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Effect of neferine on toxicodynamics of dichlorvos for inhibiting rabbit cholinesterase[1]

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ABSTRACT

AIM: To study the effect of neferine (Nef) on toxicodynamics of dichlorvos (DDVP) for inhibiting the rabbit cholinesterase (ChE) and search the relativity between *in vivo* and *in vitro* reactivating effect of Nef. **METHODS:** Calorimetric method was used to determine the blood concentraiton of DDVP, and 3P97 software was used to calculate the parameters of toxicokinetics. Ellman's method was used to determine the ChE activity in plasma. The toxicodynamics curve *in vivo* and the concentration-effect curve *in vitro* were drawn to compare the effects of Nef on these two curves. **RESULTS:** In the rabbit poisoned by ig DDVP 12.5 mg/kg there was almost no effect of Nef on the toxicokinetics of DDVP. The slope of toxicodynamics curves for depressed ChE increased about 3 times after treatment with Nef 7.5 mg/kg and pyratoxime methylchloride (2-PAM· Cl) 50 mg/kg, compared with the untreated group, there was a higher significant difference ($P<0.01$). *In vitro* Nef shifted the cumulative concentration-effect curve of DDVP on ChE to the right and decreased the maximal inhibitory effect obviously. The shift tendency of concentration-effect curve for DDVP *in vitro* was similar to that of the curve *in vivo*, difference between their value *b* was not significant ($P>0.05$). **CONCLUSION:** Nef reactivates the ChE depressed by DDVP. The experiment *in vitro* may be used as a preliminary index to observe the reactivation of ChE inhibited by anticholinesterases.

INTRODUCTION

Dichlorvos (DDVP) is one of the organophosphorus ester pesticides. It is widely used to kill the infurcous insects in farming, forestry, and environment. The poisoning accident is common occurrence, so studying its toxicology and antidotes thoroughly has important significance. There were a few reports dealing with investigation of the metabolic procedure on organophosphorus compounds *in vivo* and with the toxicodynamics for inhibiting the cholinesterase[1-4]. However, few reports about the effect of reactivator on the toxicokinetics of DDVP and the toxicodynamics of organophosphorus esters inhibiting ChE were found in the recent papers. neferine (Nef) is a dibenzylisoquinoline alkaloid extracted from the green plumule in ripe seed of lotus (Nelumbo nucifera gaerth). Its distinct cardiovascular effect was interesting for the people of medical science[5-6]. It was found in our laboratory that Nef could protect the mice poisoned by organophosphate and had the definite reactivative effect on the poisoned enzyine[7]. In the present study, DDVP was used as a toxicant to deter-

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mine the parameters of its toxicokinetics and toxicodynamics in the rabbit, and the effects of Nef and 2-PAM· Cl on the toxicokinetics and toxicodynamics of DDVP for inhibiting ChE were observed. Furthermore, the relativity between *in vivo* and *in vitro* reactivating effect of Nef on the ChE depressed by DDVP was searched.

MATERIALS AND METHODS

Experimental animal New Zealand rabbit of both sex weighing 2.4 kg±0.3 kg were supplied by Experimental Animal Center, Jiangxi Medical College (Certificate No 021-9702).

Drugs DDVP (80 % emulsion, Shandong Agriculture Pharmaceutical Factory), Nef was obtained from Department of Pharmacology, Tongji Medical University. Pyratoxime methylchloride (2-PAM·Cl, injection of 250 g/L), was purchased from Shanghai 13th Pharmaceutical Factory. Dithiobisnitro benzoic acid (DTNB), and acetylthiocholine iodide (ATCh) were purchased from Sigma Chemical Co.

Preparation of solutions The stock of DDVP was prepared from mixing its 80 % emulsion (1.5 mL) with Tween-80 (2.0 mL) and diluted with distilled water into 10 % solution before use. Nef was dissolved with hydrochloride acid (0.03 mmol/L) to make up 3 % solution. The DDVP stock and the 2-PAM· Cl injection were diluted with normal saline to make up the required concentration of each solution corresponding to the volume of 5 mL/kg body weight. DTNB solution (10 mmol/L) of 1.0 mL mixing with 30 mL of PBS (100 mmol/L) was taken as the solution R_1. ATCh (50 mmol/L) of 1.0 mL diluting with distilled water into 10 mL was taken as solution R_2.

Experiment *in vitro* Rabbit blood samples were added with DDVP in a serial concentration of 0.03- 10 μg/L. Ten minutes later the ChE activity of each sample was determined. Then Nef (1.2 and 4.8 mg/L) and 2-PAM· Cl (5 and 20 mg/L) were added into each sample, respectively. Thirty minutes later the activity of ChE in the samples was determined again. The concentration-effect curves corresponding to these two drugs were recorded. The blood sample added with hydrochloric acid (0.03 mmol/L) was used as a control to rule out the possible interference from solvent of Nef.

Echelon design and dosage regimen DDVP administered orally was the most common route for DDVP poisoning sufferer. So administration of DDVP through gastroenteric tract (ig) was more reasonable to observe the effect of Nef and 2-PAM· Cl on toxicodynamics of DDVP. According to the report, T_{max} of DDVP concentration in blood was 7- 10 min[8], *in vivo* the antidote Nef or 2-PAM· Cl was given at 12 min after ig DDVP.

Eighteen rabbits were randomly divided into 3 groups. Six rabbits of ig DDVP (12.5 mg/kg) were the poisoning group. Another 6 rabbits were Nef-treated group, Nef (7.5 mg/kg) was given at 12 min after ig DDVP. Other 6 rabbits were 2-PAM· Cl-treated group, 2-PAM· Cl (50 mg/kg) was given (iv) at 12 min after ig DDVP.

Collection of rabbit blood sample Rabbit anesthetized with sodium iso-pentobarbital was fixed in a supine posture. Heparin (10 mg/kg) was given (iv) for anticoagulation. One carotid artery was ligated and the blood sample was collected from a polyethylene tube with a three-way valve insetting in the proximal end of the artery. Before ig DDVP, 1-2 mL of the blood sample was collected and taken for the blank to determine the activity of ChE as a reference standard. After 3, 5, 7, 9, 15, 30, 60, 120, 240, and 360 min of DDVP ig, 1.2 mL blood was collected at each time. The volume of bleeding for a rabbit did not exceed 15 mL in all. It approximately accounted for 11 percent of the blood volume in a rabbit.

Determination of the ChE activity and DDVP concentration in the blood Ellman's DTNB colorimetric method was used to determine the activity of ChE[9]. The parameters of biochemical analyzer used in the present study were as follows: wave length, 410 nm; volume of sample, 20 μL; and volume of R_1 and R_2, 500 and 50 μL, respectively. Blood concentration of DDVP was determined simultaneously with the method[10].

Data analysis The saturation binding parameters were determined by the linear regression analysis of Hill's method. All values were expressed as mean±SD and statistical evaluation of the data was examined by *t* test. $P<0.05$ was considered statistically significant.

RESULTS

Concentration-effect relationship of Nef and 2-PAM· Cl in reactivating the ChE inhibited by DDVP *in vitro* Activity of ChE in the rabbit blood was inhibited obviously by DDVP 0.03- 10 μg/L. Its inhibitory effect increased with the doses. The maximal inhibitory effect was more than 90 percent. After treatment with Nef of 1.2 and 4.8 mg/L, the cumulative concentration-effect curve was shifted to the right and

the maximal inhibitory effect was decreased remarkably (Fig 1A). 2-PAM·Cl also shifted the concentration-effect curve to the right and decreased the maximal inhibitory effect (Fig 1B). Nef at a 1:3 dose of 2-PAM·Cl appeared to have the same efficacy level as the latter. The linear regression in logarithmic concentration and logit percentage of ChE inhibited by DDVP after antagonization with Nef 4.8 mg/L were r=0.984, P<0.01, b=1.161±0.231, a=0.665±0.118. There was no notable effect of the solvent hydrochloric acid (0.03 mmol/L) on the activity of ChE inhibited by DDVP.

Toxicokinetics of DDVP and toxicodynamics of ChE inhibited by DDVP The curve of time-concentration from 6 rabbits treated with single ig DDVP (12.5 mg/kg) was shown as the lower curve in Fig 2. It appeared as a 2-compartment open model with first order absorption and its parameters of toxicokinetics could be calculated with the software 3P97 as shown in Tab 1. While the toxicodynamics of ig DDVP which was expressed with the curve of time-mean inhibitory percentage of ChE from 6 rabbits, was shown as the upper curve in Fig 2. Toxicokinetics of DDVP and toxicodynamics of DDVP for inhibiting ChE were similar to each other and their trends of shift were synchronous in the main. They were all the curves of trinomial exponential functions. However, the slope of toxicodynamics was evidently less than that of toxicokinetics.

Effect of Nef or 2-PAM· Cl on toxicodynamics and toxicokinetics of DDVP Toxicokinetics of DDVP

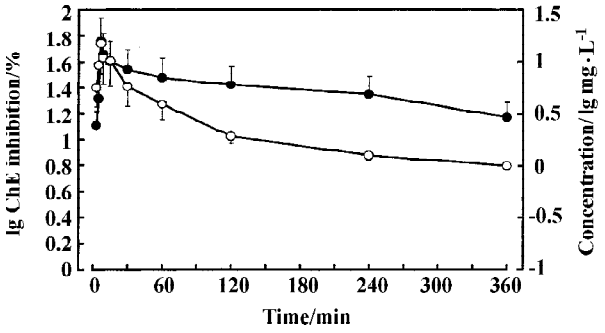

Fig 2. Toxicokinetics and toxicodynamics of DDVP for inhibiting rabbit ChE. (○) DDVP of different concentrations; (●) Inhibition of ChE after DDVP (12.5 mg/kg , ig). n=6. Mean±SD.

Tab 1. Toxicokinetic parameters of DDVP (12.5 mg/kg, ig) in combination with or without Nef (7.5 mg/kg, iv) or 2-PAM· Cl (50 mg/kg, iv). n=6. Mean±SD.

Parameter	DDVP	DDVP+Nef	DDVP+ 2-PAM· Cl
K_{12}/min^{-1}	0.11±0. 09	0.30±0.25	0.2±0.3
K_{21}/min^{-1}	0.08±0.07	0.07±0.04	0.06±0.05
K_{10}/min^{-1}	0.022±0.020	0.013±0.020	0.018±0.010
$t_{1/2\alpha}$/min	8±7	8±8	8±6
$t_{1/2\beta}$/min	121±69	156±7.9	134±65
$t_{1/2k\alpha}$/min	2.3±1.5	1.9±0.6	2.1±0.9
AUC/min· mg· L^{-1}	0.30±0.07	0.31±0.06	0.29±0.07
CL/L· kg^{-1}· min^{-1}	0.047±0.010	0.045±0.020	0.048±0.010
T_{max}/min	9±3	9±4	8±4
C_{max}/mg· L^{-1}	3.2±1.3	2.9±2.1	3.4±2.3

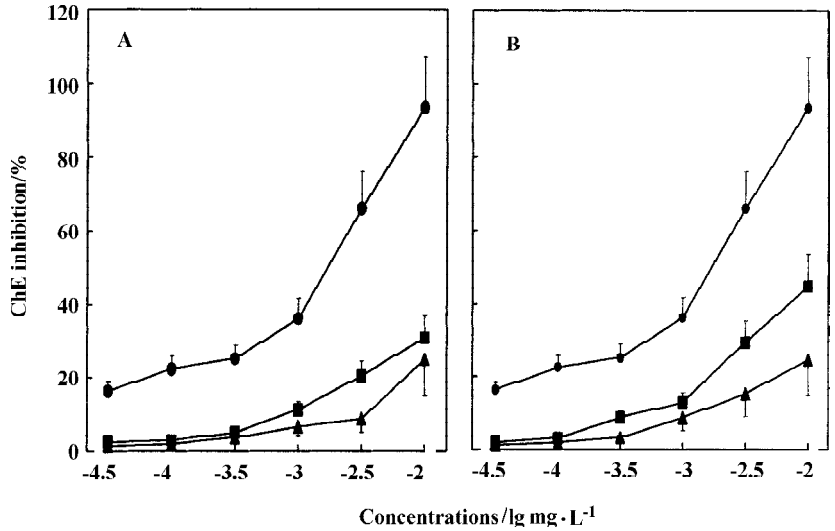

Fig 1. Concentration-effect relationship of DDVP, Nef+DDVP, and 2-PAMxCl+DDVP *in vitro*. A: (●) DDVP (0.03-10 mg/L); (■) DDVP+Nef (1.2 mg/L); (▲) DDVP+Nef (4.8 mg/L). B: (●) DDVP (0.03-10 mg/L^{-1}); (■) DDVP + 2-PAMxCl (5 mg/L); (▲) DDVP + 2-PAMxCl (20 mg/L). n=4. Mean±SD.

(ig) after treatment or untreatment with antidotes were both 2-compartment open models. As shown in Tab 1 the parameters of toxicodynamics for DDVP and DDVP+Nef or DDVP+2-PAM· Cl were essentially consistent. There was almost no effect of Nef and 2-PAM· Cl on the toxicokinetics of DDVP. The curve diagram drawn from the values of toxicodynamics for poisoned groups treated or untreated with antidotes (Fig 3), showed that the slope of toxicodynamics curve for ChE inhibited by DDVP obviously increased after treatment with Nef and 2-PAM· Cl. Since the first 4 dots of the curve were the values before treating, so only 6 dots on the right of the curve were used to calculate the logarithmic concentration of DDVP and the percentage for inhibition of ChE by DDVP. The values obtained from linear regression after logit transformation was shown in Tab 2. For the same reasons, in simply poisoning with DDVP group, the linear regression was also calculated from the latter 6 dots of the curve since the full absorption of ig toxicant would reach.

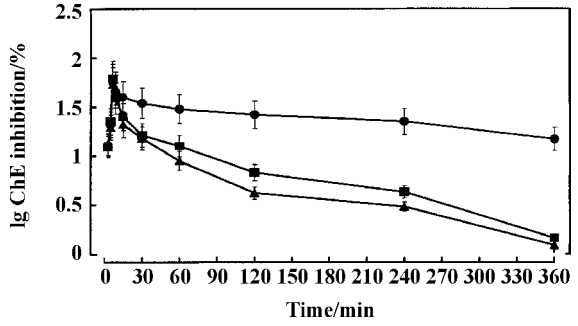

Fig 3. Toxicokinetics and toxicodynamics of DDVP for inhibiting rabbit ChE and effect of Nef or 2-PAM· Cl. (○) DDVP of different concentrations; (●) Inhibition of ChE after DDVP (12.5 mg/kg, ig); (■) 2-PAM· Cl (50 mg/kg, iv) at 12th min after ig DDVP (12.5 mg/kg); (▲) Nef iv (7.5 mg/kg) at the 12th min after ig DDVP (12.5 mg/kg). *n*=6. Mean±SD.

Relationship between toxicodynamics of DDVP *in vitro* and *in vivo* The concentration-effect relationship of DDVP *in vitro* and the dose-effect relationship of latter 6 dots on toxicodynamics curve were compared with coordinate chart. The chart was drawn from the logarithmic percentage or logit transformation for inhibition of ChE as ordinate and the logarithmic concentration of DDVP as abscissa. It showed that there was a significant correlation ($P<0.01$). The features of linearization with these two methods were almost coincident completely (Fig 4). The difference between *b* values of linear regression *in vitro* and *in vivo* had no significance ($P>0.05$). It suggested that the concentration-effect relationship *in vitro* and *in vivo* was coincident.

Tab 2. Effect of Nef and 2-PAM· Cl on the toxicodynamics of DDVP (*in vivo*: 12.5 mg/kg; *in vitro*: 0.03-10 mg/L) or inhibiting ChE. The values *b* and *a* were calculated from linear regression by Hill's method. *n*=5. Mean±SD. [c]*P*<0.01 *vs* DDVP (*in vivo*). [f]*P*<0.01 *vs* DDVP (*in vitro*).

Group	*b*	*a*
In vivo:	0.38±0.13	
DDVP (12.5 mg/kg)		1.54±0.13
Nef (7.5 mg/kg)+DDVP	1.30±0.18[c]	1.20±0.11[c]
2-PAM· Cl (50 mg/kg)+ DDVP	1.25±0.21[c]	1.16±0.15[c]
In vitro:		
DDVP (0.03-10 μg/L)	0.42±0.20	0 9±0.4
Nef (4.8 mg/L)+DDVP	1.16±0.23[f]	0.66±0.12[f]
2-PAM· Cl (20 mg/L)+DDVP	1.3±0.3[f]	0.86±0.25[f]

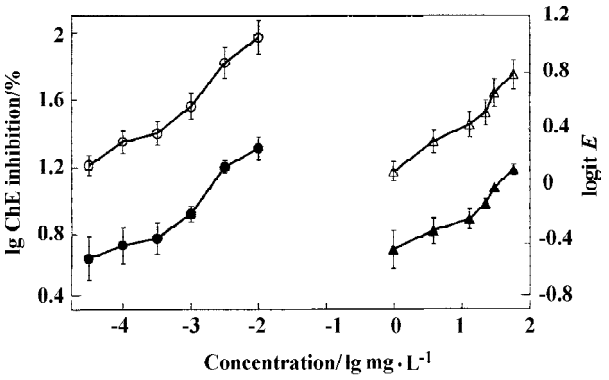

Fig 4. Concentration-effect relationship of lg percentage for ChE inhibited by DDVP *in vitro* (○) and *in vivo* (△), and logit transformation for ChE inhibited by DDVP, logit E=lg[E/(E_{max}-E)] *in vitro* (●) and *in vivo* (▲). *n*=4. Mean±SD.

DISCUSSION

Nef is a Ca^{2+} antagonist of wide actions[11]. It was demonstrated in the observation about effect of Nef on toxicodynamics of DDVP for inhibiting rabbit ChE that Nef could significantly decrease the inhibitory effect of DDVP on the activity of ChE, but there was almost no effect of Nef on the toxicokinetics of DDVP. The slope of toxicodynamics curve increased remarkably. The *b* value in the group treated with Nef was 3 times more

than that in the untreated group. There was a significant difference between them ($P<0.01$). It was evident that Nef had a definite reactivative effect on the inhibited ChE. This was coincident with the result we obtained before[5]. It was revealed in the present experiment that 2-PAM· Cl had the similar effect to Nef but its dosage was 3 times more than that of Nef. It appeared that Nef was more effective for reactivating the depressed ChE than the traditional reactivator 2-PAM· Cl. 2-PAM· Cl reactivated ChE through dissociating phosphoryl from the poisoned enzyme by its active oxime. Whether this stronger reactivative effect of Nef on ChE dealing with the nueleophilic effect of multi-nucleophilic mucor blastophore in Nef structure interacting on phosphorylase, is still obscure.

Generally, the phosphorylase could be reactivated by the reactivator of ChE before fully aging. Essence of oximic reactivator to reactivate the phosphorylase is dissociation of phosphoryl from the poisoned enzyme to form the phosphoryloxime, then to release the active enzyme. Phosphoryl can not be dissociated again, if the phosphorylase is dealkyl[12]. In this case the poisoned or "aged" enzyme, would not be reactivated with reactivator. Nef did not shift the toxicodynamics curve downward parallelly. It suggests that Nef has not reactivated the activity of ChE completely and that there may be part of the enzyme aged. Therefore, it is necessary to use the reactivator early for treating organophosphate poisoning. At present, oximic compounds are still the reactivators most in use for clinicians at home and abroad. However, at high dosage they can block the transmission of neuromuscular junction, even paralyse the respiratory muscles till death. These toxic reactions of oximic reactivators restrict them to use in sufficient dosage. Present study shows that the reactivative effect of Nef on DDVP poisoned rabbit ChE can last long since the increased *b* value does not decrease again in the course of observation. While there is no inhibition of respiration during treatment with Nef. These results found the theoretical and experimental basis for using Nef as a reactivator in sufficient dosage early to treat organophosphate poisoning.

In vivo, ChE is poisoned by DDVP through its phosphorylation in high affinity. It results in defunction of ChE for hydrolysing ACh and cumulation of ACh, then the cholinergic crisis is evoked and endangers the life of the patient. 2-PAM· Cl reactivates the poisoned enzyme and antagonizes the poisoning etiologically. Therefore, to determine the activity of ChE can learn the therapeutic effect direct[13]. *In vitro* Nef is of powerful reactivative effect on ChE inhibited by DDVP (Fig 1). Its linear relationship in logarithmic concentration and logit percentage of ChE inhibited by DDVP after antagonization with Nef is coincident with the tendency of the 6 dotes after antagonization with Nef *in vivo* as shown in Fig 3. Difference between their value *b* is not significant ($P>0.05$). The data demonstrated that the reactivative concentration-effect relationship of reactivator *in vivo* could be shown in the experiment *in vitro*. Therefore, using the method *in vitro* to study the pharmacological effect of new reactivators on ChE is of practical significance.

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