Oxidative damage in intermediate syndrome of acute organophosphorous poisoning

M. Dandapani, A. Zachariah, M.R. Kavitha*, L. Jeyaseelan* & A. Oommen**

Departments of Medicine Unit I, *Biostatistics & **Neurochemistry Laboratory, Christian Medical College & Hospital, Vellore, India

Received October 1, 2002

Background & objectives: The main cause of morbidity due to organophosphate poisoning is intermediate syndrome (Type II paralysis) that can occur 48-72 h after poisoning. Mechanisms that underlie the intermediate syndrome are not known. This study investigates the role of oxidative damage to muscles as a possible mechanism underlying the development of the intermediate syndrome.

Methods: Nineteen patients with acute organophosphate poisoning were evaluated from admission to discharge from intensive care for the severity of poisoning and the development and duration of the intermediate syndrome. Blood cholinesterases and parameters of oxidative stress were studied daily and their temporal profiles analysed according to the severity of poisoning and the development and duration of the intermediate syndrome.

Results: Fifteen patients had severe poisoning and 16 developed intermediate syndrome. There was a positive association between the severity of poisoning and the occurrence of intermediate syndrome. There was no association between the organophosphate ingested and the development of intermediate syndrome. Erythrocyte membrane acetylcholinesterase and serum butyrylcholinesterase levels at admission and over the course of poisoning were significantly ($P < 0.001$) reduced in patients compared to controls. There were significantly ($P < 0.05$) higher levels of lipid peroxidation, conjugated dienes and protein thiols in erythrocyte membranes of patients who developed the intermediate syndrome compared to healthy controls, in patients who developed intermediate syndrome compared to those who did not and in patients with long compared to short duration intermediate syndrome.

Interpretation & conclusion: In acute organophosphate poisoning, severe and prolonged acetylcholinesterase inhibition is associated with oxidative stress, detected in erythrocyte membranes, that occurs early in the course of poisoning and may contribute to the development and severity of intermediate syndrome.

Key words Acetylcholinesterase - intermediate syndrome - organophosphate poisoning - oxidative stress

Deliberate self-harm due to acute organophosphate poisoning is a well recognized emergency in India and a common cause of death\(^1\,2\). In the Christian Medical College and Hospital, Vellore (a 1800 bed tertiary referral centre), acute organophosphate poisoning is the commonest reason for admission to the medical intensive care unit\(^3\,4\) and for the initiation of mechanical ventilation (Personal communication).

Acute organophosphate poisoning (OPP) can result in three types of paralysis\(^5\,7\). Type I paralysis
accompanying the cholinergic crisis in the first 48 h of poisoning; Type II paralysis (intermediate syndrome) 48 to 72 h after poisoning associated with cranial, proximal limb and respiratory muscle weakness, and Type III paralysis, the organophosphate induced delayed neuropathy. While the cholinergic crisis is the earliest manifestation of acute OPP, it is the intermediate syndrome (IS) which necessitates prolonged mechanical ventilation and results in increased mortality.

The pathogenic mechanisms that lead to IS have not been clearly elucidated. De Bleecker et al suggested that the slow release of organophosphates from deep tissues and the persistent inhibition of acetylcholinesterase may underlie the development of IS. Electrophysiological studies of De Bleecker demonstrated both pre- and post-synaptic defects in OPP while Avasthi and Singh suggested desensitization of acetylcholine receptors being responsible for IS.

From studies in cultured rat muscle cells, Yang et al implicated a disruption of energy metabolism and calcium homeostasis in the occurrence of IS. Mathew et al reported severe muscle damage in OPP patients with the magnitude of muscle damage that occurs during the cholinergic crises determining the occurrence and severity of IS. Certain studies showed muscle necrosis in animals and humans after acute organophosphate exposure, although not of sufficient magnitude to explain the muscle weakness of IS.

The events following acute OPP provide a conducive setting for free radical generation. Free radicals mediate muscle damage and inflammation after strenuous exercise as well as the cellular injury of ischaemia reperfusion. Therefore the extensive muscle fasciculations and overactivity that occur in the cholinergic crisis of acute OPP and the paralysis following muscle overactivity which simulate ischaemia re-perfusion can both lead to free radical production and muscle damage. Studies in rats show that organophosphate induced muscle hyperactivity leads to free radical production with lipid peroxidation, a contributing factor to organophosphate induced cell injury. Similar injury to the muscle may take place in acute OPP and contribute to the development of IS.

To investigate this possible mechanism, serum butyrylcholinesterase (BuChE), RBC acetylcholinesterase (AChE) and parameters of oxidative stress in erythrocyte membranes were studied over the course of poisoning in organophosphate poisoned patients and compared between patients who developed IS with those who did not develop IS.

**Material & Methods**

**Patients:** Nineteen patients with acute OPP admitted between January and July 2000 to the Medical Intensive Care Unit (ICU) under one medical unit at the CMCH, Vellore were included in the study after obtaining informed consent from the attending relative. Criteria for diagnosis of OPP included history of OP ingestion, signs of acute OPP and low BuChE.

**Controls:** Five healthy, normal individuals (hospital staff) studied over 10 days and 10 healthy individuals (hospital staff) providing blood once were taken as controls.

This study was approved by the Research Committee of the Institution.

**Clinical studies:** Patients were seen at admission and on a daily basis in the Medical ICU until discharge. The period from ingestion of compound to presentation at hospital (lag time) was noted. Clinical assessment of the severity of poisoning (using the scale of Namba et al) was made at admission. During the course of hospitalization, detailed muscle power assessment including evaluation of facial, extra-ocular and neck muscle weakness, Medical Research Council (MRC) grading of hip and shoulder muscle power and the measurement of forced vital capacity (FVC) by spirometry were carried out. Patients were assessed daily for the Glasgow coma scale, atropine dose and ventilator requirement, and followed up for the development of intermediate syndrome. Patients were treated according to standard protocol for
management of acute OPP except for the administration of oximes.

Intermediate syndrome was defined as proximal muscle paralysis of grade 3 or less, 72 h after ingestion of organophosphate, with or without the requirement of mechanical ventilation along with extraocular, neck and respiratory muscle weakness\textsuperscript{12,19}. The grade 3 cut-off for the definition of intermediate syndrome is used to increase specificity of intermediate syndrome diagnosis.

The duration of intermediate syndrome was defined as the time from onset of intermediate syndrome to regaining proximal muscle power of both shoulder and hip of grade 4\textsuperscript{12,19}. In view of the multiple muscles involved, the differences in rate of improvement and difficulties in accurate assessment of neck, extraocular, facial and respiratory muscle weakness, the resolution of IS was defined by improvement in shoulder and hip muscle power.

The severity of intermediate syndrome was assessed by the duration of IS categorized into IS ≤1 wk or >1 wk\textsuperscript{12}.

\textit{Laboratory studies: (i) Chemicals :} Tris [hydroxymethyl] aminomethane, acetylthiocholine iodide, butyrylthiocholine iodide, 5’5’ dithiobis (2-nitrobenzoic acid) (DTNB), eserine sulphate, 2-thiobarbituric acid and 1,1,3,3 tetramethoxy propane (TEP) were from Sigma Chemical Co, USA. All other chemicals used were of the highest purity available.

Blood (5 ml in acid citrate dextrose and 1 ml for serum) was collected from 16 patients on day 1 and from 3 patients on day 2 and then on alternate days from all patients until discharge from the ICU. Three samples of blood were collected over 10 days from each of 5 controls and once from 10 other controls.

\textit{(ii) Erythrocyte membranes:} Erythrocytes from 5 ml blood were washed thrice with 12 mM phosphate buffered saline \textit{pH} 7.4, lyzed with cold water for 5 min and ghosts spun at 10,000 \textit{x} \textit{g} for 10 min. Erythrocyte membranes were re-suspended in 10 mM Tris HCl, \textit{pH} 7.4 and washed until haemoglobin contamination was minimal\textsuperscript{20}. Membranes were re-suspended in the buffer and stored at -20°C until assayed.

\textit{(iii) Cholinesterase assay:} \textit{ACHE} in erythrocyte membranes and \textit{BuChE} in serum were assayed by the method of Ellman et al\textsuperscript{21} using acetylthiocholine or butyrylthiocholine as substrates. The molar extinction co-efficient of 5-mercapto-2- nitro benzoic acid is 13.6x10\textsuperscript{3}.

\textit{(iv) Markers of oxidative stress in erythrocyte membranes:} Lipid peroxidation was estimated by assaying malonaldehyde formed by reaction of the sample with thiobarbituric acid according to the method of Okhawa et al\textsuperscript{22}. Lipid peroxides were read against a standard of 1,1,3,3 tetramethoxy propane (TEP) and units expressed as nmoles TEP/mg protein.

Conjugated dienes were extracted from 1-2 mg membrane protein with methanol : chloroform (2:1) and methanol : chloroform : water (2:1:0.8). Pooled supernatants were extracted with chloroform : water (1:1) and the lower chloroform phase dried under \textit{N}\textsubscript{2}, re-suspended in 1 ml heptane and read at 233 nm. The conjugated dienes formed were calculated\textsuperscript{23} using a molar absorption co-efficient of 2.52x10\textsuperscript{4}.

Protein thiols were estimated by the method of Habeeb\textsuperscript{24}. Membranes (1-2 mg protein) were deproteinized and the precipitates suspended in 100 \mu 100 mM Tris-HCl, \textit{pH} 8.6 containing 2mM EDTA and 0.5 per cent SDS. This was added to 200\mu 200mM Tris- HCl, \textit{pH}-8.6 containing 2mM EDTA and reacted with 30 \mu 10mM DTNB for 15 min at 25°C in the dark. Nitromercaptobenzoate anion formed from the reaction of protein thiol with DTNB was read at 412 nm which has an absorption coefficient of 13.6/mM/cm. The values are expressed as units/mg protein.

\textit{Statistical analysis:} Data were analyzed using SPSS 6.0. All continuous variables were analyzed using Mann Whitney U test, discrete variables using Chi-square test (Fisher’s Exact Probability Test) and significance of all parameters calculated for clinical and laboratory data at admission between patients who developed and did not develop IS.
To analyze the response of biochemical parameters over time (the temporal profiles), the area under the curve (AUC) was calculated as summary statistics for all parameters. Comparison between groups was made using the AUC with Mann Whitney U test\textsuperscript{25}. Levels of cholinesterases and of oxidative stress markers over the course of poisoning were compared between (i) All OP patients and controls; (ii) patients who developed IS and controls; (iii) patients who did not develop IS and controls; (iv) patients who developed IS and those who did not and (v) patients who had long duration (>1 wk) and short duration (≤1 wk) IS.

Comparisons at admission were performed between patient groups and the 15 controls whose samples were obtained once. Temporal profiles of all parameters were compared between patient groups and the 5 controls whose samples were obtained thrice over 10 days.

**Results**

**Clinical studies:** Nineteen patients, 18 men and 1 woman, with acute OPP were included in the study. The mean age of the patients was 27.95±9.25 yr. The mean age of the 15 controls was 30.8±9.96 yr, 6 were men and 9 were women.

Nine patients consumed dimethyl[1E] 1-methyl 2(methylcarbamoyl) vinylphosphate, 3 consumed O,O-dimethyl-S-methylcarbamoylmethyl phosphorodithioate and 2 consumed O,O diethyl O (3,5,6 - trichloro-2 - pyridyl) phosphorothioate. In 5 patients the compound ingested could not be identified but was probably an organophosphate.

Sixteen patients developed IS (84.2\%) and 3 did not. The mean lag time of patients who developed IS was 4.05 h and that of patients who did not develop IS was 7.15 h. Fifteen patients were severely poisoned of whom 14 developed IS. There is a significant positive association between increasing severity of poisoning at admission and the occurrence of IS (\(P<0.05\)). The mean duration of IS was 7.75 (SD 6.19) days. Nine patients had short duration IS (≤1 wk) and 7 had long duration IS (>1 wk). The duration of mechanical ventilation was significantly longer in patients who developed IS (13.25±5.54 days) compared to 4.5±0.71 days in patients who did not develop IS (\(P < 0.05\)). There is no association between compound ingested and the occurrence of IS. Of the 16 patients who developed IS, 2 patients died.

**Laboratory studies**

The cholinesterases: At admission mean AChE and BuChE levels were significantly lower in OPP patients than in healthy controls (\(P<0.001\)), in patients who developed IS than in healthy controls (\(P<0.001\)), and in patients who did not develop IS compared to healthy controls (\(P<0.001\)) but did not differ between patients who developed and did not develop IS (Table I).

The summary statistics of the area under the curve of the temporal profiles of AChE and BuChE were significantly (\(P<0.001\)) lower in patients compared to healthy controls, in patients who developed IS compared to healthy controls (\(P<0.001\)) and in patients who did not develop IS compared to healthy controls (\(P<0.001\)) but did not differ between patients who developed and did not develop IS nor between patients with short and long duration IS (Table II).

Oxidative stress: At admission mean values of lipid peroxidation, conjugated dienes and protein thiols in membranes of OPP patients did not differ significantly from healthy controls or between patients who developed IS and healthy controls or between patients who developed and did not develop IS (Table I). The summary statistics of the area under the curve of the temporal profiles of all markers of oxidative stress in erythrocyte membranes did not differ significantly between all OPP patients and controls (Table III). The summary statistics of the area under the curve of temporal profiles of lipid peroxidation and conjugated dienes in erythrocyte membranes differed significantly (\(P<0.05\)) between patients who developed IS and healthy controls with increased oxidative damage noted in patients with IS. Significant oxidative damage was not noted in patients who did not develop IS compared to healthy controls (Table III). The summary statistics of the area under the curve of temporal profiles of mean
levels of lipid peroxidation and protein thiols in erythrocyte membranes in patients with and without IS differed significantly (P<0.05) showing increased oxidation of lipids and proteins in patients who developed IS (Table III). The summary statistics of the area under the curve of temporal profiles of lipid peroxidation, conjugated dienes and protein thiols in erythrocyte membranes of patients with IS ≤ 1 wk and those with IS > 1 wk differed significantly (P<0.05) with oxidative damage greater in patients with IS > 1 wk (Table III).

**Discussion**

The high rate of intermediate syndrome in this study and the association between severity of poisoning and the development of intermediate syndrome are consistent with earlier observations from our centre\(^1\). However, the proportion of severely poisoned patients and those with intermediate syndrome are higher than other published observations\(^1\). The higher rates of intermediate syndrome may reflect the proportion of patients who are severely poisoned\(^1\), inclusion of only ICU patients and the non use of oximes. Despite the high rate of severity of poisoning and occurrence of intermediate syndrome the overall mortality was low.

The persistent severe inhibition of BuChE and AChE through the course of poisoning, which was associated with the severity of intermediate syndrome, supports the suggestion that AChE inhibition underlies both the cholinergic crises and intermediate syndrome\(^9,19\). However, failure of AChE to increase with improvement in muscle power and the similar levels of inhibited AChE between patients who developed IS and those who did not, and between patients with short and long duration IS, implicate additional mechanisms in the development of IS. The presence of only nicotinic signs during IS is not explained by the ‘AChE inhibition theory’\(^19\). AChE inhibition therefore may initiate other processes which result in cellular dysfunction.
Our results show that oxidative damage occurs soon after ingestion of the organophosphate and during the course of poisoning. There is indication of greater damage to membrane lipids and proteins in patients with IS and in patients with longer duration IS. However, these results need to be interpreted with caution in view of the small number of patients who did not develop IS. Further work to substantiate these findings, in the context of the small number of patients who did not develop IS, is required in a group of less severely poisoned patients not needing ICU care.

The source of free radicals in acute OPP is probably muscles. Oxidative damage of erythrocyte membrane lipid and protein may reflect similar reactions occurring in the myocyte. Free radical damage to myocytes may contribute to the muscle weakness in acute OPP.

Recent work of Yang et al. show the induction of heat shock protein 70 (HSP70) in cultured muscle cells exposed to dimethoate. Heat shock proteins are known to protect cells from oxidative stress and if induced in OPP patients may partly explain the low levels of oxidative stress observed in our patients despite their severe poisoning.

We suggest two possible mechanisms that may underlie IS of acute OPP: (i) persistent inhibition of acetylcholinesterase; (ii) acetylcholine induced oxidative damage to muscle membranes during the acute phase of poisoning. Additional factors that may contribute to IS need to be explored in studies of larger sample size.

Acknowledgment

This study was funded by a Fluid Research Grant of the Christian Medical College & Hospital, Vellore. The expert laboratory assistance of Rebecca Cherian and Roy Cherian is acknowledged.

References


Reprint requests : Dr Anna Oommen, Neurochemistry Laboratory, Christian Medical College & Hospital
Vellore 632004, India

e-mail : anna@cmcvellore.ac.in