time from 6 weeks onwards in both groups. Average retention time was reduced by 44% and 66% in low and high dose group animals respectively 6 weeks post-exposure as compared to the controls. Whereas, at 12 weeks post-exposure these animals showed 46% and 70% reduction in the retention time respectively. The treated animals showed neuromuscular incoordination and seemed confused during training period as compared to the control animals. None of the dichlorvos treated animals could maintain themselves on the rotating rod for the full quota of the cut off time (180 s).

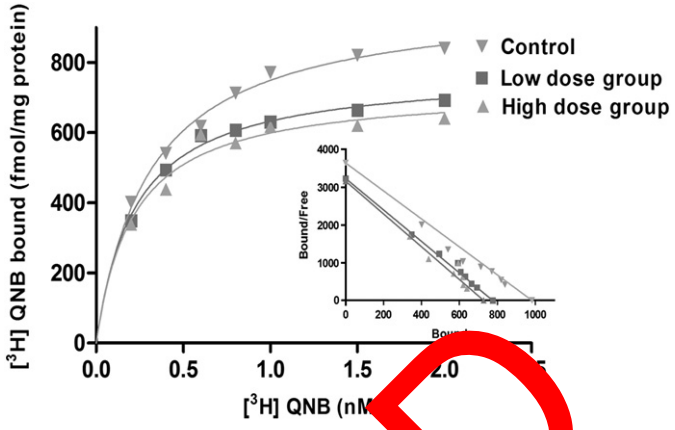

Fig. 4. Specific binding of [^{3}H] QNB to muscarinic receptors in SPMs prepared from rat brain. The values are mean±SD of 6 animals.

3.2. Effect of chronic dichlorvos exposure on passive avoidance tests

The passive avoidance response decreased significantly ($p<0.05$) in terms of latency time in both dichlorvos treated groups in comparison to control animals (Fig. 2). The latency period of the low dose group animals was about 22% less than controls whereas, for animals in high dose group it was about 44% less than controls, after 12 weeks of exposure. The animals exposed to 1 mg/kg b. wt./day of dichlorvos could keep themselves for an average of 128 s in the illuminated chamber, whereas those exposed to 6 mg/kg b. wt./day remained there for about 87.5 s following 12 weeks of exposure.

3.3. Effect of chronic dichlorvos exposure on spatial memory (acquisition test)

Morris Water Maze (MWM) was carried out to test memory function. The latencies to locate a hidden platform in the water maze beginning 24 h after the last dichlorvos exposure are illustrated in Fig. 3. The results indicate that after exposure to vehicle or different doses of dichlorvos for 12 weeks, the rats learned to locate the hidden platform with progressively shorter latencies across the 4 days of training. Time taken to locate the platform for control group animals was 51.6 s on day one and only 7.40 s on day 4 whereas; low dose group animals took 64.6 s and 16.36 s respectively. Time taken by high dose group animals was 70.22 s and 34.16 s respectively. Thus, animals from both the

Table 2
Effect of chronic dichlorvos exposure on brain acetylcholinesterase activity in rat

	Acetylcholinesterase activity (nmol product formed/min/mg protein)
Control group	9.10±1.5
Low dose group (1.0 mg/kg b. wt)	7.08±1.38^{NS}
High dose group (6.0 mg/kg b. wt)	2.59±0.24***, ###

The values are mean±SD of 6 animals (One way ANOVA, with Student–Newman–Keuls post-hoc analysis). *F*-value (55.54).
***$p<0.001$, significantly different from the control group.
###$p<0.001$, significantly different from the low dose group.
^{NS}not significant.

Table 3
Effect of chronic dichlorvos exposure on binding constants for muscarinic receptors in synaptic plasma membranes prepared from rat brain

	B_{max} (fmol/mg protein)	K_d (nM)	K_i value		
			Pirenzepine	Himbacine	4-DAMP
Control group	899.1±30	0.25	6.3	0.039	0.13
Low dose group (1.0 mg/kg b. wt)	776.8±10**	0.23	0.3	0.02	0.01
High dose group (6.0 mg/kg b. wt)	759.8±30**, NS	0.24	0.12	0.002	0.006

The values are mean±SD of 6 animals.
**$p<0.01$, significantly different from the control group.
^{NS}not significant.

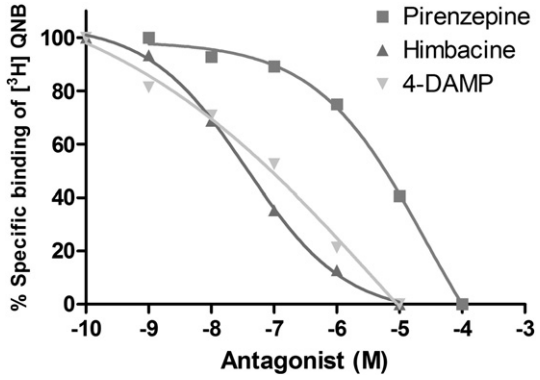

Fig. 5. Displacement of specific [^{3}H] QNB binding by the selective antagonists pirenzepine, himbacine and 4-DAMP. Each point represents the mean±SE of four experiments each performed in duplicates. Error bars are smaller than the area of the symbols used to denote a point.

groups demonstrated significant ($p<0.05$) impairment in performance of the task which is clearly indicated by higher mean latencies in locating hidden platform as compared to controls.

3.4. Effect of chronic dichlorvos exposure on spatial memory (retrieval test)

The effects of dichlorvos on spatial bias are presented in Table 1 as the time spent in the quadrant, where the platform had been located during the first 4 days of testing. Both treatment groups preferred the previous target quadrant (as opposed to the other three quadrants), as indicated by a greater-than-chance (i.e., 25%) time spent in this region of the pool ($p<0.05$, one-tailed t-tests). The higher dose of dichlorvos (6.0 mg/kg) was associated with inferior performance of retrieval test. The 1.0 mg/kg dose group animals spent about 40% less time than controls in target quadrant. Similar, but higher degree impairment was observed in the 6.0 mg/kg dose group animals. These animals significantly spent (45%) less time than controls in target quadrant.

3.5. Effect of chronic dichlorvos exposure acetylcholinesterase activity in rat brain

AChE activity was measured in the synaptic plasma membrane prepared from the whole brain of both dichlorvos treated animals. The brain acetylcholinesterase activity was significantly decreased (71%) in high dose group animals after 12 weeks of exposure. However, the decrease in AChE activity was statistically insignificant in low dose group (Table 2). These results suggest that during chronic low level exposure to dichlorvos AChE activity is inhibited to an insignificant level without any clinical symptoms and there may be other targets for dichlorvos induced neurotoxicity.

3.6. Effect of chronic dichlorvos exposure on specific binding of [^{3}H] QNB to muscarinic receptors

Investigations into the QNB binding to the muscarinic receptors were carried out in the synaptic plasma membranes of the rat brain in the presence of varying concentrations of [^{3}H] QNB (0–2 nM). Non-specific binding was carried out in the presence of atropine (1 μM). Saturation of specific QNB binding sites in the synaptosomal membrane preparation is evident at approximately 1.0 nM [^{3}H] QNB (Fig. 4). At both the doses,

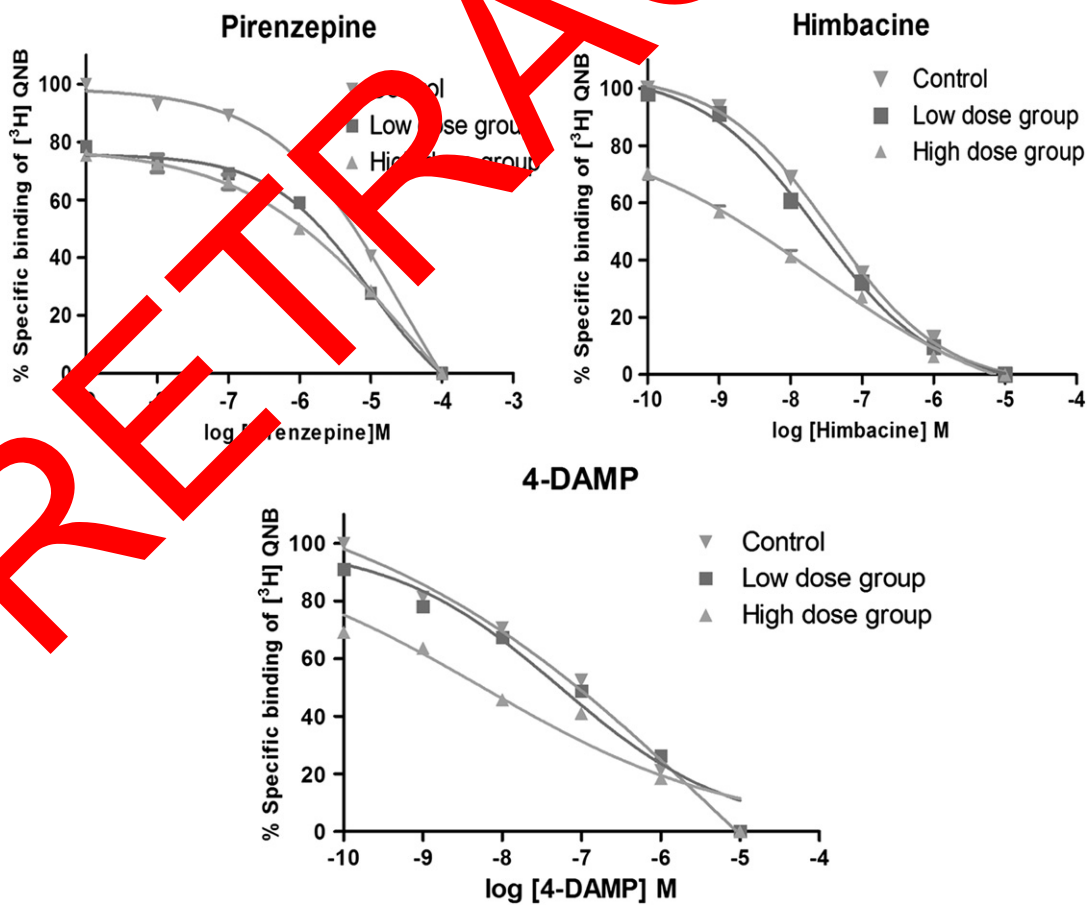

Fig. 6. Displacement of [^{3}H] QNB by the selective antagonists (a) pirenzepine, (b) himbacine and (c) 4-DAMP. Each points represents the mean±SE of four experiments each performed in duplicates. Error bars are smaller than the area of the symbols used to denote a point.

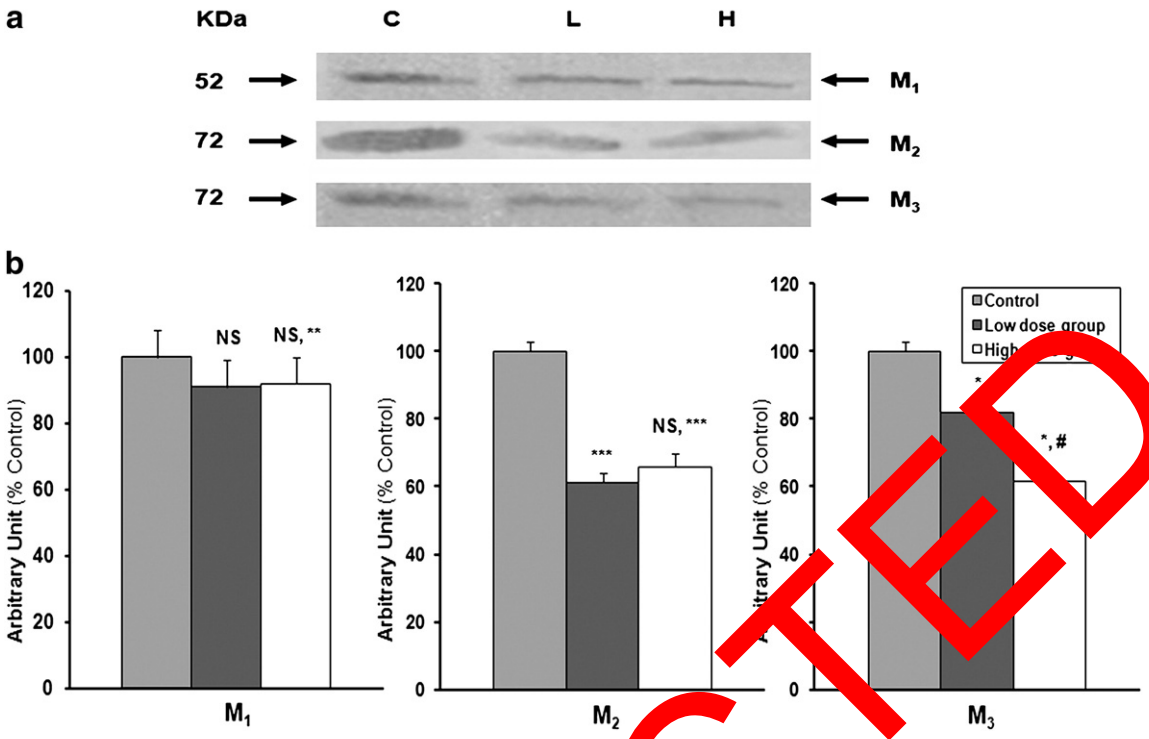

Fig. 7. Effect of chronic dichlorvos exposure on the expression of muscarinic acetylcholine receptor subtypes by western blot analysis using selective antibodies. Lane C — control, Lane L — low dose group and lane H — high dose group. a) Western blot analysis of muscarinic receptors in rat brain. b) Densitometry analysis representing the relative change in protein levels.

nonspecific binding was less than 5% of total binding. The values of constants K_d (binding affinity) and B_{max} (number of binding sites) were obtained form Scatchard plots (Fig. 4 in inset). The B_{max} for the 6.0 mg/kg b. wt. dichlorvos exposed group was 26% (Table 3) and [illegible] mg/kg b. wt. dichlorvos exposed group was 22% (Table 3) lower than the control group (982.8 fmol/mg) animals. The affinity constants (K_d) for control, low and high dose group animals were 0.23 nM, 0.2[illegible] M and 0.022 [illegible] respectively (Table 3).

7. Displacement of [^{3}H] QNB binding by the selective antagonists

The displacement curves which resulted from the binding of [^{3}H] QNB in the presence of the selective ligands, as pirenzepine, himbacine and 4-DAMP are shown in Fig. 5. For each ligand, the IC_{50} values were determined and converted to an apparent K_i value using the approximation of Cheng and Prusoff (1973). In order to determine the effect of dichlorvos on the ability of each selective ligand to

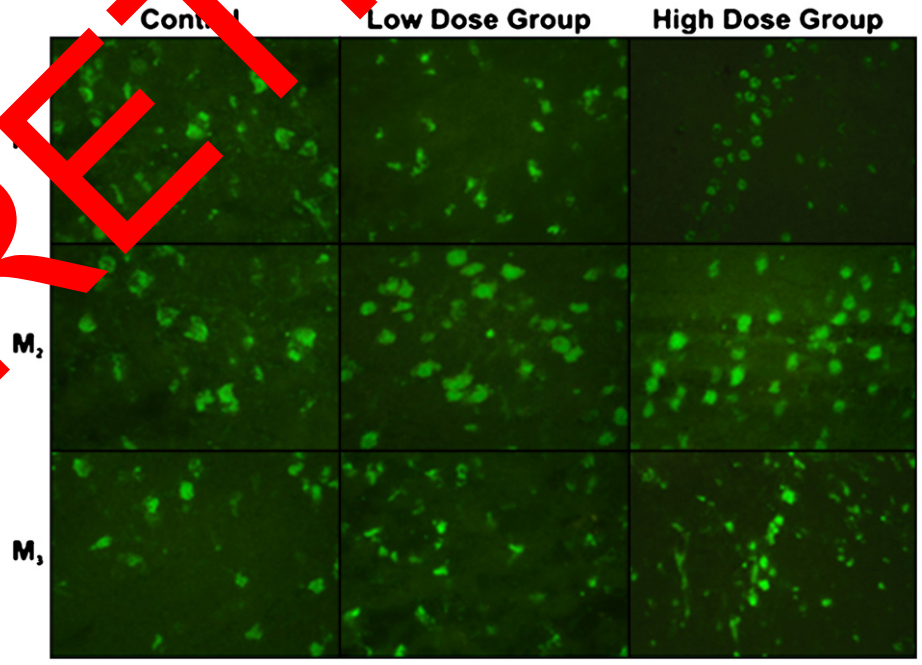

Fig. 8. Effect of chronic dichlorvos exposure on the expression of muscarinic acetylcholine receptor subtypes in hippocampus of rat brain.

displace [^{3}H] QNB from muscarinic receptor, binding of [^{3}H] QNB was determined in the presence of varying concentration of each selective ligand (Fig. 6). In all cases, dichlorvos treatment increased the ability of each selective ligand to displace [^{3}H] QNB binding as evident by decrease in the apparent K_i for each ligand. The order of magnitude of the shifts in apparent K_i values in low dose group animals was pirenzepine>4-DAMP>himbacine. High dose of dichlorvos did not show selectivity to a particular muscarinic receptor.

3.8. Effect of chronic dichlorvos exposure on the expression of muscarinic acetylcholine receptor subtypes

For the quantitative determination of the receptor subtypes, western blot analysis was performed using selective muscarinic receptor subtype specific antibodies in the crude synaptic plasma membranes of control and dichlorvos treated animals (Fig. 7a). The densitometry analysis of the protein band revealed a 46% and 44% decrease in M_2 muscarinic receptor expression in rats treated with 1.0 and 6.0 mg/kg b. wt. dichlorvos, respectively, as compared to the controls (Fig. 7b). M_3 receptor subtypes was also decreased significantly both in low and high dose group animals but in low dose group the magnitude of the reduction of expression of M_3 receptors was very small. Decreased expression of M_1 receptor was found only in animals treated with 6.0 mg/kg b. wt. of dichlorvos and no significant alterations was seen in the animals treated with 1.0 mg/kg b. wt. dichlorvos.

3.9. Immunofluorescence studies

In order to further confirm these findings, we performed the immunofluorescence staining of all the three receptor subtypes in the cerebral cortex and hippocampus of rat brain. The control group animals showed strong positivity in cytosol of both areas with all the three receptor subtypes (Fig. 8). The low dose group animals showed reduction in fluorescence with M_2 receptor subtype only in both regions i.e. cerebral cortex (data not shown) and hippocampus. Moreover, in high dose group animals, expression of all the receptor subtypes was reduced in both the brain regions.

4. Discussion

Chronic dichlorvos exposure (1.0 and 6.0 mg/kg b. wt./day) for a period of 12 weeks, significantly decreased typical exploratory behavior of experimental animals. Daily doses of 6.0 mg/kg dichlorvos did not elicit overt signs related to cholinergic hyper stimulation, however high dose of dichlorvos caused symptoms associated with cholinergic stimulation. Chronic dichlorvos exposure at both doses had a detrimental effect on the body weight of rats as compared to the controls (data not shown). Similar impact on the body weight of rats was observed following chronic exposure to dichlorvos for a period of 8 weeks (Baheti and Gill, 2002). This decrease in body weight might be due to enhanced synthesis of glucose from non-carbohydrate sources thereby leading to mobilization of fat deposits henceforth causing decreased body weight.

Chronic dichlorvos administration resulted in a marked impairment of muscle strength and motor coordination of animals as revealed by the significant reduction in retention time on the rota rod apparatus in both low and high dose group animals respectively. These observations are in line with those of Karczmar (1984) and Aggarwal et al. (1988) who reported a significant degree of hypokinesia and diminution of muscle tone following chronic exposure to OP pesticides. Decreased locomotor frequency and increased immobility following OPs intoxication have also been reported by Lazarini et al. (2004) and Sun et al. (2006). Further development of locomotor ataxia following OPs exposure has been associated with impaired axonal transport of essential enzymes and metabolites (Moretto et al., 1987); an energy dependent phenomenon, which requires the involvement of glycolytic enzymes. Sarin and Gill (1999) have earlier reported an altered glucose metabolism in rat brain after chronic dichlorvos exposure.

The MWM task has often been used for the validation of rodent models for neurocognitive disorders and the evaluation of possible neurocognitive treatments. In our study, both dichlorvos treatments resulted in poor performance of animals on MWM. This impairment was not due to the defect in motor function, as indicated by the fact that all the groups clearly preferred the target quadrant (over the other three quadrants) in probe trials at day 5. In a previous study Prendergast et al. (1997) have also reported that rats previously exposed to a sub-toxic dose of DFP, showed marked impairment to learn the spatial clues to locate the hidden platform relative to control animals. Our data also suggests that chronic low-level exposure to dichlorvos (1 mg/kg b wt, 12 weeks) produces subtle effects associated with impaired ability to learn a novel task. Here, it is important to mention that low dose group animals, which did not show any impairment in cholinesterase activity, showed significant impairment in neurobehavioral indices. The observed impairment of learning and/or memory processes may be because of neurotoxic effect of dichlorvos on acetylcholine receptors. It has been reported that pharmacological blockade of neuronal muscarinic receptors impairs performance of tasks which assess learning and memory processes (Timofeeva et al., 2003; Seeger et al., 2004). Our results are in accordance with the many other observations, which demonstrated that in rats, low-level exposure to OPs impaired their cognitive functions without any significant effect on acetylcholinesterase activity (Jett et al., 2001; Canadas et al., 2005). It has been suggested that the behavioral effects following chronic exposure to OP pesticides may be related to altered AChE activity or decreased number of muscarinic acetylcholine receptors, especially the M_2 receptors (Mundy et al., 1988). Therefore, we carried out the muscarinic acetylcholine receptor binding studies.

Results presented in the study indicated the decrease in the activity of AChE at 6 weeks interval in rats, following chronic dichlorvos exposure. Significant decrease was observed only in high dose group animals, from 6 weeks exposure onwards which continued till 12 weeks. This is in conformity with the observations of Bhatnagar et al. (1994) and Plumlee et al. (1994), who also observed substantial AChE inhibition following chronic high level dichlorvos exposure. The decrease in the AChE activity is indicative of the fact that there may be a cholinergic dysfunction following chronic dichlorvos exposure. This further supports the fact that dichlorvos may be implicated in the etiology of neurodegenerative diseases, since most of these diseases characteristically show a decrease in AChE activity (Fibiger, 1991). Our findings of low dose group are in accordance with the findings of Farahat et al. (2003) who have shown that moderate chronic OP exposure may not only affect visuomotor speed but also verbal abstraction, allocation of memory, without any effect on acetylcholinesterase activity. Desi and Nagymajtenyi (1999) also showed impaired neurobehavioral indices after OP exposure, although AChE activity was unaffected. Thus these results support the existence of some alternative mechanism (in addition to AChE inhibition) for dichlorvos induced neurobehavioral deficits at chronic low level exposure.

Numerous *in vivo* studies report a reduction in the number of muscarinic receptors in certain brain areas as a result of chronic exposure to OPs (Zheng et al., 2000). *In vitro* studies have also reported decreased muscarinic receptor binding without changes in the receptor affinity in the presence of low-levels of organophosphates (Bomser and Casida, 2001; Viana et al., 1988). Our results as shown in Fig. 4 make it clear that both high dose and low dose group animals showed a significant alteration in [^{3}H] QNB binding with muscarinic receptors as compared to control animals. In both treated groups, B_{max} values were significantly decreased although the binding affinities (K_d) were not significantly altered. These results are consistent with the earlier observations, which

demonstrate the decreased QNB binding after exposure to the dichlorvos and other OP's (Betancourt and Carr, 2004). Such down regulation of muscarinic receptor number has been shown to be the primary mechanism of adaptation to elevated synaptic ACh levels (Zheng et al., 2000). The possible mechanism for the observed effects of low concentration of organophosphates on the B_{max} for antagonist binding to muscarinic receptors is loss of muscarinic receptors under phosphorylating conditions in synaptic membranes (Burgoyne, 1981).

The use of selective muscarinic receptor antagonists in the present study indicated that chronic low-level exposure to dichlorvos differentially modulated subpopulation of muscarinic receptors. Synaptic plasma membranes (muscarinic receptor membranes) prepared from low dose group animals shifted the displacement curves for the selective ligands, reflecting regulatory changes in the affinity of these selective ligands for the remaining pool of receptors (Fig. 6). Maximum shift in apparent affinity was seen with the M_1 selective ligand, pirenzepine followed by M_3 selective ligand 4-DAMP but selective ligand for M_2 receptor subtype i.e. himbacine did not show any change in displacement curve. These results suggested a significant loss of low affinity M_2 and slightly M_3 sites due to dichlorvos exposure. It is important to note that at the high dose (6.0 mg/kg b. wt.) dichlorvos did not have specificity to a particular muscarinic receptor subtype and all the selective ligands show a significant displacement (Fig. 6). The effects observed at higher dose (6.0 mg/kg b. wt.) can be explained on the basis of the fact that pesticide at higher doses down regulates the expression of all the muscarinic receptor subtypes (i.e. total pool of receptors) and thus receptors are not available to bind to the antagonist (QNB) (Damodaran et al., 2006). The present results support the hypothesis put forth by Liu et al. (2002), which says that the decrease in the presynaptic auto receptors by OPs leads to a spontaneous release of acetylcholine, which causes a further down-regulation of postsynaptic receptors.

These findings were further confirmed by western blot analysis of various subtypes of muscarinic receptors. Densitometry analysis revealed that at very low concentration of dichlorvos, M_2 muscarinic receptors were significantly reduced and to some extent M_3 receptors also. Moreover, at higher dose, all the three receptor subtypes were affected. The immunofluorescence study showed the cytosolic localization of various receptors. Dichlorvos treatment affected the distribution of these receptors and the most affected receptor subtype was M_2 in both low and high dose group animals.

So, all the above results suggest an alternative mechanism which is independent of AChE inhibition, for the action of dichlorvos at very low concentration *in vivo*. Therefore, future studies on the signal transduction cascade associated with muscarinic receptor subtypes are needed to have a clear view of the mechanism of action for low level chronic dichlorvos exposure at molecular level.

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