Supralethal poisoning by any of the classical nerve agents is effectively counteracted by procyclidine regimens in rats

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ABSTRACT

A treatment regimen consisting of HI-6, levetiracetam, and procyclidine (termed the triple regimen) has previously been shown to work as a universal therapy against soman poisoning in rats, since it has capacities to function as both prophylactic and therapeutic measure. The purpose of the present study was to examine whether the triple regimen may have antidotal efficacy against intoxication by other classical nerve agents than soman. The treatment was given 1 and 5 min after exposure to a supralethal dose of nerve agents, and the results showed that the triple regimen successfully prevented or terminated seizures and preserved the lives of rats exposed to 5 x LD_{50} of soman, sarin, cyclosarin, or VX, but solely 3 x LD_{50} of tabun was managed by this regimen. To meet the particular antidotal requirements of tabun, the triple regimen was reinforced with obidoxime and was made to a quadruple regimen that effectively treated rats intoxicated by 5 x LD_{50} of tabun. The rats recovered very well and the majority gained pre-exposure body weight within 7 days. Neuropathology was seen in all groups regardless of whether the rats seized or not. The most extensive damage was produced by sarin and cyclosarin. Differentiation between the nerve agents' potency to cause lesions was probably seen because the efficacious treatments ensured survival of supralethal poisoning. A combination of 2 oximes and 2 anticonvulsants may be a prerequisite to counteract effectively high levels of poisoning by any classical nerve agent.

1. Introduction

Organophosphorus nerve agents, such as tabun, sarin, soman, cyclosarin, and VX, are categorized as the most toxic of chemical warfare means. Exposure to nerve agent can induce increased salivation, respiratory distress, tremor, seizures/convulsions, coma, and death. The destructive effects of nerve agents have repeatedly been demonstrated in recent years. Sarin was used twice in Japan by terrorists (Okumura et al. 2003, 2005), and sarin was dispersed in the suburbs of Damascus in Syria in 2013 resulting in massive casualty (Rosman et al. 2014).

The primary mechanism of action of nerve agents is inhibition of acetylcholinesterase (AChE), the enzyme that hydrolyzes acetylcholine. Accumulation of acetylcholine results in excessive stimulation of muscarinic and nicotinic receptors. Increased cholinergic activity in the brain is probably related to the initial phase of seizures, whereas sustained seizures are probably associated with increased glutamatergic activity leading to neuronal damage (McDonough and Shih 1997).

Exposure to nerve agent requires immediate medical treatment. For this purpose, military personnel are issued with autoinjectors containing countermeasures for self-administration or "buddy aid". Antidotes against nerve agents are based on drugs acting at the muscarinic receptors and $GABA_A$ receptors (McDonough and Shih 1997). In addition, partial protection against nerve agents can be obtained by the use of reversible (carbamate) AChE inhibitors shielding a portion of AChE from irreversible inhibition by nerve agents prior to nerve agent exposure. Furthermore, reactivation of any unaged AChE by an oxime is regarded as important immediate treatment after nerve agent exposure.

A number of armed forces have based their therapy against nerve agent intoxication on an oxime (obidoxime, 2-PAM, HI-6), an anticholinergic agent (atropine) combined with carbamate (pyridostigmine) pretreatment (Aas 2003). Such treatment regimens can, however, reduce immediate lethality, but they do not attenuate the occurrence of nerve agent-induced seizure activity and concomitant convulsions, unless atropine is given early and at a high dose (McDonough and Shih 1997). To overcome this shortcoming, several nations have provided their personnel with autoinjectors containing diazepam or avizafone (both benzodiazepine analogues with similar anticonvulsant action).

The choice of oxime in autoinjector therapy differs among countries. Currently, there is no universal oxime that effectively can reactivate AChE inhibited by any known nerve agent. This oxime-nerve agent specificity makes the choice of a single oxime difficult; alternatively plural oximes have to be considered. Most studies indicate that obidoxime is more efficient than HI-6 (1-[([4-(aminocarbonyl)pyridinio]methoxy)methyl]-2-

[(hydroxyimino)methyl]pyridinium) against tabun, whereas HI-6 is a better drug than obidoxime against soman. However, HI-6 is assessed by many as the most promising broad spectrum oxime against nerve agent intoxication (Aas 2003; Kassa 2002), although in a recent comprehensive study examining a variety of oximes against all classical nerve agents and a number of organophosphorus pesticides in guinea pigs it was concluded that MMB-4 and HLö-7 are the most efficacious oximes (Wilhelm et al., 2014). Some countries use atropine along with obidoxime (e.g., Germany, Norway, The Netherlands, and Finland). A future autoinjector regimen containing atropine, HI-6, and diazepam (presently used by Canada) is proposed by several nations within NATO (Aas 2003).

After the Gulf War, the prevalent opinion has been to reduce reliance on pretreatment against nerve agent. However, in the present situation, an adequate stand-alone post-poisoning treatment does not exist. As a substitute, it has been claimed that it is reassuring and necessary to have an adequate pretreatment in place (van Helden et al. 2011). In the search for an effective stand-alone therapy, we have performed a series of lesion and microinfusion studies to identify critical control sites for soman-induced seizure activity in the forebrain of rats. Subsequent specification of important pharmacological subreceptors in the sites identified can provide information for the selection of drugs affecting the control sites in the forebrain and avoiding the brainstem with the control center for respiration (Myhrer 2010). Through this process, procyclidine turned out to have the highest impact of the drugs tested in seizure controlling sites in the forebrain of rats (Table 4 in Myhrer 2010). Enhancement of procyclidine`s outstanding antidotal properties (anticholinergic and antiglutamatergic) would make up a novel and interesting approach. Levetiracetam with a unique profile in preclinical models of epilepsy has been shown to increase the potency of other antiepileptic drugs up to 19-fold (Kaminski et al. 2009).

In a recent study, it was demonstrated that a triple regimen consisting of HI-6, levetiracetam, and procyclidine is able to prevent or terminate seizures and conserve lives when given 1 and 5 min after poisoning with supralethal doses of soman (3, 4, and 5 x LD_{50}) (Myhrer et al. 2013b). In order to investigate whether the triple regimen may have a more extensive applicability, the present study was designed to test the anticonvulsant and life-saving properties of the regimen in rats challenged with supralethal doses of tabun, sarin, soman, cyclosarin, or VX. Results from pilot experimentation showed that a quadruple regimen consisting of HI-6, obidoxime, levetiracetam, and procyclidine was required to effectively manage intoxication by 5 x LD_{50} of tabun, but for the other nerve agents (5 x LD_{50}) the triple regimen was effective (Fig. 1).

Experimental design

Seizures

0 min	1 min	5 min
↑	↑	↑

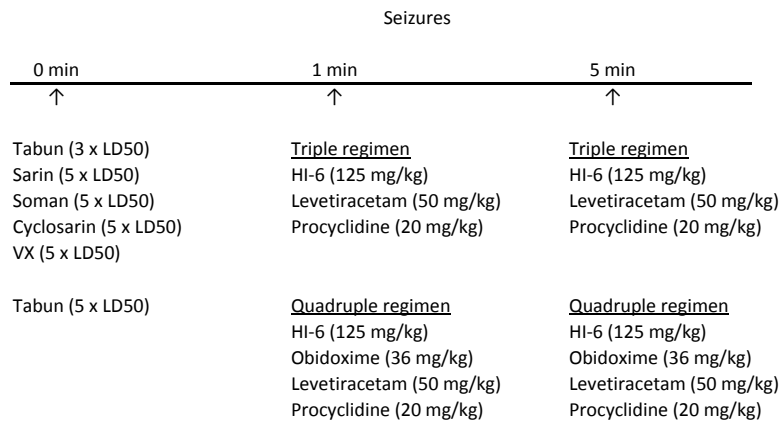| Tabun (3 x LD50) | Triple regimen | Triple regimen |
Sarin (5 x LD50)	HI-6 (125 mg/kg)	HI-6 (125 mg/kg)
Soman (5 x LD50)	Levetiracetam (50 mg/kg)	Levetiracetam (50 mg/kg)
Cyclosarin (5 x LD50)	Procyclidine (20 mg/kg)	Procyclidine (20 mg/kg)
VX (5 x LD50)		
Tabun (5 x LD50)	Quadruple regimen	Quadruple regimen
	HI-6 (125 mg/kg)	HI-6 (125 mg/kg)
	Obidoxime (36 mg/kg)	Obidoxime (36 mg/kg)
	Levetiracetam (50 mg/kg)	Levetiracetam (50 mg/kg)
	Procyclidine (20 mg/kg)	Procyclidine (20 mg/kg)

Fig. 1.

Schematic overview of the experimental design. Because the triple regimen was only able to counteract poisoning by a tabun dose of 3 xLD_{50}, obidoxime was added to make up a quadruple regimen that effectively managed survival following a tabun dose of $5 \times LD_{50}$.

2.   Materials and methods

2.1. Animals

Male Wistar rats from a commercial supplier (Taconic Breeding Laboratories, Denmark) weighing 300-330 g served as subjects. The experiments were approved by the National Animal Research Authority. The animals were housed individually and had free access to commercial rat pellets and water. The rats were handled individually 3 days preoperatively and 3 days postoperatively, being allowed to explore a table top (80 x 60 cm) for 3 min a day. The climatized vivarium (21ºC) was illuminated from 0700 to 1900 h.

2.2. Surgery

The rats were anesthetized ip with diazepam (4.5 mg/kg) and fentanyl fluanisone (2 mg/kg). Stainless screws were implanted bilaterally in the parietal cortex (1 mm behind bregma, 3 mm lateral to midline); one served as recording electrode and the other one served as ground. The screws were fixed with dental cement (Durelon; ESPE, Seefeldt, Germany). The rats were given a recovery period of 7 days.

2.3. Drugs

The drug doses chosen were derived from previous studies of anticonvulsant effects against soman-evoked seizures in rats; HI-6 dimethanesulphonate 125 mg/kg, obidoxime 36 mg/kg, levetiracetam 50 mg/kg, procyclidine hydrochloride 20 mg/kg (Kassa and Koupilová 2000; Myhrer et al. 2011, 2013b). The drugs were

dissolved in 0.9% saline and were administered intramuscularly. The injection site alternated between the left and right muscle in the hind leg. The drugs were given successively in the following order: oxime(s), levetiracetam, and procyclidine. Because only LD_{50} doses of soman are known for our strain of male Wistar rats, we calculated the conversion factor for the toxicity relation between soman and each of the other nerve agents to reach at the exact doses for our rats. The relevant information is available in previous studies in which soman was used in addition to 1 or several of the other nerve agents (Bajgar 1992; Hamilton and Lundy 1989; Hoskins et al. 1986; Kassa et al. 2012; Shih and McDonough 1999; Sivam et al. 1984).The mean conversion factor based on 2-3 studies was used for the toxicity between soman and each of the other nerve agents. According to the calculations, soman is 2.3, 1.32, 1.95, and 0.17 times more toxic than tabun, sarin, cyclosarin, and VX, respectively. The nerve agent dose of 5 x LD_{50} was for tabun 920 µg/kg, sarin 528 µg/kg, cyclosarin 780 µg/kg, soman 400 µg/kg, and VX 68 µg/kg. The 3 and 4 x LD_{50} doses for tabun were 552 µg/kg and 736 µg/kg. The nerve agents were injected subcutaneously. The treatment regimens were given 1 min (injections started at 50 sec) and 5 min (injections started at 4.7 min) after exposure to nerve agent. Levetiracetam is commercially available (Keppra®). Procyclidine was purchased from Sigma (St Louis, MO, USA), and obidoxime was purchased from Merck (Darmstadt, Germany). HI-6 dimethanesulphonate was a gift from Defence Research and Development (Suffield, Medicine Hat, Canada). Tabun, sarin, soman, cyclosarin, and VX were purchased from TNO (Netherlands Organization for Applied Scientific Research, The Netherlands).

2.4. Histology

The rats lived for 8 days after challenge with nerve agent. They were anesthetized as described for surgery, perfused intracardially with 10% formalin, and the brains were post-fixed in 10% formalin for at least 24 h. The brains were dehydrated and embedded in paraffin (Schmued et al. 1997). The sections were cut 5 µm thick and dried in an incubator (37ºC) for 12 h before they were stained with Fluoro-Jade B (Schmued and Hopkins 2000). Since Fluoro-Jade staining requires perfusion of the brain, only live rats could be used for this purpose. Fluoro-Jade has been considered to be the compound most suitable for the detection of neuronal degeneration (Schmued et al. 1997). A degenerating neuron presumably expresses a strong basic molecule, since it has an affinity for the strongly acidic Fluoro-Jade (Schmued et al. 1997). The Fluoro-Jade method has previously been described in detail (Schmued et al. 1997; Schmued and Hopkins 2000). Fluoro-Jade B staining is seen with a blue excitation filter. A digital microscope camera (AxioCam, Zeiss, Jena, Germany) was used to make photomicrographs. This

technique allows processing of the photographs so that elements of particular interest can be made clearer by adjusting contrasts.

2.5. Evaluation of neuropathology

A grading system of 0-4 previously described (McDonough et al. 1995), was used to determine severity of neuronal damage in the piriform cortex, the hippocampal CA1 region, and the basolateral amygdala based on the approximate percentage of tissue involvement: 0 – no lesion; 1 – minimal, 1-10%; 2 – mild, 11-25%; 3 – moderate, 26-45%; 4 – severe > 45%. Each animal was given bilateral neuropathology scores for the 3 individual brain areas chosen. The criterion used to characterize the pathology was neuronal degeneration.

2.6. EEG

The electrodes were connected with the polygraph (Grass Model 79E) with alligator clips and leads. The use of a swivel allowed the rats to move freely. Seizure activity was defined as terminated when epileptiform waves had ceased (absence of continuous high amplitude rhythmic spike or sharp wave activity). EEG recording was made while the animals were situated in their home cages (50 x 30 x 15 cm). Measures were made 24 h prior to drug treatment, immediately after, and during 10 min at 24, 48, or 144 h after treatment.

2.7. Observation of animals

The rats were observed for convulsions and visible signs of intoxication continuously for the first 2-3 h and then for 10 min at 24 and 48 h after soman injection.

2.8. Statistics

Overall analyses were carried out by using parametric or nonparametric one-way analysis of variance (ANOVA). Group comparisons were made with Newman-Keuls or Dunn's post hoc test, two-tailed Mann-Whitney U test, or with two-sided Fisher's exact test. Use of the grading system of neuropathology resulted in nonparametric data. Computations were made with the Prism statistical software program (GraphPad Software, Sand Diego, CA, USA).

3. Results

3.1. Pilot experimentation

The high level of toxicity used in the present study has previously been seen to produce rather homogeneous reactions within groups in terms of convulsant responses and lethality by use of 5 x LD_{50} of soman (Myhrer et al. 2006). Hence, for guidance of responses, 2-3 test rats were challenged with a given nerve agent (5 x LD_{50}), and the rats were treated with the triple regimen immediately (5 sec) after onset of seizures. The results show latency to onset of seizures and lethality rate after intoxication by tabun, sarin, soman, cyclosarin, or VX (Table 1). All rats except 1 poisoned by sarin died within short time after administration of the triple regimen. One-way ANOVA did not reveal a reliable overall effect for latency to onset of seizures (H (5) = 6.759, P = 0.1492).

Table 1.
Strongly reduced life preserving capacity of a triple regimen (HI-6, levetiracetam, procyclidine) when given immediately after onset of seizures induced by high level of nerve agent poisoning in rats.

Nerve agent	Dose	N	Median latency to seizure onset (min) range	Convulsing rate	Lethality rate (min after exposure)
Tabun	5 × LD_{50}	2	1.9 1.4–2.4	2/2	2/2 (3.0–4.4)
Sarin	5 × LD_{50}	2	1.8 1.5–2.1	2/2	1/2 (3.2)
Soman	5 × LD_{50}	3	1.4 1.1–1.7	3/3	3/3 (2.8–5.4)
Cyclosarin	5 × LD_{50}	2	1.3 1.2–1.4	2/2	2/2 (3.2–3.3)
VX	5 × LD_{50}	2	6.1 5.0–7.2	2/2	2/2 (8.3–9.2)

Table options ▼

Early during experimentation with the regular rats, it became clear that tabun appeared difficult to manage. The first rat given 5 x LD_{50} of tabun and treated at 1 and 5 min died 12.20 min after poisoning. The next rat given 4 x LD_{50} of tabun and treated lived for 15 min. Then it was established that the triple regimen was able to counteract 3 x LD_{50} of tabun without death shortly after exposure. However, when obidoxime was given along with HI-6, levetiracetam, and procyclidine, poisoning by 5 x LD_{50} of tabun was effectively counteracted. For this reason, rats poisoned by 3 x LD_{50} were treated with the triple regimen, whereas rats poisoned by 5 x LD_{50} of tabun were treated with a quadruple regimen (HI-6, obidoxime, levetiracetam, procyclidine).

3.2. Anticonvulsant and life-saving efficacy

When treatment was given 1 and 5 min after a nerve agent dose of 3 or 5 x LD_{50}, several rats in most groups responded with convulsions and seizure after 1-5 min (Table 2). Both the triple and quadruple regimens prevented or stopped seizures, and none of the rats displayed epileptiform activity 10 min after soman exposure

(Fig. 2). Furthermore, no incidents of epileptiform activity were observed when EEG was recorded during 10 min at 24, 48, and 144 h. In some cases when seizures started after a latency of more than 2.5 min, the convulsions appeared moderate because of the impact of the anticonvulsant drugs. The rats poisoned by VX did not convulse at all. Because of the late onset of seizures induced by VX, the treatment was able to prevent seizure activity. ANOVA did not reveal a reliable treatment effect in latency to onset of seizures among the nerve agent groups ($F (4,18) = 1.258$, $P > 0.05$). Fisher's exact test showed that the convulsing rate was significantly higher for the groups that received 3 x LD_{50} of tabun *(P = 0.0152)* or sarin and cyclosarin *(P = 0.0022)* compared with the VX group. The respiration of the rats poisoned by tabun, sarin, soman, or cyclosarin was severely depressed 1-3 min after exposure. As the drugs were gradually absorbed, the rate of respiratory movements increased and became more evident.
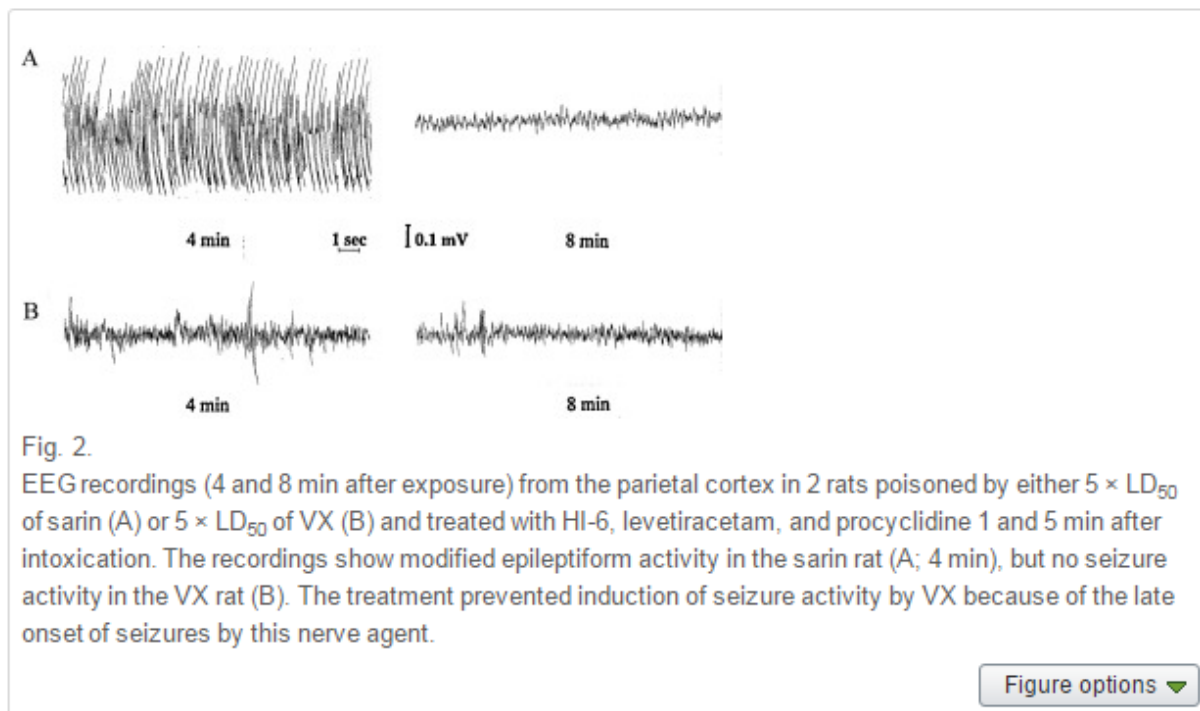
Table 2.
Anticonvulsant and life preserving effects of a triple regimen (HI-6, levetiracetam, procyclidine) or a quadruple regimen (HI-6, obidoxime, levetiracetam, procyclidine) administered 1 and 5 min after exposure to 3 or 5 × LD_{50} of various nerve agents in rats.

Nerve agent	Dose	Treatment regimen	N	Mean (±SEM) latency to seizure onset (min)	Convulsing rate	Epileptiform EEG 7– 10 min after exposure	Lethality rate 24 h after exposure
Tabun	3 × LD_{50}	Triple	6	2.8 ± 0.43	5/6[*]	0/6	2/6
Sarin	5 × LD_{50}	Triple	6	2.5 ± 0.37	6/6[**]	0/6	0/6
Soman	5 × LD_{50}	Triple	6	3.2 ± 0.08	4/6	0/6	0/6
Cyclosarin	5 × LD_{50}	Triple	6	2.4 ± 0.27	6/6[**]	0/6	0/6
VX	5 × LD_{50}	Triple	6	–	0/6	0/6	0/6
Tabun	5 × LD_{50}	Quadruple	6	1.9 ± 0.60	2/6	0/6	0/6

Significantly different from the VX group in rate of convulsing; [*]$P = 0.0152$, [**]$P = 0.002$. Two rats in the group poisoned by 3 × LD_{50} of tabun and treated with the triple regimen died 8 and 30 min after exposure.

Table options ▼

Fig. 2.

EEG recordings (4 and 8 min after exposure) from the parietal cortex in 2 rats poisoned by either 5 × LD$_{50}$ of sarin (A) or 5 × LD$_{50}$ of VX (B) and treated with HI-6, levetiracetam, and procyclidine 1 and 5 min after intoxication. The recordings show modified epileptiform activity in the sarin rat (A; 4 min), but no seizure activity in the VX rat (B). The treatment prevented induction of seizure activity by VX because of the late onset of seizures by this nerve agent.

Figure options ▼

3.3. Recovery and body weight

The course of recovery after high levels of nerve agent poisoning (3 or 5 x LD$_{50}$) was reflected in restoring the body weight for all nerve agent groups (Table 3). The rats displayed a weight loss of 5-7% during the 2 first days after exposure, but otherwise seemed almost unaffected by the supralethal intoxication. ANOVA revealed a significant treatment effect among the groups in regaining the original body weight (F (5,28) = 7.321, P = 0.0002). Group comparisons showed that the tabun 3 x LD$_{50}$ group had a reliably lower body weight than the following groups; sarin ($P < 0.01$), cyclosarin ($P < 0.05$), and VX ($P < 0.001$). Also the tabun 5 x LD$_{50}$ group had a significantly lower body weight than the sarin $(P < 0.05)$ and VX ($P < 0.01$) groups. The body weight of the soman group was reliably lower than for the VX group ($P < 0.05$). The rats were incapacitated for a brief period of time (about 10 min), but started to walk soon after. Following 3 h, they were able to rear and started to eat and drink. At 24 h, all surviving rats appeared to be in a very good condition. The rats displayed no overt signs of intoxication and remained very calm throughout the study.

Table 3.
Mean (±SEM) percent of original body weight 7 days after intoxication by different nerve agents in rats treated with a triple regimen (HI-6, levetiracetam, procyclidine) or a quadruple regimen (HI-6, obidoxime, levetiracetam, procyclidine).

Nerve agent	Dose	N	Treatment regimen	Percent of pre-exposure body weight
Tabun	$3 \times LD_{50}$	4	Triple	97.5 ± 0.87
Sarin	$5 \times LD_{50}$	6	Triple	$102.2 \pm 0.75^{*\P}$
Soman	$5 \times LD_{50}$	6	Triple	99.8 ± 0.54
Cyclosarin	$5 \times LD_{50}$	6	Triple	$101.3 \pm 0.80^{*}$
VX	$5 \times LD_{50}$	6	Triple	$103.2 \pm 1.01^{*\P}$
Tabun	$5 \times LD_{50}$	6	Quadruple	98.7 ± 0.56

Significantly different from the tabun $3 \times LD_{50}$ group ($^{*}P < 0.05$). Significantly different from the tabun $5 \times LD_{50}$ group ($^{\P}P < 0.05$).

Table options ▼

3.4. Histology

Small marks from the recording and ground screws appeared similar in both parietal cortices. Even if pharmacological treatments given 1 and 5 min after soman poisoning prevented or terminated epileptiform activity within 10 min in all rats, evident neuropathology was discovered in the index areas piriform cortex and amygdala, but less evident in the hippocampal CA1 field (Table 4, Fig. 3). Fluorescent staining was seen in all animals in 2 or 3 of the index areas. The neuronal injury was unrelated to whether the rats convulsed or not. Group comparisons only comprised the rats that received 5 x LD_{50} of nerve agents. One-way ANOVA of the total score of neuropathology revealed a reliable treatment effect ($H (5) = 17.17$, $P = 0.0018$). Group comparisons showed that both the sarin and cyclosarin groups had significantly more extensive neuropathology than the soman and VX groups ($P < 0 05$). Hemispheric differences in terms of significantly higher levels of neuronal damage in the left versus the right side occurred. This was seen in both the piriform cortex and amygdala in the tabun 3 x LD_{50} group, and in the amygdala only in the cyclosarin and VX groups ($P < 0.05$, with two-tailed U test).
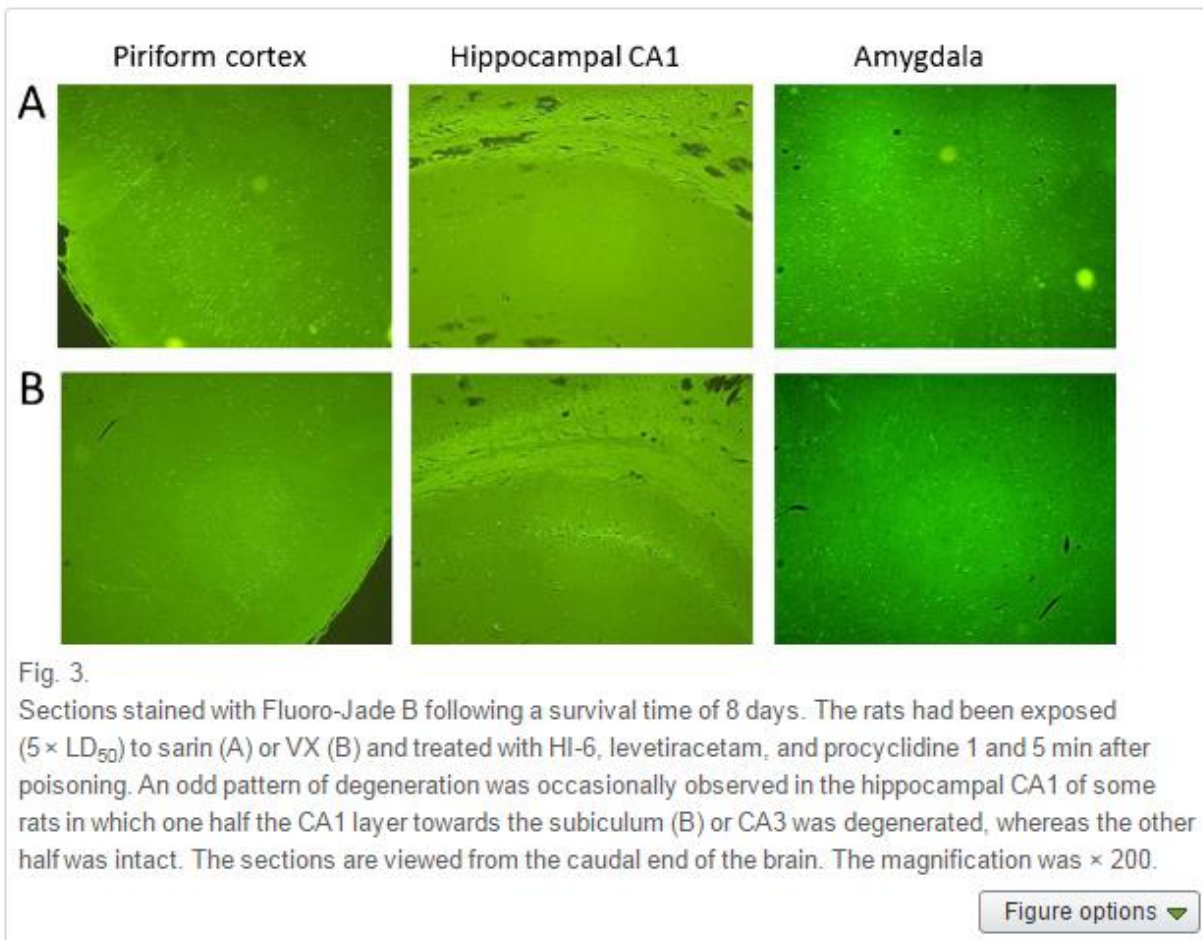
Table 4.
Median (range) neuropathology scores of rats that lived for 8 days after exposure to a given nerve agent and the treatment regimen used.

Nerve agent	Dose	N	Treatment regimen	Piriform cortex		CA1		Amygdala		Total
				Left	Right	Left	Right	Left	Right	
Tabun	$3 \times LD_{50}$	4	Triple	2.5* (1–3)	0.0 (0–0)	1.0 (1–1)	1.0 (0–3)	2.0* (1–4)	0.0 (0–0)	3.8 (2.0–5.0)
Sarin	$5 \times LD_{50}$	6	Triple	3.5 (1–4)	3.0 (0–4)	0.5 (0–1)	1.0 (0–3)	3.0 (1–4)	1.5 (1–4)	6.0¶ (4.5–8.0)
Soman	$5 \times LD_{50}$	6	Triple	1.5 (0–4)	0.5 (0–2)	0.0 (0–1)	0.0 (0–2)	2.5 (0–3)	0.5 (0–1)	2.8 (1.0–4.0)
Cyclosarin	$5 \times LD_{50}$	6	Triple	3.5 (2–4)	1.0 (0–3)	0.0 (0–4)	2.0 (0–3)	3.5* (2–4)	1.0 (0–2)	6.3¶ (4.5–9.5)
VX	$5 \times LD_{50}$	6	Triple	1.0 (0–4)	1.5 (0–4)	0.0 (0–1)	0.0 (0–4)	2.5* (0–4)	1.0 (0–1)	3.0 (1.5–4.0)
Tabun	$5 \times LD_{50}$	6	Quadruple	2.5 (1–4)	2.0 (1–3)	0.0 (0–4)	1.0 (0–4)	2.0 (0–4)	1.5 (1–3)	4.5 (3.0–8.5)

The tabun $3 \times LD_{50}$ group was not included in group comparisons. Significantly different from the soman group and VX group (¶$P < 0.05$). Significantly different from the contralateral side (*$P < 0.05$).

Table options ▼

Fig. 3.

Sections stained with Fluoro-Jade B following a survival time of 8 days. The rats had been exposed ($5 \times LD_{50}$) to sarin (A) or VX (B) and treated with HI-6, levetiracetam, and procyclidine 1 and 5 min after poisoning. An odd pattern of degeneration was occasionally observed in the hippocampal CA1 of some rats in which one half the CA1 layer towards the subiculum (B) or CA3 was degenerated, whereas the other half was intact. The sections are viewed from the caudal end of the brain. The magnification was × 200.

Figure options ▼

A peculiar lesion pattern was occasionally observed in the hippocampal CA1 layer. Half of the CA1 ending in the subiculum or the CA3 was demolished, whereas the other half was intact (Fig. 3B). This phenomenon was seen bilaterally in 1 rat in the tabun 5 x LD_{50} group, in the left side of 2 rats in the cyclosarin group, and in the right side of 1 rat in the VX group.

4. Discussion

The results from the present study showed that a triple regimen consisting of HI-6, levetiracetam, and procyclidine was able to prevent or terminate seizures and save the lives of rats when given 1 and 5 min after exposure to 5 x LD_{50} of sarin, soman, cyclosarin, or VX, but only 3 x LD_{50} of tabun was counteracted. However, when the triple regimen was made a quadruple regimen by adding obidoxime, 5 x LD_{50} of tabun was successfully managed. The rats recovered very well, and no late toxic signs were observed, but still ample neuropathology was detected, even in the VX rats that did not convulse at all. The high level of lethality of the nerve agents (5 x LD_{50}) was demonstrated in a separate set of rats in which all rats but 1died when the treatment was postponed to immediately (5 sec) after onset of seizures.

Arrhythmias in nerve agent poisoning have been demonstrated in anesthetized guinea pigs exposed to high doses (5-10 x LD_{50}) of tabun, sarin, soman, or VX. Respiratory arrest occurs 2-3 min after intoxication, followed by circulatory arrest a few minutes later in non-treated animals. Atropine restores respiration in sarin poisoning, improves it partly in soman and VX poisoning and is completely ineffective in tabun poisoning, whereas the combination of atropine plus HI-6 or HLö 7 has a reasonable therapeutic effect against soman and tabun and restores respiration almost completely in sarin and VX poisoning (Worek et al. 1995). In the present rats, the respiratory movements were hardly observable 2-3 min after intoxication by all nerve agents except VX, but gradually became more obvious in response to the pharmacological effects of the triple regimen. The addition of obidoxime made it also possible to counteract 5 x LD_{50} of tabun poisoning. The rescue potential of the triple and quadruple regimens appeared impressive and suggests that if the right antidotal medication is at hand, rapid relief can be achieved even in severe intoxication, but minutes or even seconds may count. The results showed that both sets of injections at 1 and 5 min after exposure were necessary for efficacious treatment.

The 5 nerve agents applied in the present study have been shown to exert differential effects on the AChE enzyme activity in guinea pigs at a dose of 1 x LD_{50}. All 5 nerve agents cause maximum inhibition of AChE activity in red blood cells between 5 and 10% of the control within 10 min after exposure. In whole blood, sarin, soman, and cyclosarin produce more rapid and greater inhibition than do tabun and VX. Cyclosarin is the most rapid, producing a maximum inhibition to 5% of the control in 5 min, whereas VX is slower reaching maximum inhibition to 30% of the control at 15 min (Shih et al. 2005). Even if the time scale was probably shorter for the 5 x LD_{50} doses in the present study, the differential effects of the various nerve agents seem to be reflected in the latencies to seizure onset in the rats treated after this point of time (Table 1) and also in the rats treated at 1 and 5 min after exposure. The latencies to seizure onset for tabun, sarin, soman, and cyclosarin are very short and rather long for VX.

When the doses of tabun, sarin, soman, cyclosarin, or VX have moderate lethality levels for rats (1.6 x LD_{50}) and guinea pigs (2 x LD_{50}), tabun poisoning does not appear as particularly problematic to counteract (Shih and McDonough 1999, 2000; Shih et al. 2003). However, when supralethal doses are used, tabun intoxication seems to create antidotal problems. In a study of guinea pigs, 5 x LD_{50} doses of each of the 5 classical nerve agents were given, and treatment with HI-6, physostigmine, and scopolamine was administered 1 min after exposure. All animals survived and recovered well, except for the guinea pigs in the tabun group. Their maximal body weight loss was more than 20%, and only 1 of 6 animals lived for 7 days (Wetherell et al. 2006). The survival time is considerably shorter after tabun poisoning (5 x LD_{50}) than after soman poisoning (5 x LD_{50})

in guinea pigs treated with atropine and HI-6 or atropine and HLö-7 (Worek et al. 1995). Tabun differs from other toxic organophosphates in its chemical structure and by the fact that commonly used antidotes are not able to sufficiently prevent tabun-induced toxic effects. The deleterious effects of tabun are extraordinarily difficult to antagonize because of the changes in hydrogen binding and conformational changes of AChE-tabun complex in the AChE active site that make the nucleophilic attack of oximes very difficult (Kassa et al. 2014).

Rats poisoned by $1.6 \times LD_{50}$ of soman usually develop convulsions in 4-5 min and have a weight loss of about 17% during the following 1-5 days if no anticonvulsant treatment is given. Such weight loss is due to variable periods of aphagia/adipsia (McDonough and Shih 1993). Rats treated with obidoxime, atropine, and diazepam 1 and 5 min after challenge with $2 \times LD_{50}$ of soman either died or were in a very poor health condition 24 h later. When treated with HI-6, atropine, and avizafone 1 and 5 min after challenge with $4 \times LD_{50}$ of soman, 7 rats died within 24 h and 1 rat was euthanized because of a very bad health condition (Myhrer et al. 2013a). In contrast to the results in the latter study, the rats in the present study exposed to $5 \times LD_{50}$ of the classical nerve agents recovered very well and started to eat and drink 3-4 h after poisoning. The latter finding may be associated with the ability of the treatment regimens to restore normal cholinergic input to the hypothalamus that is putatively adversely affected during soman poisoning (Myhrer 2007). The small, but significantly slower restoring of the body weight among the rats exposed to tabun (3-4%) is not readily accounted for.

Neuropathology occurred in the groups where all rats seized and had the most extensive brain damage (sarin, cyclosarin) and in the groups where only some rats seized (tabun, soman). The seizure activity, however, lasted less than 8 min. Morphological changes in all rats were also seen when none of the animals convulsed (VX). It has previously been maintained that the initial signs of neuronal damage are detectable about 20 min after onset of soman-induced seizure activity that is accompanied by glutamatergic excitotoxicity (Lallaement et al. 1994; McDonough et al. 1995). From the present results, it is apparent that the development of neuropathology can follow an alternative avenue when the degree of nerve agent intoxication is very high. The severe poisoning might have induced a long-lasting "silent" excitotoxic process. Even if no EEG abnormalities were detected by the present use of superficial cortical recordings, depth electrodes in critical structures (e.g., perirhinal cortex) might have revealed epileptiform activity. Hence, it is important that adjuvant treatment (e.g., glutamatergic antagonists, GABA modulators) is administered after the pharmacological actions of the triple or quadruple regimens have ceased in order to prevent or reduce neuronal injury. More extensive neuropathology in the left versus the right hemisphere was seen in some groups (Table 4). This phenomenon has previously been encountered and has tentatively been associated with local asymmetric excitotoxicity (Myhrer et al., 2013a).

HI-6 has evidently a life preserving action, because treatment with levetiracetam and procyclidine alone following 3 x LD_{50} of soman results in death among all rats within 14 min after exposure (Myhrer et al. 2013b). HI-6 has been reported to protect respiratory function, both centrally and peripherally during nerve agent poisoning. The oxime causes recovery of neuronal transmission in the respiratory center possibly by affecting GABAergic mechanisms and causes recovery of neuromuscular transmission in the diaphragm (van Helden et al. 1996). Levetiracetam is an antiepileptic drug that strongly enhances the anticonvulsant effects of compounds affecting either glutamatergic or GABAergic neurotransmission (Kaminski et al. 2009). Although the exact mechanisms are not well known, levetiracetam probably reduces release of glutamate by which the effects of glutamatergic antagonists are highly increased (Kaminski et al. 2009). Levetiracetam has been reported to increase GABAergic inhibitory activity (Meehan et al. 2012). Reduced releases of acetylcholine and reduced postsynaptic responsiveness in cholinergic synapses have been seen to follow administration of levetiracetam (Oliveira et al. 2005). Procyclidine has a powerful capability to antagonize a lethal dose of NMDA in mice (McDonough and Shih 1995; Raveh et al. 1999). Procyclidine inhibits the phencyclidine site at the NMDA receptor very potently (Reynolds and Miller 1988) in a concentration-dependent manner (Myhrer et al. 2004). In addition, procyclidine binds to the muscarinic receptor subtypes m1 and m2 (Waelbroeck et al. 1992). The half-life in plasma of rats for HI-6 is 24 min (Garrigue et al. 1990), for levetiracetam 150 min (Löscher et al. 1998), and for procyclidine 120 min (Jang et al. 2001). For details about actions of HI-6, levetiracetam, and procyclidine, see Myhrer and Aas (2014).

Obidoxime is the only oxime hitherto known to be able to reactivate tabun-inhibited AChE (10%) in rat brain tissue, whereas pralidoxime and HLö-7 are completely ineffective and HI-6 is almost ineffective in the reactivation process (Kuča et al. 2005). Newly developed oximes are not suitable for the replacement of obidoxime in the treatment of acute tabun poisoning (Kassa et al. 2014). A comparison of the therapeutic efficacy of obidoxime and HI-6 when each is combined with atropine and diazepam show that the protective effect of HI-6 is a little higher than for obidoxime in tabun-poisoned mice, although the difference between the protective effects of HI-6 and obidoxime is not significant. Since HI-6 is a really weak reactivator of tabun-inhibited AChE, it is suggested that the beneficial effects may originate from the secondary antidotal effects (Kassa and Vachek 2002). The effectiveness of oximes is dependent on their reactivation properties as probably reflected by the use obidoxime in the present case of tabun poisoning.

A treatment regimen consisting of HI-6, scopolamine, and physostigmine (termed the physostigmine regimen) has been shown to successfully counteract a dose of 5 x LD_{50} of sarin, soman, cyclosarin, and VX, but

not tabun when administered 1 min following exposure of guinea pigs (Wetherell et al. 2006). The latter regimen, however, has a very limited time window (< 10 min). In comparison of the antidotal effects of the physostigmine regimen and our triple regimen (HI-6, levetiracetam, procyclidine) in counteracting supralethal doses of soman (3, 4, 5 x LD_{50}) both regimens were effective when given 1 and 5 min after intoxication. When the treatments were administered 10 and 14 or 20 and 24 min after soman exposure (1.6 and 1.3 x LD_{50}, respectively), only the triple regimen was able to terminate seizures and preserve lives (Myhrer et al. 2013b). The antidotal efficacy of the triple regimen has been seen to be very good against supralethal poisoning by the classical nerve agents except tabun. The death of 2 rats in the group poisoned by 3 x LD_{50} of tabun may indicate that the triple regimen's upper limit is in the area of 3 x LD_{50} of tabun. The inclusion of obidoxime makes a quadruple regimen able to effectively counteract 5 x LD_{50} of tabun. The quadruple regimen will probably be somewhat more complex to administer due to the number of drugs. On the other hand, in lack of a single broad spectrum oxime the quadruple regimen may possess properties to meet challenges with organophosphates other than the classical nerve agents. The quadruple regimen may represent a future stand-alone therapy, since no pretreatment is necessary for a successful result.

In conclusion, the triple regimen effectively counteracted intoxication by 5 x LD_{50} of all classical nerve agents but tabun that was managed at 3 x LD_{50}. The quadruple regimen was able to successively treat poisoning by 5 x LD_{50} by tabun. The triple regimen may be more convenient to administer than the quadruple regimen. The quadruple regimen, however, will probably have a wider spectrum of action than the triple regimen. A robust medical therapy against a given classical nerve agent may require the combination of 2 oximes and 2 anticonvulsants.

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Figure legends

Fig. 1. Schematic overview of the experimental design. Because the triple regimen was only able to counteract poisoning by a tabun dose of 3 xLD_{50}, obidoxime was added to make up a quadruple regimen that effectively managed survival following a tabun dose of 5 x LD_{50}.

Fig. 2. EEG recordings (4 and 8 min after exposure) from the parietal cortex in 2 rats poisoned by either 5 x LD_{50} of sarin (A) or 5 x LD_{50} of VX (B) and treated with HI-6, levetiracetam, and procyclidine 1 and 5 min after intoxication. The recordings show modified epileptiform activity in the sarin rat (A; 4 min), but no seizure activity in the VX rat (B). The treatment prevented induction of seizure activity by VX because of the late onset of seizures by this nerve agent.

Fig. 3. Sections stained with Fluoro-Jade B following a survival time of 8 days. The rats had been exposed (5 x LD_{50}) to sarin (A) or VX (B) and treated with HI-6, levetiracetam, and procyclidine 1 and 5 min after poisoning. An odd pattern of degeneration was occasionally observed in the hippocampal CA1 of some rats in which one half the CA1 layer towards the subiculum (B) or CA3 was degenerated, whereas the other half was intact. The sections are viewed from the caudal end of the brain. The magnification was x 200.