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Protective effect of nimodipine on dichlorvos-induced delayed neurotoxicity in rat brain

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Abstract

The effect of dichlorvos (200 mg/kg body weight) with or without nimodipine (6 mg/kg body weight/day for 3 days, starting 1 day prior to the administration of dichlorvos) on calcium homeostasis was studied in the rat brain. The delayed neurotoxic potential of dichlorvos was assessed in terms of neuropathy target esterase (NTE) inhibition in the brain and the subsequent development of motor incoordination at 21 days post-exposure. NTE activity had recovered up to 84% at the time of clinical manifestations. No signs of motor deficit were present when nimodipine was given with dichlorvos. The administration of dichlorvos alone caused an increase in intrasynaptosomal Ca^{2+} with a concomitant increase in calpain activity. These increases in calpain activity and in the levels of intracellular Ca^{2+} were not observed when nimodipine was administered to rats treated with dichlorvos. Also, the inhibition of calcium ATPase following the exposure to dichlorvos was reduced when animals received nimodipine. This indicates that nimodipine, a centrally acting calcium channel blocker, may contribute to the amelioration of dichlorvos-induced neurotoxicity by attenuation of calcium-mediated disruption of cytoskeletal homeostasis, without preventing NTE inhibition.

Keywords: Dichlorvos; Nimodipine; OPIDN; Calcium channel blocker; Calcium homeostasis; NTE

1. Introduction

Dichlorvos, an OP pesticide, is being used throughout the world for the protection of stored products and crops and also as a public health insecticide. The clinical signs and symptoms associated with acute dichlorvos poisoning are generally attributable to acetylcholine accumulation following the inhibition of acetylcholinesterase. In addition to acute cholinergic effects, some OP compounds are also capable of causing an irreversible, progressive delayed neurological deficit called OPIDN in both humans and animals [1]. It is characterized by the development of locomotor ataxia 2–3 weeks after a single exposure or following repeated low-level exposure to an OP pesticide [2,3]. Typical symptoms of OPIDN include hyperexcitability, weakness and flaccid paralysis of the extremities, and eventually complete paralysis [4]. Inhibition of NTE, a neuronal enzyme, has been reported to be the primary biochemical event associated with the onset of OPIDN [5]. However, thus far it has not been possible to elucidate the exact sequence of events between NTE inhibition and the onset of OPIDN following exposure to OPs, including dichlorvos [6].

The delayed neurotoxic potential of dichlorvos remains controversial and needs to be clearly established. Whereas Johnson [7] and Abou-Donia [1] reported that dichlorvos has no OPIDN potential, Caroldi and Lotti [8] and Wadia *et al.* [9] reported the induction of ataxia in hens and humans following acute dichlorvos exposure. Recently, we determined that administration of dichlorvos (200 mg/kg body weight, s.c., single dose) inhibited NTE in the rat brain at various post-exposure intervals. The delayed neurotoxic potential of dichlorvos was evident by a rota-rod test, which revealed severe motor deficits in all the exposed animals at 21 days post-exposure [10].

It has been proposed that NTE inhibition by OP compounds is associated with an increase in $[Ca^{2+}]_i$, which, in turn, may activate Ca^{2+}/calmodulin-dependent kinase lead-

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Abbreviations: OP, organophosphate; NTE, neuropathy target esterase; OPIDN, organophosphate-induced delayed neuropathy; PAM, 2-pyridine aldoxime methiodide; SPM, synaptic plasma membranes; VOCC, voltage-operated calcium channels; TOCP, tri-*o*-cresyl phosphate; PSP, phenyl saligenin phosphate; and $[Ca^{2+}]_i$, intracellular calcium.

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ing to aberrant phosphorylation of cytoskeletal proteins and their accumulation, a common feature of many neurodegenerative diseases [11]. Although the precise mechanism of OPIDN has not been defined, the breakdown of cytoskeletal proteins accompanied with the increased level of axonal free calcium could be an important contributing factor for OPIDN.

Increased $[Ca^{2+}]_i$ may also lead to neuronal degeneration through the activation of calcium-activated proteases, mainly calpain [12,13]. Proteases, including calpain, have been shown to be involved in the breakdown of cytoskeletal elements in Wallarian degeneration, a typical characteristic of OP toxicity [14]. In this context, calcium channel blockers like verapamil and nifedipine have been shown to ameliorate some of the functional and morphological deficits of OPIDN observed in the hen [15,16]. However, it remains to be seen if other centrally acting calcium channel blockers, viz. nimodipine which crosses the blood–brain barrier easily, may provide better protection against OPIDN.

In the present study, an attempt was made to examine the involvement of the calcium messenger system in the development of dichlorvos-induced delayed neurotoxicity. Also, the protective efficacy of nimodipine, a new calcium channel blocker, was assessed so as to have a better therapeutic regimen for OPIDN in the future.

2. Materials and methods

2.1. Animals and treatment

Male Wistar strain rats in the weight range of 150–200 g were used throughout the study. Animals were kept in hygienic conditions and fed a standard rat pellet diet and water ad lib.

Rats were divided into the following four groups (6–8 animals/group):

(i) Control group. Rats were injected with 100 mg PAM/kg body weight and 20 mg atropine sulphate/kg body weight (i.p), and then were administered an equal volume of corn oil (s.c).
(ii) Dichlorvos-treated group. Rats were injected with 100 mg PAM/kg body weight and 20 mg atropine/kg body weight (i.p). After 15 min, the animals received 200 mg dichlorvos/kg body weight (s.c.) with corn oil as the vehicle.
(iii) Nimodipine-treated group. Rats in this group were given nimodipine at a dose volume of 6 mg/kg body weight (i.m.), for 3 days, 15 min after receiving i.p. injections of PAM and atropine.
(iv) Nimodipine + dichlorvos-treated group. Rats were injected with PAM and atropine as above and 15 min later received 6 mg nimodipine/kg body weight (i.m.) for 3 days beginning 1 day prior to the administration of dichlorvos (200 mg/kg body weight) to ensure adequate bioavailability.

Animals were examined for behavioural and biochemical alterations on days 1, 7, 15, and 21 post-pesticide exposure.

2.2. Chemicals

Adenosine triphosphate, sodium lauryl sulphate, EGTA, DEAE cellulose, Fura-2AM, and paraoxon were purchased from the Sigma Chemical Co. $^{45}Ca^{2+}$ was obtained from BRIT. Dichlorvos (2,2-dichlorovenyl dimethyl phosphate, 99% pure) was a gift from Hindustan Ciba Geigy Ltd. Phenyl valerate and mipafox were gifts from the Defense Research and Development Establishment (DRDE). Nimodipine was a gift from Torrent Pharmaceuticals. All other chemicals used were commercial products of the highest grade.

2.3. Analytical procedures

2.3.1. NTE assay

NTE was assayed by the method of Johnson [17]. An appropriate amount of brain homogenate was incubated for 20 min at 37° with 40 μM paraoxon and either (a) buffer or (b) mipafox (50 μM) in a final volume of 2 mL. The substrate phenyl valerate (2 mL) was then added to a final concentration of 1 mM to all samples except the blank, and the samples were incubated for another 15 min at 37° followed by the addition of SDS (1%, w/v), 4-aminoantipyrene (0.025%), and potassium ferricyanide (0.4%). The resultant red colour was read at 510 nm. NTE activity was calculated as the difference between (a) and (b) and is expressed in terms of micromoles of product formed per minute per milligram protein.

2.3.2. Motor function

Motor function was assessed by the rota-rod test as described by Dunham and Miya [18], which involves the assessment of the muscle strength and coordinated movements of the animals. Rats were placed on a metallic rod (5 cm in diameter) turning at the rate of 8 rpm. The rats were initially trained to maintain themselves on the rotating rod for more than 3 min (15 trials). Subsequently, i.e. after 24 hr, rats were checked for their ability to maintain themselves on the rotating rod for 3 successive trials of 180 sec each. In the event of the rats being unable to do so, the test was considered positive, i.e. motor incoordination was said to have been produced.

2.4. Preparation of synaptosomes

Synaptosomes were prepared from rat brain by the discontinuous sucrose gradient centrifugation method of Gray and Whittaker [19]. In brief, rat brain homogenate (10%, w/v) was prepared in 0.32 M sucrose in 20 mM Tris–HCl, pH 7.4, and centrifuged at 1000 *g* for 20 min at 4° to obtain a crude mitochondrial pellet. The pellet was resuspended in

0.32 M sucrose in 20 mM Tris–HCl, pH 7.4, and layered upon a gradient containing 0.8 M, 1.0 M, and 1.2 M sucrose. The gradient was centrifuged at 80,000 *g* for 2 hr at 4° to get the synaptosomal fraction at the interface over the 1.2 M sucrose layer.

2.5. Intrasynaptosomal calcium assay

Synaptosomal free calcium was determined by using a fluorescent calcium indicator dye, Fura-2AM, according to the method of Adamson *et al.* [20] Synaptosomal suspensions were loaded with 1 mM Fura-2AM at 37° for 1 hr. The excess of Fura-2AM was removed by centrifugation at 20,000 *g* for 20 min at 4°. A saturated solution of dantrolene (25 μM) was used to trap intraorganellar stored calcium. Fluorescence (F) values at 340/380 nm excitation and 510 nm emission were measured. Maximal fluorescence (F_{max}) and minimal fluorescence (F_{min}) were measured after the addition of 10 μL of 20% SDS and 5 mM EGTA, respectively. Intracellular free calcium was calculated by the formula: $[Ca^{2+}]_i = [(F\text{-}F_{min})/(F_{max}\text{-}F)] \times K_d$. The K_d value for Fura-2AM is 225 nM.

2.6. $^{45}Ca^{2+}$ uptake in synaptosomes

$^{45}Ca^{2+}$ uptake was measured in unstimulated (low K^+, 4.9 mM KCl) and stimulated (high K^+, 106 mM KCl) synaptosomes according to the method of Edelfors and Ravn-Jonsen [21]. Uptake was terminated with 3 mL of stop solution (1.8 mM $LaCl_3$ in 0.154 mM NaCl, 1 mM EGTA, 20 mM Tris–HCl, pH 7.4) followed by filtration of the samples through nitrocellulose filters (Whatman). Nonspecific binding was represented by the uptake at zero time. The net K^+ stimulated uptake was expressed as the difference between $^{45}Ca^{2+}$ uptake across the synaptosomal membrane under unstimulated and stimulated conditions.

2.7. Preparation of the synaptic plasma membranes from rat brain

For the calcium ATPase assay, synaptic plasma membranes were isolated from homogenized rat brain by the discontinuous gradient centrifugation method of Jones and Matus [22]. The crude mitochondrial pellet was lysed in hypotonic buffer followed by the addition of sucrose to a concentration of 34% (w/v). A discontinuous sucrose gradient was formed by layering 28.5% and then 10% sucrose over the sample. The density gradient was centrifuged at 60,000 *g* for 110 min at 4°. The middle gray band corresponded to the synaptic plasma membrane portion. The purity of the plasma membrane was checked by assaying for acetylcholinesterase activity in the different fractions by the method of Ellman *et al.* [23].

2.8. Calcium ATPase assay

Calcium ATPase was assayed in SPM according to the method described by Desaiah *et al.* [24]. The reaction mixture (1.5 mL) contained 40 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.05 mM $CaCl_2$, an appropriate amount of SPM, and 2.5 mM ATP. Mg^{2+}-ATPase activity was determined in the presence of 1 mM EGTA, and this was subtracted from the total Ca^{2+}/Mg^{2+}-ATPase activity, in order to obtain the net calcium ATPase activity. The reaction mixture was incubated for 15 min at 37°, and trichloroacetic acid (10% TCA) was added to stop the reaction followed by centrifugation at around 2057 *g* (using an REMI R-8C centrifuge) for 10 min at room temperature. Inorganic phosphorus released by the action of ATPase was estimated in the supernatant by the method of Fiske and Subbarow [25].

2.9. Calpain assay

Calpain was partially purified from rat brain homogenate [homogenizing buffer contained 50 mM Tris–HCl (pH 7.4), 10 mM β-mercaptoethanol, and 5 mM EDTA] by passing it through a DEAE cellulose column as described by El-Fawal *et al.* [16]. Calpain was assayed in the fractions collected after equilibrating the column with elution buffer containing 200 mM NaCl and elution with 300 mM NaCl. The calpain assay in the microplate system (with casein as a substrate) was performed by the method of Wang *et al.* [26].

3. Results

3.1. Brain NTE activity

Inhibition of brain NTE has been reported to be a sensitive marker of the potential of OP compounds in producing OPIDN. Therefore, serial estimations of NTE activity were carried out in brain homogenates following exposure to a single dose of dichlorvos. As evident from Table 1, a subcutaneous dose (200 mg/kg body weight) of dichlorvos inhibited brain NTE up to 65.2% compared with that of the control after 24 hr of pesticide exposure. A progressive recovery of NTE activity was observed throughout the study period, showing 49.8 and 26.4% inhibition on days 7 and 15, respectively. However, even after 21 days of exposure to dichlorvos, NTE activity was inhibited by 16.4%. Similarly, animals that received nimodipine along with dichlorvos also exhibited a similar extent of initial brain NTE inhibition (65% after 24 hr), and a similar rate of enzyme recovery (40, 11.9, and 17.9% inhibition on day 7, 15, and 21 post-exposure, respectively) as compared with the controls. No significant difference in the values of NTE activity was observed when compared with the group treated with only dichlorvos, which suggests that nimodipine may not prevent NTE inhibition, an initial event in the onset of delayed neurotoxicity.

Table 1
Brain NTE activity after administration of dichlorvos and nimodipine

Groups	NTE activity (μmol/min/mg protein)
Control	2.01 ± 0.05
Nimodipine	1.97 ± 0.12
Dichlorvos	
Day 2	0.70 ± 0.06*
Day 7	1.01 ± 0.14*
Day 15	1.48 ± 0.23*
Day 21	1.68 ± 0.07*
Nimodipine + dichlorvos	
Day 2	0.86 ± 0.12*
Day 7	1.20 ± 0.15*
Day 15	1.77 ± 0.17*
Day 21	1.65 ± 0.16*

Control rats were given a corn oil vehicle. Dichlorvos-treated rats received 200 mg dichlorvos/kg body weight, subcutaneously. The nimodipine-treated group received 6 mg nimodipine/kg body weight, intramuscularly for 3 days. The dichlorvos + nimodipine-treated group received nimodipine (6 mg/kg body weight, intramuscularly) and dichlorvos (200 mg/kg body weight, subcutaneously, administered the day after initiation of nimodipine treatment). Values are means ± SD (N = 6). Values listed on days 7, 15, and 21 were obtained from samples taken on respective days after dichlorvos treatment.

* Significantly different from the control group ($P < 0.05$, Dunnett's test for multiple comparison).

3.2. Rota-rod test

This test was performed in order to assess the development of any motor incoordination following dichlorvos exposure. As shown in Table 2, until day 15 of pesticide exposure, all the animals performed similarly on the rota-rod; however, severe motor deficit appeared on day 21 in the group of animals that received only dichlorvos, as evident by a 69.8% decrease in retention time, compared with the controls. The rats that received nimodipine and dichlorvos together did not show any sign of motor incoordination throughout the study period. Even on day 21 post-exposure, significantly higher values (2.96-fold) of retention time were observed in this group as compared with that of the dichlorvos-treated group, suggesting the possibility of a protective role for nimodipine against dichlorvos-induced motor incoordination.

Table 2
Rota-rod performance after administration of dichlorvos alone or along with nimodipine

	Retention time (sec)		
Treatment	Day 7	Day 15	Day 21
Control	168 ± 17.1	180 ± 00	179 ± 1.2
Dichlorvos	159.8 ± 13.9	164 ± 13.7	54 ± 24.8*
Nimodipine	175.8 ± 9.3	176 ± 7.3	180 ± 00
Nimodipine + dichlorvos	177.1 ± 6.3	180 ± 00	160 ± 19**

Control rats were given a corn oil vehicle. Dichlorvos-treated rats received 200 mg dichlorvos/kg body weight subcutaneously. The nimodipine-treated group received 6 mg nimodipine/kg body weight intramuscularly for 3 days. The dichlorvos + nimodipine-treated group received nimodipine (6 mg/kg body weight, intramuscularly) and dichlorvos (200 mg/kg body weight, subcutaneously, administered the day after initiation of nimodipine treatment). Values are means ± SD (N = 6). Values listed on days 7, 15, and 21 were obtained from samples taken on respective days after dichlorvos treatment.

* Significantly different from the control group ($P < 0.05$, Dunnett's test for multiple comparison).

** Significantly different from the dichlorvos-treated group ($P < 0.05$), ANOVA with the Newman-Keuls test for multiple comparison).

Table 3
Calpain activity, calcium ATPase activity, and intracellular calcium concentration in rat brain after administration of dichlorvos and nimodipine

Groups	Intracellular free calcium (nM)	Calpain activity (absorbance units/30 min/mg protein)	Calcium ATPase activity (nmol P_i liberated/min/mg protein)
Control	158.7 ± 33.25	16.40 ± 1.30	5.46 ± 0.47
Nimodipine	114.9 ± 32.89	14.05 ± 0.61	5.23 ± 0.97
Dichlorvos			
Day 7	332.7 ± 38.70*	44.80 ± 1.46*	3.00 ± 0.77*
Day 15	434.7 ± 35.14*	61.05 ± 6.32*	1.93 ± 0.69*
Day 21	395.5 ± 45.77*	42.60 ± 0.81*	1.41 ± 0.41*
Nimodipine + dichlorvos			
Day 7	257.5 ± 17.28*	18.26 ± 0.38**	3.75 ± 0.48*
Day 15	288.5 ± 47.77*,**	16.45 ± 2.00**	3.19 ± 0.13*,**
Day 21	232.0 ± 31.95*,**	23.25 ± 3.63**	4.03 ± 0.97*,**

Control rats were given a corn oil vehicle. Dichlorvos-treated rats received 200 mg dichlorvos/kg body weight subcutaneously. The nimodipine-treated group received 6 mg nimodipine/kg body weight intramuscularly for 3 days. The dichlorvos + nimodipine-treated group received nimodipine (6 mg/kg body weight, intramuscularly) and dichlorvos (200 mg/kg body weight, subcutaneously, administered the day after initiation of nimodipine treatment). Values are means ± SD (N = 6). Values listed on days 7, 15, and 21 were obtained from samples taken on respective days after dichlorvos treatment.

* Significantly different from the control group ($P < 0.05$, Dunnett's test for multiple comparison).

** Significantly different from the dichlorvos-treated group ($P < 0.05$), ANOVA with the Newman-Keuls test for multiple comparison).

3.3. $[Ca^{2+}]_i$

Free ionic calcium ($[Ca^{2+}]_i$), present in the cytosol, has been reported to be involved in the etiopathology of a variety of neurodegenerative diseases. In this context, synaptosomes were prepared from whole brain by density gradient ultracentrifugation, and free ionic synaptosomal calcium was estimated by using a fluorescent dye, Fura-2AM. A significant increase over control values in the $[Ca^{2+}]_i$ level was observed at all the post-exposure intervals (Table 3), following a single dose of dichlorvos. Initially, on day 7, a 2.1-fold increase was observed followed by a 2.5-fold increase on day 21. However, the maximal increase (2.74-fold) was observed on day 15 post-exposure. On the contrary, the group receiving nimodipine along with dichlorvos showed markedly lower $[Ca^{2+}]_i$ levels at all intervals com-

Table 4
Effect of dichlorvos with or without nimodipine on depolarization-induced calcium influx in synaptosomes

Groups	K^+-induced $^{45}Ca^{2+}$ influx (nmol $^{45}Ca^{2+}$ uptake/min/mg protein)
Control	32.92 ± 14.02
Nimodipine	28.64 ± 8.32
Dichlorvos	
Day 7	29.59 ± 12.13
Day 15	27.13 ± 5.69
Day 21	26.71 ± 15.83
Nimodipine + dichlorvos	
Day 7	29.24 ± 17.04
Day 15	26.27 ± 6.98
Day 21	25.40 ± 12.98

Control rats were given a corn oil vehicle. Dichlorvos-treated rats received 200 mg dichlorvos/kg body weight, subcutaneously. The nimodipine-treated group received 6 mg nimodipine/kg body weight, intramuscularly for 3 days. The dichlorvos + nimodipine-treated group received nimodipine (6 mg/kg body weight, intramuscularly) and dichlorvos (200 mg/kg body weight, subcutaneously, administered the day after initiation of nimodipine treatment). Values are means ± SD (N = 6). Values listed on days 7, 15, and 21 were obtained from samples taken on respective days after dichlorvos treatment. Non-significant changes were observed when compared by using the Dunnett or Neuman-Keuls test for multiple comparison.

pared with the group treated with dichlorvos alone. This suggests that nimodipine, at the dose used in this study, may accord protection against a dichlorvos-induced increase in $[Ca^{2+}]_i$.

3.4. Ca^{-2+} influxing and effluxing enzymes

In response to a variety of physiological stimuli or following toxic insult, cytosolic free calcium levels can increase either through enhanced entry of Ca2+ from the extracellular fluid into the cytoplasm or via release of Ca^{2+} from intracellular stores. An excess load of cytosolic calcium can be removed by calcium ATPase either by sequestering the excess Ca^{2+} into intracellular stores like mitochondria/endoplasmic reticulum or pumping it out of the cytosol to the extracellular medium. In view of an earlier report [27] indicating that delayed neurotoxicants may interfere with either one or both sites of intracellular calcium regulation, calcium ATPase activity (Table 3) and $^{45}Ca^{2+}$ uptake (Table 4) through VOCC were studied after a single exposure to dichlorvos. For this, synaptosomes were incubated with polarizing (low K^+ containing) and depolarizing (high K^+ containing) medium containing $^{45}Ca^{2+}$. As indicated by our data (Table 4), no significant change in $^{45}Ca^{2+}$ uptake through VOCC was observed at any of the post-exposure intervals. Similar results were observed in the group of animals that received both nimodipine and dichlorvos.

To assess the effect of dichlorvos on calcium ATPase, the major Ca^{2+} effluxing enzyme, SPM fractionated from whole brain were used. Table 3 shows a progressive and statistically significant decrease in the activity of calcium ATPase at all intervals. As is evident from the data, a 45% decrease in enzyme activity was observed on day 7; the activity was found to be further reduced to 65 and 74% on days 15 and 21 post-exposure, respectively. This could possibly be the reason for the increase in the $[Ca^{2+}]_i$ levels observed following exposure to dichlorvos. Not only was the decrease in enzyme activity in rats receiving nimodipine and dichlorvos simultaneously comparable to the values observed in the group treated with only dichlorvos on day 7, but the activity was also seen to approach control values on days 15 and 21, which further demonstrates the protective effect of nimodipine against dichlorvos-induced delayed neurotoxicity.

3.5. Calpain activity

Calpain, a cysteine protease whose activity depends upon the status of cytosolic calcium, reportedly is activated at higher levels of $[Ca^{2+}]_i$ [13]. Table 3 depicts a consistent and significant increase in calpain activity at all the intervals following dichlorvos exposure. A 2.8-fold increase in enzyme activity was observed on day 7, which increased further to 3.8-fold on day 15. Even on day 21, a 2.66-fold increase was still persistent in animals treated with dichlorvos. In the group given nimodipine and dichlorvos, very interesting results were obtained. Calpain activity in this group was found to be close to that of the control group up to day 15, with a nonsignificant increase in the activity of calpain on day 21. However, calpain activity in this group was significantly lower than the value in animals administered just dichlorvos. This suggests that nimodipine could attenuate the dichlorvos-induced increase in calpain activity.

4. Discussion

The initial event in the onset of OPIDN has been attributed to the inhibition of NTE. However, there is a general lack of information regarding the ability of dichlorvos to cause delayed neurotoxicity. Our studies have shown that a single subcutaneous dose (200 mg/kg body weight) of dichlorvos leads to a 65.2% inhibition of brain NTE after 24 hr of exposure. An early reduction in NTE activity to 20–30% of control has been considered predictive of OPIDN [28,29]. Inhibition of NTE is necessary but not sufficient to induce OPIDN in sensitive species. NTE inhibition must be followed by a second important step, i.e. aging of the organophosphorylated enzyme [30,31], for the induction of OPIDN. Numerous studies have reported a good correlation between inhibition and aging of NTE in the presence of OP compounds and the ability of these compounds to produce OPIDN. We have already reported [10] that dichlorovos given as a single dose of 200 mg/kg body weight can cause

OPIDN. We also reported the formation of an aged complex of NTE with dichlorvos following *in vitro* inhibition of NTE, which failed to be reactivated by potassium fluoride. Thus, this observation clearly fulfills the criteria for the initiation of OPIDN by dichlorvos. NTE activity significantly recovers prior to the clinical appearance of ataxia after the administration of neuropathic OP [1,16,32]. The present study also showed NTE recovery to 50% of control 7 days after dichlorvos exposure, and on day 21 it reached up to 84% before the appearance of clinical signs.

The OPIDN potential of dichlorvos was further substantiated by the performance of animals on the rota-rod apparatus. Apart from the hyperexcitability of animals to tactile stimulus, no other clinical symptoms of OPIDN were overtly visible. Nevertheless, a significant amount of motor dysfunction was evident in dichlorvos- treated animals. Further, the appearance of a motor deficit only on day 21 post-exposure could be due to a high degree of peripheral nerve regeneration or excessive axonal sprouting, which may prolong muscle strength and sensory motor function and thus account for the preservation of hind limb function in the rat, until late in the disease [33].

Although, the hen has generally been considered the most appropriate animal model for studies of OPIDN [1], rats are also routinely used for general toxicity testing. Various studies have indicated that rats are susceptible to both NTE inhibition and delayed neuropathy after administration of certain OP compounds [34,35]. A report by Ehrich *et al.* [36] further confirms that rats do develop OPIDN. The absence of the onset of paralysis in the rat that is in contrast to the appearance of visible locomotor ataxia in the hen and humans following dichlorvos exposure could be due to either a difference in the distribution and pharmacokinetics of dichlorvos in the rat or to a more rapid synthesis of NTE in the rat brain [37,38] rather than to differential sensitivity of rat brain NTE to dealkylation. Significant inhibition of NTE at various post-exposure intervals in brains of animals and the appearance of clinical signs (hyperexcitability, weakness in hind limb, and motor incoordination) all confirm the delayed neurotoxic potential of dichlorvos.

Events that may occur between NTE inhibition and the appearance of clinical deficits are not completely understood. Moretto *et al.* [39] correlated NTE inhibition and aging with a 70% reduction in retrograde transport. As reported by Ochs and Iqbal [40], axonal transport can be blocked in the presence of excess calcium. Abou-Donia [11] proposed that increased intracellular calcium and the resultant altered calcium homeostasis could be an intervening step in the development of OPIDN. In this study, we observed a consistent increase in $[Ca^{2+}]_i$ compared with the control, with the maximum increase being observed on day 15. A similar increase in the $[Ca^{2+}]_i$ concentration in axoplasm as well as in mitochondria after TOCP exposure was reported earlier, and it was proposed that the increase in the intra-axonal calcium concentration might be involved in the pathogenesis of TOCP-induced OPIDN [41]. Furthermore, PSP, another neurotoxic OP compound whose delayed neurotoxic activity has been well characterized, has also been shown to increase total calcium in the sciatic nerve of the hen [16].

It was speculated that the increase in $[Ca^{2+}]_i$ following dichlorvos exposure could be due to an increase in Ca^{2+} influx through the calcium channels or a dichlorvos-induced release of Ca^{2+} from intracellular stores. Although we did not observe any alteration in $^{45}Ca^{2+}$ uptake through the VOCC, a massive inhibition of calcium ATPase was observed throughout the study period of 21 days following dichlorvos exposure, which suggests that dichlorvos may not be acting at the level of VOCC, but may be mobilizing an increased amount of calcium from intracellular stores such as mitochondria and endoplasmic reticulum, resulting in its accumulation in the cytosol in the presence of inhibited calcium ATPase. Similar results were reported with diisopropyl phosphorofluoridate, a delayed neurotoxic OP, which caused a persistent decrease in Ca^{2+}-ATPase activity in the hen until the complete development of neurotoxic symptoms [27].

The breakdown of the axonal cytoskeletal elements has been associated with an increase in the level of $[Ca^{2+}]_i$ in various neurodegenerative states [42]. Calcium appears to contribute to such degenerative changes by facilitating the activities of Ca^{2+}-associated proteases and kinases [12,43]. Changes in these enzymes have been noted following administration of OP compounds known to induce delayed neurotoxicity [44,45]. In this context, the increase in calcium-activated neutral protease, calpain, activity leading to Wallerian degeneration has been demonstrated to be intimately associated with an increase in intracellular free calcium [46]. In our study, we have shown that a single exposure to dichlorvos led to a progressive increase in calpain activity with a corresponding increase in $[Ca^{2+}]_i$. Although the maximum increase in calpain activity following dichlorvos exposure was observed on day 15, its activity remained elevated as clinical deficits progressed. This is in line with an earlier report by El-Fawal *et al.* [16], who demonstrated a similar increase in calpain activity after PSP and TOCP exposure. Hence, it can be proposed that enhanced calpain activity could be an important intervening step between NTE inhibition and the onset of clinical signs of dichlorvos-induced delayed neurotoxicity.

Although the hypercholinergic effects of OP poisoning can be arrested by the use of antidotes such as atropine and PAM, these very patients and animals have been reported to develop delayed neurotoxicity after 2–3 weeks of exposure. To date, no therapeutic regimen is available to counter this syndrome, due to the lack of complete understanding of the mechanism behind OPIDN. Our result suggests that an increase in $[Ca^{2+}]_i$ following NTE inhibition could be a key step in the onset of OPIDN. In this context, El-Fawal *et al.* [15,16] have shown that calcium channel blockers like verapamil and nifedipine can ameliorate some of the functional and morphological deficits associated with PSP- and

TOCP-induced delayed neurotoxicity in hens. Nimodipine, another calcium channel blocker, was preferred in our study (at a dose of 6 mg/kg body weight), since it is a centrally acting drug and is able to cross the blood–brain barrier more effectively [47]. Although nimodipine could not prevent NTE inhibition following exposure of these animals to dichlorvos, it could accord protection against the development of motor incoordination. On the other hand, dichlorvos produced severe motor incoordination as evident from the performance of the rats on the rota-rod apparatus. These animals were lethargic and could not even stand properly on the rod by day 21 post-exposure. However, when nimodipine was administered along with dichlorvos, the rats did not show any sign of dichlorvos intoxication and even on day 21, the day on which delayed neurotoxic signs of dichlorvos manifest, these animals did not show any motor deficit. Animals from this group remained active throughout the study period. This finding is consistent with an earlier report [16] and suggests that NTE inhibition precedes the increase in $[Ca^{2+}]_i$. This further emphasizes the involvement of altered calcium homeostasis in the development of dichlorvos-induced delayed neurotoxicity. Since no alteration in the Ca^{2+} influx through VOCC following dichlorvos treatment was observed in our study, nimodipine could be expected to produce its protective effects by inhibiting intracellular mobilization of Ca^{2+}, which, in turn, could be responsible for the amelioration of delayed neurotoxicity.

In this study, we have demonstrated that a single dose of dichlorvos (200 mg/kg body weight) can produce NTE inhibition followed by the subsequent development of motor incoordination in rats after 3 weeks of exposure, which is a classical feature of OPIDN. Biochemical changes between the NTE inhibition and the onset of clinical symptoms of OPIDN could be an increase in $[Ca^{2+}]_i$ followed by a subsequent increase in calpain activity. Although the use of nimodipine could prevent the deleterious biochemical and behavioural changes induced by dichlorvos, it was unable to prevent the initial event of NTE inhibition in dichlorvos-treated rats. The protective efficacy of nimodipine for OPIDN is thus clearly evident by virtue of its ability to reduce cytosolic free Ca^{2+}, thereby preventing the dichlorvos-induced increase in calpain activity which may be one of the reasons for axonal degeneration.

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