Pharmacotherapy to protect the neuromuscular junction after acute organophosphorus pesticide poisoning

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Organophosphorus (OP) pesticide poisoning is a leading cause of morbidity and mortality in the developing world, affecting an estimated three million people annually. Much of the morbidity is directly related to muscle weakness, which develops 1–4 days after poisoning. This muscle weakness, termed the intermediate syndrome (IMS), leads to respiratory, bulbar, and proximal limb weakness and frequently necessitates the use of mechanical ventilation. While not entirely understood, the IMS is most likely due to persistently elevated acetylcholine (ACh), which activates nicotinic ACh receptors at the neuromuscular junction (NMJ). Thus, the NMJ is potentially a target-rich area for the development of new therapies for acute OP poisoning. In this manuscript, we discuss what is known about the IMS and studies investigating the use of nicotinic ACh receptor antagonists to prevent or mitigate NMJ dysfunction after acute OP poisoning.

Keywords: pesticide; neuromuscular junction; poisoning

Background

Organophosphorus (OP) pesticide poisoning is a leading cause of morbidity and mortality in the developing world. The World Health Organization has estimated that approximately three million people suffer from OP poisoning annually, with an overall mortality rate of roughly 10%. Most of these poisonings are intentional, where the OPs are a common method of self-harm and suicide. In the United States and developed countries, the risk of OP poisoning is much lower. However, the common pathophysiologic mechanism between OPs and the military nerve agents (namely the inhibition of acetylcholinesterase (AChE)) and with their real and potential use as a terrorist weapon has led to a substantial increase in clinical and research interests into AChE inhibitors.

Acute OP poisoning leads to three main clinical syndromes: (1) acute cholinergic syndrome, (2) OP-induced delayed neuropathy, and (3) the intermediate syndrome (IMS). Virtually all OP-related research has focused on the first clinical entity, and that treatment includes the provision of oxygen, atropine, an AChE reactivator (such as pralidoxime), and benzodiazepines. The IMS, however, is a common complication that remains a major contributor to the high morbidity and mortality in OP poisoning. The IMS was first described as a syndrome of paralysis occurring 1–4 days after resolution of the acute cholinergic syndrome. Recent work suggests that the IMS occurs much sooner. Weakness predominantly affects the limb muscles, respiratory musculature, and muscles supplied by the cranial nerves. The pathophysiology of the IMS is not understood, but is thought to be due to a persistent excess of acetylcholine (ACh) at the neuromuscular junction (NMJ). Therefore, interventions targeting the NMJ could yield tremendous dividends in mitigating the toxicity of OPs.
Respiratory complications of acute OP poisoning

Approximately 24% of OP poisoned patients require intubation; of these, more than 50% die. Most of these deaths result from acute respiratory failure (due to either central respiratory depression, respiratory muscle weakness, direct pulmonary effects, such as bronchospasm or bronchorrhea), or as a consequence of prolonged mechanical ventilation. Furthermore, the muscle weakness caused by acute OP poisoning contributes significantly to morbidity, consuming resources and hospital beds and significantly prolonging hospitalization. Targeted treatment, already associated with mortality of 10–40% for acute OP poisoning, is nonexistent for the IMS. The only therapy available for the IMS is intubation and mechanical ventilation, which is often required for a few weeks. The clinical manifestation of the IMS includes remarkable neck muscle weakness and varying degrees of proximal (rather than distal) muscle weakness. Other variable findings include either decreased or absent deep tendon reflexes and involvement of muscles innervated by cranial nerves. The respiratory and proximal muscle weakness or paralysis of the IMS lasts a variable amount of time, but typically is resolved after approximately 30 days. Because there are often minimal advanced practitioners and equipment, such as ventilators, available in the developing world, patients with the IMS are often unable to receive adequate care. Despite focused research over the last four decades, current therapy for OP poisoning is directed toward generalized reversal of excess ACh.

While it is an understudied area of research, there is some laboratory evidence from isolated mouse diaphragms that suggests that increased recovery of AChE activity leads to increased force of muscle contraction. Therefore, it seems logical that therapies directed toward improving neuromuscular transmission should provide morbidity and mortality benefits to these patients.

The NMJ effects of OPs are not well understood

The NMJ consists of a presynaptic axon terminal and a postsynaptic muscle end plate. Within the presynaptic terminal are vesicles containing ACh. Most of these vesicles are bound to the actin cytoskeleton by proteins called synapsins. When an action potential induces opening of calcium channels, increased intracellular calcium concentrations promote phosphorylation of synapsins. This phosphorylation results in release of the ACh-containing vesicles from their cytoskeletal sites.

After release from the cytoskeleton, the vesicles become bound at the presynaptic membrane terminal in areas called active zones. This docking of the vesicles allows rapid exocytosis of the vesicles and ACh. Docking is mediated by a group of proteins termed SNAREs (soluble N-ethylmaleimide-sensitive fusion-attachment protein receptors). SNAREs attached to the terminal membrane form complexes with proteins located on the vesicle. Proteins involved in SNARE complexes include vesicle-associated membrane protein, which is found on the vesicle surface, along with synaptosomal-associated protein 25 and syntaxin, proteins found at the terminal membrane. The increased calcium concentration induced by the action potential leads to phosphorylation of docking proteins, which induces SNARE complex formation, followed by exocytosis of the vesicle contents and incorporation of the vesicle membrane into the terminal membrane. Neurotransmitter vesicles are recycled when pits form in the terminal membrane and become coated with a protein termed clathrin. These clathrin-coated pits then pinch off to reform vesicles, into which ACh is synthesized and repackaged.

The postsynaptic membrane is heavily folded and invaginated. ACh receptors are found at the crests of the junctional folds, and voltage-sensitive Na+ channels are concentrated within the folds. The ACh receptors have an ideal binding constant to allow reversible binding of ACh. When bound, ion channels within the receptor are opened with an influx of Na+, and there is a transient depolarization of the end-plate region. If this end-plate potential is large enough, a muscle fiber action potential is generated, which leads to muscle contraction. ACh remaining in the synapse is rapidly degraded by AChE, and the muscle is allowed to repolarize.

The IMS may represent a neuroparalytic syndrome resulting from a collective insult to the nerve, neuromuscular transmission, and/or the muscles. In isolated rodent muscle preparations, AChE inhibitors have been shown to induce muscle

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Protecting the neuromuscular junction

More recent electrophysiological study reports on the IMS were suggestive of neuromuscular junctional defect. It has also been hypothesized that direct muscle injury due to oxidative free radical damage may be causing or contributing to the muscle weakness. More recent electrophysiological studies suggest peripheral mechanisms like NMJ dysfunction and myopathy in the causation of weakness. Recently, some investigators have theorized that the IMS is a “spectrum” disorder in which NMJ dysfunction progresses over time through a series of electrophysiological changes.

Early animal studies investigating NMJ failure following OP poisoning identified some of these electrophysiologic features. Identified features included repetitive nerve stimulation (RNS), decrement—increment changes, which were in part increased with edrophonium use and which were normalized after the use of tubocurarine. In a rat phrenic nerve and diaphragm preparation, Besser et al. found that AChE was transiently inhibited by neostigmine and that a decrement—increment response was observed. Using RNS testing (in which a series of electrical shocks are applied to certain nerves and the responses in the muscles that those nerves control are recorded), De Bleecker et al. studied rats poisoned with paraoxon (the highly toxic active metabolite of parathion) and fenthion. They found that at high frequencies there were two types of RNS decrements observed—decrement—increment and decrement alone—and that the decrement—increment phenomenon preceded the decrement phenomenon and was also associated with marginally less AChE inhibition than decrement alone.

More recent clinical research by Jayawardane et al. has sought to characterize the RNS changes that occur in patients poisoned with OP pesticides (mostly chlorpyrifos) in Sri Lanka. In their study, 78 patients with acute OP poisoning were assessed upon presentation and several times daily for clinical signs of the IMS. They also determined nerve transmission daily using RNS. Ten of their 78 patients developed the IMS. In these 10 patients, they observed several changes in the muscle responses to RNS, including some changes that were observed before the clinical manifestation of the IMS. Perhaps most important in this clinical study, in four patients diagnosed with the IMS who developed respiratory failure and required mechanical ventilation, an RNS response pattern that was called severe decrement (i.e., a reduced response to the first electrical shock and then no response to any of the subsequent shocks) was seen before respiratory failure developed. Last, providing evidence that the IMS is a spectrum disorder, there were other changes in muscle responses to RNS seen in 30 patients who developed muscle weakness that was not severe enough to receive a diagnosis of the IMS, which the investigators termed incomplete or forme fruste IMS.

The early work of Maselli and Leung showed that the sustained depolarization of the end-plate receptor is the primary cause of the failure of neuromuscular transmission during the exposure of the NMJ to low concentrations of AChE inhibitors. In the study by Jayawardane, both patients with incomplete IMS and patients with classic IMS demonstrated the decrement—increment pattern in the early stage of poisoning. In classical IMS patients, however, the decrement—increment pattern worsened to become a severe decrement pattern at stimulations of high frequency. It therefore appears that, in the early stages or perhaps in less severe cases of OP poisoning, there is a depolarization block at the NMJ. This is supported by the finding that persistent and sustained accumulation of ACh at the NMJ can lead to a form of desensitization block. It is possible that desensitization in part counteracts depolarization block, but in the presence of high concentrations of OPs, desensitization serves to intensify the depolarization block, and it likely becomes the primary or dominant mechanism by
which NMJ transmission is altered. In the Jayawar- 
dane et al. study, the authors hypothesized that “the transition of electrophysiological abnormali-
ties from severe decrement to progressive decrement 
pattern observed in our study may represent the 
transition between the two types of neuromuscular 
blockade.”

It is likely that the pathophysiology of the IMS is 
complex. This is due, in part, to the variable phys-
iochemical properties, toxicokinetics, and toxicody-
namics of the many different OP pesticides in use 
worldwide. It is also likely that the specific ther-
apy that a particular patient receives influences the 
development and severity of the IMS. For instance, 
because AChE inhibition is a prerequisite to IMS 
development, the inadequate use of oximes may 
play a role in IMS development or severity. Some 
individual susceptibility to the IMS is also possible, 
with some patients exhibiting NMJ nicotinic recep-
tor polymorphisms, altered ACh recycling, AChE 
deficiency, or some other genetic predisposition to 
the disorder.

Nicotinic receptor antagonists

Nicotinic acetylcholine receptors (nAChRs) can be 
broadly grouped into two classes: neuromuscular 
and neuronal. Neuronal nAChRs are present in the 
brain, sympathetic and parasympathetic ganglia, 
and the adrenal medulla. The role of these recep-
tors in the pathophysiology of acute OP poisoning 
is limited, and the prospects of pharmacologic gan-
glionic antagonism for OP poisoning treatment are 
guarded, mostly due to the significant hypotension 
caused by these medications.

Neuromuscular nAChRs, however, are attractive 
therapeutic targets. Because acute OP pesticide poi-
soning leads to fasciculations and progressive weak-
ness with overstimulation of these receptors, the 
development of competitive antagonists of neuro-
muscular nAChR is a logical approach to mitigating 
the effects of OP pesticides and their nAChR ago-
nism. Pharmacologic protection of the NMJ with 
nicotinic ACh receptor antagonists has only recently 
been theorized.

Breningstall et al. published a fascinating case 
report of congenital NMJ end-plate AChE deficiency 
treated with intermittent intramuscular pancuro-
nium therapy, which persisted for months. Besser 
et al. have investigated the ability of pancuronium 
to effect improvement in compound muscle action 
potentials (CMAPs) after OP poisoning. In one 
study, two patients with acute severe organophos-
phate intoxication showed single evoked CMAP 
with repetitive discharges and prominent decre-
mental responses of CMAP supramaximal nerve 
stimulation. Following the injection of a small dose 
of pancuronium, improvement in these abnormal-
ities occurred and persisted for several hours. 
Unfortunately, all of these patients were given pan-
curonium several days after OP poisoning (except 
for one patient who received pancuronium within 
5 h of poisoning).

The above findings support the proposition that 
nAChR antagonists could improve muscle strength 
after acute OP poisoning. Controlled animal stud-
ies examining functional neuromuscular response 
and NMJ architecture changes with and without 
nAChR therapy are needed to determine if this class 
of agents could serve as potential therapy for acute 
OP poisoning.

Developing animal models

One reason for the current paucity of therapeu-
tic options has been the lack of a realistic animal 
model of OP poisoning using comprehensive critical 
care treatment. As part of our ongoing Counter-
ACT National Institutes of Health studies, we have 
been studying the electrophysiology and NMJ struc-
tural effects of parathion poisoning in a critical-care 
minipig model. We have developed a minipig model 
of severe OP poisoning that utilizes comprehensive 
critical support (currently, this work in progress 
has only been presented in conference presentation 
and an abstract). The benefit of a large animal 
model is that serial blood and muscle samples can 
easily be obtained over a 24-h period of time, or 
longer as necessary. Details of the minipig model 
are as follows. Under general anesthesia, 22- to 25-
kg minipigs are intubated and undergo mechani-
cal ventilation with isoflurane (the effects of this 
anaesthetic on AChE activity, unlike many others, 
have been characterized) for up to 24 h; femoral 
venous and arterial lines are placed for venous access 
and for arterial blood pressure measurements and 
blood draws; a surface electrocardiogram is continu-
ously recorded, and constant body temperature is 
maintained with the use of a rectal thermometer.
and homeostatic blanket; air flow is continuously monitored via a pneumotachometer; and systolic and mean arterial blood pressures are continuously recorded. This animal model is based largely upon the work of Eddleston and colleagues.\textsuperscript{45,46}

To objectively detect and measure neuromuscular function during parathion poisoning, electrical train of four (TOF) stimulation at the peroneal nerve–muscle unit is performed every 30 min in one hind leg and measured via acceleromyograph. In TOF testing, four electrical stimuli are applied in rapid succession. Measurement of the first and fourth stimulus responses is determined by AMG and is expressed as a ratio, which in turn indicates the degree of NMJ function.\textsuperscript{45} Once all monitoring is initiated, a parathion dose of 75 mg/kg is administered, which is approximately 10 times the oral LD\textsubscript{50} in rats. In order to mimic a mass-poisoning scenario in which treatment would be delayed, antidotal therapy is given when the mean arterial pressure reaches 55 mmHg. A bolus dose of atropine followed by an atropine infusion is used, as are bolus doses of pralidoxime. To more closely mimic therapy in humans, we also administer intravenous diazepam every 4 hours. Norepinephrine is used as needed for arterial hypotension. Immediately before poisoning and every 4 h thereafter, a sample of muscle is taken from the hind leg tibialis anterior; the muscle tissue is flash frozen in isopentane. Previous studies have shown that NMJ function deteriorates gradually, as measured by hind leg acceleromyography or mechanomyography (Fig. 1).\textsuperscript{45} After poisoning, direct stimulation of muscle results in appropriate contraction, indicating NMJ failure at the synapse and not failure of the contractile apparatus.

Semi quantitative analyses of the postsynaptic ACh receptor density in animals are useful to determine the effect of treatments in mitigating the effects of the IMS. This method involves incubating muscle samples with α-bungarotoxin, followed by circumscribing the total end-plate region (stained areas and unstained areas interspersed within). The areas of these regions are then used to calculate NMJ dispersion percentage by dividing the end plate’s stained area (nAChR clusters) by its total area and multiplying by 100.\textsuperscript{47} An example of such NMJ semi quantitative studies is provided in Figures 2–4. These semi quantitative analyses of NMJs after OP poisoning allow investigators to follow NMJ physiologic and morphologic changes that occur acutely after poisoning and support the development of an animal model of the IMS and medical countermeasures to the IMS, such as nAChR antagonists.

**Challenges to use of NMJ therapies**

The clinical use of nAChR antagonists as a therapy to protect the NMJ would present at least one significant problem in the management of these patients. Because such drugs induce a chemical paralysis, intubation and mechanical ventilation would be necessary. This has led some investigators to question the benefit or utility of these agents in treating acutely OP-poisoned patients. However,
it must be remembered that, although they are not always available in developing countries, virtually all severely poisoned OP patients require intubation and mechanical ventilation as part of their standard treatment. Thus, the use of nAChR antagonists would not fundamentally change the acute management of these desperately ill patients. In fact, if the use of nAChR antagonists is shown to decrease the overall incidence and/or duration of the IMS, then the use of these agents concomitantly with a mechanical ventilator would likely increase the availability of this precious resource in acute and intensive care settings in developing countries.

Another challenge to the widespread acceptance of nAChR antagonist therapy for acute OP poisoning is the potential development of critical illness myopathy (CIM). CIM is still a relatively poorly understood entity found in a small percentage of intensive care unit patients after approximately 7 days of mechanical ventilation. CIM was formerly thought to be associated with the use of nAChR antagonists concomitant with corticosteroids. However, CIM is now understood to be a diverse array of myopathies, with corticosteroids thought to be the major contributing factor (with some potential component of nAChR antagonists) in a specific myopathy termed “thick-filament myopathy.” The risk of CIM should not detract from the investigation of nAChR antagonists as potential therapy for acute OP poisoning, however, as it is unlikely that an nAChR antagonist would be necessary for more than a few days, owing to the known pharmacokinetics of most OP pesticides after severe poisoning and the theoretical and potential benefits of nAChR on the duration of mechanical ventilation.

Furthermore, while once the exclusive purview of anesthesiologists, nAChR antagonists are now routinely used in everyday clinical practice by a wide range of medical specialists, including prehospital personnel, emergency physicians, and critical care specialists. Furthermore, nAChR antagonists are now stocked routinely in advanced-care life-support ambulances and used clinically by paramedics to assist with intubation of patients. Thus, concerns about developing nAChR antagonists to prevent or mitigate NMJ dysfunction in acutely OP-poisoned patients are generally not valid.

Last, despite the impressive preclinical effects of nAChR antagonists in protecting the NMJ, a rigorous randomized controlled trial of humans poisoned with OP pesticides is needed. By necessity, such a trial will need to be conducted in an area with a high prevalence of self-poisoning with OP pesticides, such as India, Sri Lanka, or China.

**Conclusions**

One of the most critical developments after OP pesticide poisoning is transient muscle weakness. The preponderance of evidence now supports the idea that this muscle weakness (the IMS) is a direct result of AChE inhibition, leading to sustained and high
concentrations of synaptic ACh. After more than 40 years of studies and antidote research, no therapy for the IMS has been developed. Rigorous and reproducible animal models, such as the minipig, can provide remarkable insight into the progression of muscle weakness and NMJ changes that occur after acute OP poisoning using conventional critical care therapies. Based upon such a minipig model, it appears that targeting nAChR with specific nAChR antagonists is a logical addition to our treatment armamentarium. Ongoing studies to develop a model of IMS with functional indices of muscle strength after recovery from the acute poisoning are needed. If successful, these data should provide impetus for conducting a human trial utilizing nAChR antagonists in acutely poisoned patients.

Conflicts of interest

The authors declare no conflicts of interest.

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