Regeneration of Red Cell Cholinesterase Activity Following Pralidoxime (2-PAM) Infusion in First 24 h in Organophosphate Poisoned Patients

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Abstract Oximes such as pralidoxime chloride reactivate acetylcholinesterase. However their role in management of organophosphate poisoning is controversial. The study was carried out to find effectiveness of pralidoxime chloride (2-PAM) in regenerating red cell acetyl cholinesterase in first 24 h following administration of it in dose recommended by WHO. Eight patients with OPP [chlorpyrifos (3), phorate (3), dichlorvos (1) and monocrotophos (1) who fulfilled the criteria for inclusion were investigated. In addition to decontamination and atropine, all these patients were administered 30 mg/kg body wt of 2-PAM as bolus dose followed by 7.5 mg/kg body wt/h with maximum dose being 500 mg/h as continuous infusion till first 24 h. Red cell AChE activity was estimated every 15 min for first 4 h, one hourly for next 4 h and then 2 hourly till 24 h and subsequently without 2-PAM every 12 h till 7 days or discharge or death which ever earlier. In all the patients maximum increase in activity was observed in first 4 h following which rise was very slow despite continued 2-PAM infusion and reaching a steady state in 20 h in all the cases. The increase in red cell AChE activity observed in diethyl group at 24 h of 2-PAM infusion was 154% vs. 81% in dimethyl group. At 7 days the increase in activity

was 215% vs. 118% respectively. However on multiple repeated ANOVA, no statistically significant difference was observed between diethyl and dimethyl groups at admission and discharge ($P > 0.05$). Similarly no significant difference was observed in three groups when patients were categorized according to WHO classification of organophosphates ($P > 0.05$). The maximum increase in red cell AChE activity occurs in first 4 h of 2-PAM administration followed by a slow increase despite 2-PAM infusion till 24 h.

Keywords Organophosphates · Red cell acetylcholinesterase · Pralidoxime chloride

Introduction

The acetylcholine esterase (AChE, EC 3.1.1.7) is a hydrolytic enzyme belonging to the serine hydrolase family which plays an important role during the course of signal transmission at cholinergic synapses. Its principal biological role is the termination of impulse transmissions by rapidly hydrolyzing the neurotransmitter, acetylcholine (ACh) [1]. The organophosphates (OP's) are either esters, amides, or thiol derivatives of phosphoric acid. The structural diversity in their side chain determines their toxicokinetic and toxicodynamic properties. As they are highly lipophilic, they get rapidly absorbed and distributed to lipophilic tissues where they accumulate with subsequent slow elimination.

The basic mechanism of action of OP's is known: They lead to irreversible AChE inhibition by phosphorylating serine 203 at the active center of the enzyme at cholinergic synapses leading to accumulation of ACh at synaptic junctions leading to initial overstimulation of cholinergic

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pathways with subsequent desensitization of cholinergic receptor sites [2, 3]. The evidence supporting AChE as the primary site of OP agents action is based on a number of observations such as correlation between AChE inhibition in vivo and vitro, good therapeutic efficacy of anticholinergics and cholinesterase reactivation and on to possibility to prevent intoxication i.e. cholinesterase inhibition) using reversible cholinesterase inhibitors e.g. carbamates and others [3]. The restoration of AChE activity is possible either by spontaneous de-phosphorylation of the inhibited enzyme or by synthesis of fresh enzyme. However as ageing occurs with time, the lost activity can only be restored by synthesis of new enzyme which occurs in bone marrow [4].

The treatment involves decontamination to reduce further absorption and using specific antidotes i.e. atropine and oximes. The oxime available in India is pralidoxime chloride (2-PAM) [5]. It exerts a nucleophilic attack on the phosphate moiety of the inhibited enzyme, removing phosphate and thereby restoring enzyme activity [4]. Several in vitro studies in animals and human erythrocytes suggest beneficial effect of oximes [6, 7]. However their efficacy is affected by ageing of AChE as well as by high concentration of OP's. Ageing takes longer with diethyl than dimethyl organophosphates. It has been suggested that treatment will be beneficial if oximes are administered within 12 h in case of dimethyl OP's and within 120 h in case of diethyl OP's and oxime administration is continued till pesticide concentration drops to the point at which reactivation surpasses inhibition [7]. According to WHO, 2-PAM should be administered as 30 mg/kg body wt as bolus followed by 7.5 mg/kg body wt/h as continuous infusion till recovery [7]. However, oxime efficacy in humans has been questioned in several clinical trials [8, 9]. In our present study we studied the regeneration of red cell AChE after administration of 2-PAM as 7.5 mg/kg body wt/h as infusion after the bolus dose for first 24 h and subsequently without it till 7 days or death or discharge whichever was earlier in eight patients with organophosphate poisoning. It would have been ideal to continue the infusion till recovery but due to financial constraints we could infuse for first 24 h only.

Patients and Methods

Patients above 18 years of age with organophosphate poisoning admitted to emergency of Nehru Hospital attached to Post Graduate Institute of Medical Education and Research, Chandigarh between January to October, 2007 and who fulfilled the inclusion criteria were included in the study. The inclusion criteria were history of ingestion or exposure to known organophosphate, presence of signs and

symptoms suggesting moderate to severe toxicity, plasma cholinesterase (PChE) activity depressed by $\geq 50\%$, reached hospital within 24 h of ingestion and had not received 2-PAM outside. Those patients who did not reach hospital within 24 h of ingestion or exposure, where nature of compound was not known, or who had any systemic illness like malignancy, chronic lung disease, renal or hepatic failure and pregnancy were excluded. The clinical features at admission, nature of compound, mode of exposure, time interval between ingestion and arrival, PChE activity, red cell AChE activity and pralidoxime dose was recorded on a predesigned proforma daily.

A central venous line was inserted at admission and 5 ml of venous blood was withdrawn of which 2 ml was collected in a sterile heparinized vial and was used for red cell AChE and PChE activity estimation where as the rest was collected in plain vial and was used for routine biochemistry. Gastric lavage, urine and blood samples were also collected for confirmation of organophosphate compound and were stored at -70°C .

Following pralidoxime infusion, one ml blood in heparinized vial was collected every 15 min for first 4 h, one hourly for next 4 h and then two hourly till first 24 h. Subsequently it was collected every 12 h till 7 days or recovery or death whichever was earlier.

Red cell AChE activity was determined by modified Ellman method [10, 11]. This is based on principle that when the rate of hydrolysis of acetylthiocholine by a red cell suspension at pH 8 is measured by the reaction between thiocholine and 5-5'-dithiobis-2-nitrobenzoic acid (DTNB), a yellow color compound (5-thio-2-nitrobenzoate anion) with molar extinction coefficient of 13.6/mol/cm is generated. The red cells were prepared by 1:100 dilution of whole blood sample with 0.1 M sodium phosphate buffer, pH 7.4 containing 0.03% triton X-100 (diluting reagent facilitating complete hemolysis of whole blood sample) was undertaken immediately after withdrawal to minimize the reaction between red cell AChE inhibitor and reactivator oxime [11]. The samples were stored in a freezer at -20°C till analysis usually in next 24 h. To 1.95 μl phosphate buffer (0.1 M, pH 8.0) and 1 ml lysed RBC's, 50 μl of DTNB was added and incubated for 5 min. The rate of appearance of thiocholine was observed at 436 nm against a reference containing buffer only. The activity was calculated after subtracting the activity using eserine sulfate as total cholinesterase inhibitor. Hemoglobin (Hb) was estimated by method of Subramanian et al. [12]. The activity was expressed as nmol/min/mg Hb.

Red cell AChE activity was measured in fifty healthy controls (men = 27; women = 23) with age between 18 and 75 year (mean $\pm$ SD 38.9 ± 12.55 in men and 37 ± 12.41 in women). They were adults attending medical outpatient for health check up and were found to be healthy. The blood

Table 1 Age, sex, nature of compound exposed to, total dose of 2-PAM in first 24 h, PChE activity at admission and red cell AChE activity at admission, 24 h, at discharge and time of peak activity in eight patients

Patient	Age/sex	Type of compound	Time between exposure and 2-PAM adm	2-PAM (total in gms)	Plasma ChE activity (adm) ^a	Red cell AChE activity ^b			Red cell AChE activity ^b	
						Adm	24 h	Discharge	Time of peak	Peak Activity ^b
1. AC	17M	Dichlorvos	6 h	13.5	0.3	2.0	4	6	4 h	4
2. PK	22F	Phorate	24 h	13.5	0.1	1	7	10	4 h	5
3 DV	30M	Monocrotophos	20 h	14	0.3	9	16	18	2 h	12.4
4 GS	23M	Phorate	7 h	14	0.3	7	14	17	4 h	14
5 VS	26M	Phorate	24 h	13.5	0.2	6	14	18	4 h	13
6 B	26M	Chlorpyrifos	5 h	14	0.2	5	15	18	3 h	12.8
7 SR	23M	Chlorpyrifos	12 h	14	0.4	8	21	26	2 h	19
8 MS	28M	Chlorpyrifos	20 h	14	0.4	7	16	19	3 h	13.1

^a μ moles/min/l^b Red cell AChE activity in healthy controls (men 38.9 ± 12.55 ; women 37 ± 12.41 nmol/min/mgHb)

and gastric lavage samples were analyzed for organophosphorus compounds by gas chromatography-capillary column technique equipped with mass spectrometry by Aggarwal et al. method at Defence Research and Development Establishment, Gwalior (India) [13].

All the chemicals used in this study were of analytic grade. Acetyl-thiocholine iodide, 5-5'-dithiobis-2-nitrobenzoic acid, quinidine sulphate, eserine sulphate, diethyl p-nitrophenyl phosphate and Tris-HCl were obtained from Sigma chemical co. USA whereas others were obtained from E. merck Ltd. Mumbai, India.

The statistical analysis was carried out using SPSS package 15.0. The comparison between diethyl and dimethyl groups and groups based on WHO classification was carried out by multiple repeated ANOVA. The other parameters were analyzed by Student *t*-test. The study was approved by institute's ethics committee. An informed consent was obtained from patients if they were fully conscious and if they were obtunded, it was obtained from an immediate closest relative.

Results

In healthy controls, the mean $\pm$ SD red cell AChE activity was 24.3 ± 3.31 nmol/min/mg Hb. Of twenty patients admitted with OP poisoning during this period, only in eight, nature of compound could be confirmed from history and examination of containers and were included in the study. All of them had symptoms and signs consistent with OPP and had significantly depressed PChE activity. Five had ingested with suicidal intent and in three it was accidental exposure during agricultural spray. The age, sex, nature of compound exposed to, time interval between ingestion and arrival to hospital, duration of hospital stay,

ventilatory support required or not and dose of 2-PAM in first 24 h and outcome are shown in Table 1.

In addition to gastric lavage and supportive measures, all patients received atropine and 2-PAM as 30 mg/kg body wt as bolus dose and subsequently infused as 7.5 mg/kg body wt/h with maximum dose of 500 mg/h for first 24 h only. PChE activity at admission and red cell AChE activity in eight patients following 2-PAM infusion at admission, 4 and 24 h of infusion and discharge are shown in Table 1. The daily dose of atropine considerably varied between patients. The mean daily dose on day 1 was 138.6 mg (range 60–380) and declined on subsequent days.

In three patients with chlorpyrifos poisoning, the mean red cell AChE at admission was 6.6 ± 1.1 nmol/min/mg Hb, at 4 h and at 24 h was 17 ± 3 nmol/min/mg Hb with % increase being 153. At discharge it was 21 ± 4.3 nmol/min/mg Hb with % increase being 23 from day 1. In three patients with phorate poisoning, mean $\pm$ SD red cell AChE activity was 5.3 nmol/min/mg Hb at admission was 5.3 ± 3.9 , at 4 h was and at 24 h was 10 ± 4.8 with % increase being 89 from that at admission. At discharge it was 15 ± 4.3 with % increase being 50. In one patient with dichlorvos poisoning, red cell AChE activity was 2 nmol/min/mg Hb, at 4 h was and at 24 h being 8 nmol with % increase being 200. At discharge it was 9 nmol with % increase being 7.5 than at admission. In one patient with monocrotophos poisoning, red cell AChE activity was 9 nmol/min/mg Hb, at 4 h was, at 24 h was 16 nmol with % increase being 77. At discharge it was 18 nmol with % increase being 7% (Fig. 1).

For comparison we divided these patients into two groups depending on nature of compound exposed to i.e. dimethyl or diethyl. Six had exposure to diethyl compounds (group 1) chlorpyrifos (*O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate) and phorate (*O,O*-diethyl *S*-ethylthiomethylphosphorodithioate) while two

Fig. 1 Kinetics of red cell AChE activity in eight patients with organophosphate poisoning

had exposure to dimethyl compounds (group 2) one with dichlorvos (2,2-dichloro vinyl dimethyl phosphate) and one with monocrotophos (dimethyl (*E*)-1 methyl-2 i.e. methyl carbamoyl) vinyl phosphate]. The increase in red cell AChE activity in diethyl group was 154% where as in dimethyl group was 81% at 24 h where as at discharge was 215 and 118% respectively. When these two groups were compared using multiple repeated ANOVA, no statistically significant difference could be observed in red cell AChE activity at admission and discharge ($P > 0.05$). Similarly when patients were categorized into three groups based on WHO Classification of the OP's, group 1 had three patients (chlorpyrifos; class II moderately hazardous), group 2 had two patients (one dichlorvos and monocrotophos each (Class Ib highly hazardous) while group 3 had three patients exposed to phorate (Class Ia extremely hazardous). On pair wise comparison using multiple repeated ANOVA, no significant difference could be observed in red cell AChE activity between the groups at admission and discharge ($P > 0.05$). However within same patient with respect to time, red cell AChE revealed highly significant increase when tested by multiple repeated ANOVA ($f = 29.26$; $P < 0.000$).

In stored blood samples we could not detect the parent compound on gas chromatography–mass spectrometry using Aggarwal's method [13] probable because of the fast rate of hydrolysis of these compounds in blood. We could not estimate their metabolites.

Discussion

In addition to measures used to enhance poison excretion and intensive resuscitation, specific therapy includes use of

anti-cholinergics, AChE reactivators (ChR), anticonvulsants and others [3]. The problem of their use is timing and duration of and achievement of sufficient levels of these antidotes after administration [14]. The use of cholinolytics is aimed at blocking the cholinergic effects resulting from rise in ACh level as well as lowering its concentration in synapses. In addition the use of reactivators (ChR) leads to restoration of inhibited enzymatic activity. Of all cholinolytics, atropine sulfate is the most widely used. It is used in high doses and administered frequently as its action ceases much sooner than OPC effects. It eliminates muscarinic effects like bronchospasm, salivation, sweating etc. Atropine sulfate however does not reverse the effects of anti-cholinesterase compounds on neuromuscular transmission. The ChR are capable of reversing the toxic effects of anti cholinesterases on nicotine receptors. The capacity of ChR to reactivate red cell AChE is dependant on their nucleophilic ability. In addition to reactivating the phosphorylated enzyme, they also de-blockade neuromuscular conduction at synaptic receptors [15]. The other actions are ganglion blocking action, M and N cholinolytic activity, AChE protection against irreversible inhibition, inhibition of AChE ageing reaction and direct chemical interaction with ACh in blood [3]. The ability of oximes to interact directly with some OPC's and with phosphorylated enzyme is considered as additional protective mechanism of their action [3]. A further role of ChR's is their ability to restore activity not only of AChE but also other serine containing enzymes e.g. ATP-ses inhibited by OPC's [3] Ultrastructural studies have revealed that after in application of reactivator of the ChR TMB-4, the neuromuscular synapses recover their normal ultrastructure with in 10 days after intoxication but without treatment full recovery of ultrastructure occurs in 30 days [16].

The basic structure of oximes is similar. They differ from each other by the number of pyridinium rings monopyridinium and bispyridiniumoximes, by the position of oxime group on the pyridinium ring and by the chemical structure of the bridge connecting the pyridinium rings. The first generation ChR include Pralidoxime (2 PAM iodide and chloride), diprioxime (TMB-4), Toxogonine (Obidoxime). The pralidoxime and obidoxime have been mainly used in clinical practice [8]. They are predominantly quaternary compounds with poor permeability for biological membranes so they have only peripheral action (blood and respiratory musculature). A significant disadvantage of these ChR's lies in their relatively high toxicity and ineffectiveness in poisonings where rapid ageing occurs. Subsequently 2nd generation ChR i.e. HI-6, HS-6, HGG-12, HGG-42 have been developed. Of these HI-6 is most efficacious and less toxic as compared to HS, H6-6, toxogonine and 2-PAM. They also cannot cross blood brain barrier and action is mainly peripheral they reduce ageing

of AChE by 2–2.5 levels. However they are unstable in aqueous solutions, have to be stored in lyophilized form and have to be administered by auto injector [17]. Subsequently 3rd generation ChR have been developed e.g. thi-hydroxime which is less toxic and stable in aqueous solution. As they have tertiary nitrogen atom in their structure, they are readily able to penetrate CNS. They also lead to rapid reactivation of synaptic AChE leading to deblocking of post synaptic membrane/reactor choline with normalization of mediator release by postsynaptic membrane.

The repeated analysis of the red cell AChE (surrogate of synaptic cholinesterase as being structurally similar to synaptic enzyme activity and easily obtainable) [18] before and after oxime administration provides a meaningful parameter for the optimization of oximes as antidotal treatment. In our present study by we tried to determine the reactivation ability of 2-PAM in regenerating red cell AChE activity after its administration in eight patients with OP poisoning in dose recommended by WHO in first 24 h. We determined red cell AChE at 15 min interval for first 4 h, 1 h interval for next 4 h, 2 hourly till first admission day after 2-PAM. In all the eight patients (diethyl 6; dimethyl 2), the maximum increase in red cell AChE activity was observed within first 4 h following which despite 2-PAM infusion till 24 h was slow almost similar to as without 2-PAM over next 6 days.

At present all acute OP poisoning cases are regarded as a homogeneous entity and no specific treatment protocol is followed for different OP's despite wide variation in their toxicity, fat solubility, metabolism, selectivity for AChE and speed of ageing observed amongst different OP agents, which might affect poisoning severity and response to treatment [7]. In our study, the increase in red cell AChE activity was 154% in diethyl group between 0 and 24 h versus 81% observed with dimethyl compounds and at discharge % increase was 215 and 118 for respective groups. We could not demonstrate any significant difference in increase in red cell AChE activity amongst the three encountered OP's according to WHO classification and also between diethyl and dimethyl group on multiple repeated ANOVA. This is unlike the study by Eddleston et al. [19]. This lack of significance is probably due to very small number of patients in different groups. Even though maximum recovery of red cell AChE was observed in 4 h, patient did not recover and thus benefit of 2-PAM chloride is not clear as observed in several clinical studies [8, 19].

We could not estimate the parent compound in stored blood and other samples by Aggarwal method using GCMS. This estimation failed as OP's hydrolyze rapidly as observed by Ageda et al. [20]. Unfortunately we could not measure the concentration of metabolites of parent compound and there by could not determine correlation

between increase in red cell AChE activity and the blood concentration of compound at different intervals. Further our study gets limited by fact that we infused 2-PAM for first 24 h only. There is need to carry out study involving more patients with proven OP poisoning with known concentration in blood with continuous infusion of 2-PAM till recovery or death to determine in which group of OP poisoning red cell AChE rises and patient improves.

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