Memantine Alleviates Toxicity Induced by Dichlorvos in Rats

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Abstract: Memantine Alleviates Dichlorvos Poisoning in Rats: Zhijun Zhou, et al. School of Public Health, Fudan University, China—The changes of N-methyl-D-aspartate (NMDA) receptor and protective efficacy of memantine (MEM) in rats poisoned with dichlorvos were studied. Dichlorvos evoked down-regulation of the affinity and density of [3H]MK-801 binding to NMDA receptor in the brain of rats receiving dichlorvos (15 and 25 mg/kg bw, i.p.). The binding capacity of NMDA receptor and acetylcholinesterase activity were determined at 4 h, 8 h, 16 h, 24 h and 48 h after treatment. When rats were given a different doses of MEM (5, 15 and 45 mg/kg bw) after poisoning (dichlorvos 25 mg/kg bw), the latency of onset of signs was postponed and the magnitude of muscular fasciculation was alleviated as the dose of MEM increased. The lower doses of MEM (5 and 15 mg/kg bw) could antagonize the dichlorvos-evoked down-regulation of NMDA receptor, while the highest dose (45 mg/kg bw) decreased the Bmax and Kd values of NMDA receptors. These results show the dichlorvos-evoked down-regulation of NMDA receptor might be self-regulation by the body to protect the central nervous system. MEM could antagonize the down-regulation of NMDA receptors, and alleviated signs of poisoning, especially reducing the magnitude of muscular fasciculation. We suggest that the role of NMDA receptor in organophosphates (OP) poisoning should receive more attention, and, that MEM treatment in acute OP poisoning, as a supplement to atropine plus oxime regimen is needed.

Key words: Memantine, Dichlorvos, NMDA-receptor

Organophosphates (OPs) are easily accessible as widely used agricultural and residential insecticides. Thousands of occupational and accidental (suicidal) intoxications and hundreds of fatalities are reported in this country annually. OPs possible use in warfare and terrorist attack attracts grave concern.

Atropine (m-cholinergic receptor antagonist) and oxime reactivators are remedies currently used for treatment of OP intoxication. Most cases respond well to this combined remedy, although fatalities in severe cases still happen frequently. Besides, the therapeutic regimen of atropine plus oxime seems to have no effect on alleviating the overt changes from n-cholinergic receptor and clinical features due to non-cholinergic mechanisms, such as impairment and death of neurons including OP induced polyneuropathy (OPPN), etc. Thus, an auxiliary remedy for such aggravating effects, as a supplementary treatment to atropine plus oxime regimen is needed.

Excitotoxicity, including seizure due to OP has been well studied in nerve gas, but not in insecticides. The excitotoxic effect of dichlorvos (DDVP) was tested in this study.

Excitatory amino acid (EAA) receptor antagonists have been shown anticonvulsive and protective effects against neurotoxins in experimental animals. It has been hypothesized that the N-methyl-D-aspartate (NMDA) receptor-associated mechanism plays a critical role in the spread and maintenance of OP-induced seizure activity. A recent study has confirmed that inhibition of acetylcholinesterase and the subsequent increment of acetylcholine might initiate seizure, resulting from a secondary increment of excitatory amino acid transmitter.

Memantine hydrochloride (MEM, Fig. 1), an amantadane derivative, has been recently suggested for the treatment of many central nervous system disorders, such as Parkinsonism, coma, convulsions, and seizures induced by OP. Masuo demonstrated that MEM reversibly blocks neuromuscular transmission. A combined treatment of MEM and atropine was found to be one of the most effective remedies against
anticholinesterase poisoning due to methamidophos and methyl-parathion.

Memantine (MEM), a NMDA receptor antagonist, has shown a capability to reduce the over-stimulation of the NMDA receptor caused by abnormally high concentrations of glutamate, and to restore the normal receptor-signal function. Meantime as a remedy would alleviate the excitotoxicity and also render neuroprotective effects by preventing the overload of calcium in the neuron. The overload of calcium has been implicated in the pathogenesis of neurodegenerative diseases.

In this study, the adverse effect of dichlorvos, an OP insecticide that is widely used in this country, on the activity of the NMDA receptor in the rat brain was examined. Then, the therapeutic effect of MEM on dichlorvos-poisoned rats through regulation of NMDA receptor was tested.

Material and Methods

Chemicals: Dichlorvos (DDVP), 95% purity in crystalline form, was a gift from the Yongfa Chemical Co, Ltd (Taiwan). MK-801 (dizocilpine maleate) and memantine hydrochloride (MEM, 1,3-dimethyl-5-aminoadantane hydro chloride) were purchased from the Sigma Chemical Company. ASCh (acetylthio-choline iodide), DTNB (5,5'-dithiobis-2-nitro-benzoic acid) and physostigmine were purchased from the Fluka Company. 

Preparation of solutions: Dichlorvos and MEM were dissolved in normal saline (0.9% NaCl, w/v). The assay buffer\(^{10}\) (118 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l MgSO\(_4\), 5 mmol/l NaHCO\(_3\), 1.2 mmol/l KH\(_2\)PO\(_4\), 2.5 mmol/l CaCl\(_2\), 11 mmol/l glucose, 20 mmol/l Hepes, pH 7.4) was a routine laboratory preparation. \([^{1}H]MK-801\) (Specific activity=28.9 Ci/mmol) was purchased from the NEN Company. All the substances used were of the highest chemical purity available.

Animals: Adult male Sprague-Dawley rats, weighing 200–240 g, were supplied by the Department of Animal Science of Fudan University. The animals were acclimatized in our laboratory for 5 d before use in the experiment. Rats were housed 5 per cage at a temperature of 21 ± 1°C, 50 ± 10% humidity, and light (12 h light/dark cycle) with free access to standard laboratory food and water. The animals were distributed randomly to the experimental groups.

Observation of muscle fasciculation and toxic signs: Animals were observed 2 h after the administration of dichlorvos. Muscle fasciculation was assessed according to the following qualitative staging system described by Yu\(^{11}\): stage 0, no abnormal muscle fasciculation; stage 1, gentle tremor of the face and limbs without movement of the joints; stage 2, light muscle fasciculation of limbs and horizontal movement of the joints; stage 3, muscle fasciculation of limbs and vertical movement of joints.

Measurement of acetylcholinesterase activity: The tested animals were sacrificed by decapitation. Whole brains were rapidly dissected and placed on ice-cold slides. Fresh, unfrozen tissues were analyzed immediately after collection. The tissues were homogenized using a glass homogenizer in 5 ml ice-cold 0.32 mol/l sucrose buffer (pH 7.4). A 30-µl aliquot of homogenate was added to 3 ml phosphate buffer (pH 7.4) containing DTNB and ASCh.

The acetylcholinesterase activity was determined with Ellman’s colorimetric method\(^{12}\) at a wavelength of 412 nm. The blood acetylcholinesterase activity was determined as \(µmol\) acetylthiocholine iodide hydrolyzed/ ml blood/h. The brain acetylcholinesterase activity was determined as \(µmol\) acetylthiocholine iodide hydrolyzed/ mg protein/h.

Determination of activity of NMDA receptor: For the in vitro binding assay, whole brains from dichlorvos-poisoned rats were homogenized in 9 volumes of ice-cold 0.32 mol/l sucrose with a glass homogenizer. The homogenate was centrifuged for 15 min at 1,000 × g, the supernatant was re-centrifuged at 13,000 × g for 25 min at 4°C. The pellet was suspended in assay buffer and incubated at 23°C for 30 min prior to final centrifugation at 13,000 × g for 20 min at 4°C. The pellet was re-suspended in assay buffer\(^{16}\). Protein concentration was determined with the method reported by Lowry et al.\(^{13}\)

Binding of \([^{1}H]MK-801\) was measured by incubating 150 µl of duplicate aliquots of the crude membrane suspension (0.15 mg of protein) with 10 µl different concentration of \([^{1}H]MK-801\) (final concentration between 0.5–24 nmol/l), and 140 µl of assay buffer for 60 min at 23°C; this was total binding. Nonspecific binding was defined by 100 µl of unlabeled MK-801 (0.1

Fig. 1. Structure of memantine.
Incubation was terminated by rapid filtration through Type 49 filters, which were washed immediately with two 5 ml portions of ice-cold assay buffer in a ZT-II 12-R cell harvester. The complete filtration and wash processes were finished in less than 10 s. Radioactivity of the filters was determined by liquid scintillation counting (Beckman LS6500) in vials with 5 ml scintillate solution and 2 ml ethyl alcohol absolute at 20% counting efficiency. Maximum binding capacity (Bmax) and equilibrium dissociation constant (Kd) were determined by Scatchard analysis.

**Experiment design:** To study the time-course of changes of the NMDA receptor in dichlorvos-poisoned rats, 55 male rats were used and divided into 11 experimental groups. One group was treated with saline as a control. Five groups were treated with dichlorvos at a dose of 15 mg/kg bw. The other five groups were given dichlorvos at a dose of 25 mg/kg bw. Dichlorvos was given intraperitoneally in a volume of 10 ml/kg bw. Each group was sacrificed at different times after dichlorvos injection. The sacrifice times were 4 h, 8 h, 16 h, 24 h and 48 h respectively.

To study the protective effect of MEM, the following experiment was designed. One group of rats served as control, another was given dichlorvos only, and three groups at different concentrations were given dichlorvos plus MEM. The dose of dichlorvos was 25 mg/kg bw. The doses of MEM in the three MEM treatment groups were 5, 15 and 45 mg/kg bw, respectively. All injections were given intraperitoneally. The rats were sacrificed 16 h after intoxication.

**Statistical analysis:** The data are presented as mean ± SD and compared through variance analysis or Student’s *t*-test. The intensity of muscle fasciculation was presented as the median and compared with the Mann-Whitney *U*-test. The level of significance for all analyses was set at *p*<0.05.

**Results**

The male Sprague-Dawley rats receiving acute sublethal doses of dichlorvos (15 mg/kg and 25 mg/kg) developed cholinergic toxic signs within 15–20 min, but none of the rats were dead at the end of experiment. Signs with increasing propensity to maximal severity, including hyper-secretion, respiratory distress, tremor, generalized muscle fasciculation and convulsion, were evident during 30 min to 1 h and lasted for 1 h. Thereafter, signs were seen up to 4 h with declining intensity. By the end of 5 h the toxic signs had subsided completely.

The blood and brain acetylcholinesterase activity in control (saline-treated) rats were 47.082 ± 4.031 and 5.451 ± 0.299, respectively. Compared with controls, *p*<0.05 **p*<0.01.

### Table 1. Time-course changes of AChE activities in dichlorvos-poisoned rats (mean ± SD)

<table>
<thead>
<tr>
<th>Dichlorvos (mg/kg)</th>
<th>Indicator</th>
<th>4 h</th>
<th>8 h</th>
<th>16 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.731 ± 0.055**</td>
<td>0.631 ± 0.040**</td>
<td>1.212 ± 0.045**</td>
<td>1.305 ± 0.094**</td>
<td>1.4537 ± 0.103**</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>16.890 ± 1.299**</td>
<td>13.969 ± 1.530**</td>
<td>18.235 ± 1.668**</td>
<td>23.585 ± 4.515**</td>
<td>30.055 ± 1.992**</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>0.7567 ± 0.058**</td>
<td>1.097 ± 0.099**</td>
<td>1.431 ± 0.132**</td>
<td>1.511 ± 0.207**</td>
<td>1.633 ± 0.114**</td>
<td></td>
</tr>
</tbody>
</table>

Rat blood and brain acetylcholinesterase activities in control (saline-treated) rats were 47.082 ± 4.031 and 5.451 ± 0.299, respectively. Compared with controls, *p*<0.05 **p*<0.01.

### Table 2. Time-course changes of NMDA receptor activities in dichlorvos-poisoned rats (mean±SD)

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Dichlorvos (mg/kg)</th>
<th>4 h</th>
<th>8 h</th>
<th>16 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmax</td>
<td>25</td>
<td>0.517 ± 0.053*</td>
<td>0.478 ± 0.050**</td>
<td>0.456 ± 0.057**</td>
<td>0.426 ± 0.072**</td>
<td>0.515 ± 0.094*</td>
</tr>
<tr>
<td>15</td>
<td>0.528 ± 0.058*</td>
<td>0.499 ± 0.043**</td>
<td>0.476 ± 0.063**</td>
<td>0.410 ± 0.036**</td>
<td>0.550 ± 0.080</td>
<td></td>
</tr>
<tr>
<td>Kd</td>
<td>25</td>
<td>49.401 ± 3.438**</td>
<td>58.774 ± 5.156**</td>
<td>75.548 ± 7.869**</td>
<td>77.568 ± 6.152**</td>
<td>63.432 ± 4.134**</td>
</tr>
<tr>
<td>15</td>
<td>47.353 ± 4.786**</td>
<td>55.910 ± 5.935**</td>
<td>66.917 ± 5.340**</td>
<td>71.003 ± 7.135**</td>
<td>60.905 ± 6.375**</td>
<td></td>
</tr>
</tbody>
</table>

#Mean Bmax and Kd values in control (saline-treated) rats were 0.615 ± 0.043 pmol/mg protein and 37.368 ± 4.167 nmol/l, respectively. Compared with control, *p*<0.05 **p*<0.01.
Dichlorvos are presented in Table 1. The acetylcholinesterase activities were rapidly inhibited by dichlorvos, while brain acetylcholinesterase activity decreased more severely and recovered much slower. The peak of inhibition of acetylcholinesterase both in blood and brain was 8 h after administration of dichlorvos.

The rat brain NMDA receptors’ $K_d$ values and $B_{max}$ in the controls were $37.368\pm4.167$ nmol/l and $0.615\pm0.043$ pmol/mg pro, respectively. $B_{max}$ of NMDA receptor decreased significantly after the rats received dichlorvos. $B_{max}$ value reached the lowest level at 24 h and then began to recover. Contrarily, $K_d$ became significantly higher at 4 h after dichlorvos injection and reached its peak by the time of 24 h, then decreased gradually (Table 2).

The latency of onset of signs, the intensity of muscle fasciculation, and the sum of score of signs in the four tested groups are listed in Table 3. Variance analysis showed that the difference of these three parameters among three groups were statistically significant. This showed that MEM treatment (15, 45 mg/kg) could postpone the latency of onset of signs, and alleviated the intensity of muscle fasciculation and the total score of signs.

The acetylcholinesterase activities both in blood and brain samples were still at a low level after MEM administration. No differences were found in comparison with the control group receiving dichlorvos only.

Dichlorvos evoked down-regulation of $[^3H]$MK-801 binding to NMDA receptor in the rat brain with a significant decrease in the affinity, which was characterized as increment of the NMDA receptor $K_d$ value ($75.548\pm7.869$ nmol/l) and reduction of the $B_{max}$ value ($0.456\pm0.057$ pmol/mg pro). The NMDA receptor $K_d$ value in the normal rat brain and $B_{max}$ in the control were $37.368\pm4.167$ nmol/l and $0.615\pm0.043$ pmol/mg protein, respectively.

In groups receiving low doses of MEM (5 and 25 mg/kg bw), MEM alleviated dichlorvos-evoked down-regulation of $[^3H]$MK-801 binding to NMDA receptor in rat brain; while in groups receiving the highest dose of MEM (45 mg/kg bw) the $B_{max}$ ($0.454\pm0.062$ pmol/mg protein), and $K_d$ ($22.884\pm4.421$ nmol/l) of NMDA receptor were decreased significantly ($p<0.01$) (Table 4).

**Discussion**

The results show that the $B_{max}$ value of the NMDA receptor was decreased and the $K_d$ value was raised after dichlorvos administration. These changes were statistically significant (Table 4). It was demonstrated that dichlorvos evoked down-regulation of $[^3H]$MK-801 binding to the NMDA receptor in the rat brain. Such down-regulation may be self-regulation by the body to protect the central nervous system from organophosphate intoxication.

The NMDA receptor is one of the excitatory amino acid subtypes and plays a key role in the mechanism evoking excitotoxic effects. The excitatory amino acids
are the important excitatory neural-transmitters. Some neurodegenerative diseases and trauma of the central nervous system have been demonstrated to be associated with excitotoxic effects through the action of excessive glutamic acid or analogues\(^\text{14, 15}\). Shih et al. reported that acetylcholine levels in the rat brain increased quickly within 3 min after soman treatment. Norepinephrine levels decreased while dopamine and its metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid were subsequently elevated. The levels of excitatory amino acids (aspartate and glutamate) in the brain were markedly decreased, while the inhibitor amino acids, such as gamma-aminobutyric acid (GABA), were significantly increased following the foregoing change of the cholinergic system\(^8\).

Binding studies have revealed the direct interaction between OP and the NMDA receptor protein complex. For example, Johnson et al. demonstrated that several OPs such as diisopropylfluorophosphate (DFP) could inhibit the specific binding to brain synaptic membranes at the NMDA receptor of \(^{\text{[H]CPP}},\) a selective antagonist of NMDA receptors\(^16\).

The NMDA receptors were significantly decreased after combination with the excessive extracellular excitatory amino acid. It is down-regulation of NMDA receptors, a kind of compensatory mechanism, which could protect the central nervous system against damage from excess stimulation of the excitatory amino acids. The activation of NMDA receptors increases the permeability of Ca\(^{2+}\), Na\(^{+}\), K\(^{+}\) and Mg\(^{2+}\), which combine with the receptors. Then Ca\(^{2+}\) overloads and Na\(^{+}\) accumulates in the nerve cells. High concentrations of these ions cause the damage in the cells. Secondary brain damage might initialize the apoptosis of nerve cells. It also suppresses the synthesis and accelerates the decomposition of NMDA receptors simultaneously. The excessive activation of the receptors would also secondarily reduce their life span and decrease the concentration of the receptors\(^17\). Eventually this would lead to the reduction of the number and the affinity of the NMDA receptors on the nerve cell membrane.

NMDA receptors exist widely in the brain. They are involved in learning, memory and other physiological activities. Under pathological conditions, the rapid increment of excitatory amino acids and metabolism disorders would result in the over activation of NMDA receptors, which would further cause secondary brain damage\(^6\). The implication of the NMDA receptor in OP poisoning should be noted. Moreover, the changes of NMDA receptor could serve as guidance for the use of NMDA receptor antagonists.

Based upon the above findings, experimental therapy with MEM was conducted. The results demonstrate emphatically that MEM could postpone the latency of excitatory toxic signs, and alleviate the intensity of muscle fasciculation. Total score of clinical features decreased as the dose of MEM increased (Table 3). The improvement is in accordance with the change of NMDA receptors. MEM rehabilitates the down-regulation of NMDA receptors, i.e. the affinity and density of NMDA receptors recovers to normal level (Table 4).

A number of studies have shown that MEM could alleviate several neurological disorders, such as coma, convulsion and spasm caused by intoxication with certain OP and carbamate insecticides\(^19\text{–}21\). The potential mechanism of this antagonism against nicotinic symptoms is that MEM irreversibly inhibits the communication of neuromuscular junctions\(^8, 22\). MEM has rendered dopamine effects and could alleviate high-frequency tremor of peripheral nerves through inhibition of monoamine oxidase activity. MEM could abate the responsibility of the flexor and the extensor, too\(^20\). NMDA antagonists remarkably reduced the high permeability of the blood-brain barrier resulting from brain injury\(^18\).

However, the administration dose of MEM is critical. We noticed in our study that 45 mg/kg MEM caused the reduction of both Bmax and Kd, while 5 mg/kg MEM showed no effect. It has been reported that over-dose of NMDA receptor antagonist caused respiratory inhibition and finally resulted in the death of rats. The reason may be that it interacted with the related receptors involved in the modulation of respiration\(^23\). This indicates that the therapeutic dose of MEM should be below 45 mg/kg in the rats. A dose of 15 mg/kg MEM would be appropriate, for in our study this dose kept the NMDA receptors within normal range and alleviated toxic symptoms, especially muscular fasciculation.

All man-made adamantane derivatives cannot reactivate or suppress the erythrocyte acetylcholinesterase inhibited by OP\(^24\). We also demonstrated that MEM possesses no effect on the blood or brain acetylcholinesterase in rats poisoned with dichlorvos. These results remind us that MEM is insufficient to treat OP poisoning alone. Our previous experiment has validated this point also. It showed that a combination of MEM with atropine achieved better therapeutic effect than using MEM solely\(^25\). However, the molecular mechanism of MEM in OP poisoning needs more thorough elucidation before raising a suggestive protocol of treatment regimen for OP poisoning.

In conclusion, dichlorvos evoked down-regulation of \(^{\text{[H]MK-801}}\) binding to NMDA receptors in the rat brain with decrease in affinity and density of NMDA receptor. MEM could alleviate certain signs, especially reducing the intensity and magnitude of muscle fasciculation. The mechanism of MEM was not relevant to the inhibition of acetylcholinesterase and its consequences, but protected the NMDA receptors in the rat brain from organophosphate attack. Although, MEM alone was far from effective in treating acute organophosphate poisoning.
poisoning, the administration of MEM as a supplement to atropine and oxime (cholinesterase reactivator) in treatment of acute organophosphate poisoning is recommended for alleviating the neuro-excitatory signs.

Acknowledgments: This work was supported by a grant from the National 973 project (2002CB512905) and a grant from the Shanghai Shu-guang Program. The author thanks Prof. Shou-zheng XUE for his help in correcting the English writing.

References