ORGAN TOXICITY AND MECHANISMS

Treatment efficacy in a soman-poisoned guinea pig model: added value of physostigmine?

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Abstract Current treatment of organophosphate poisoning is insufficient, and survivors may suffer from long-lasting adverse effects, such as cognitive deficits and sleep-wake disturbances. In the present study, we aimed at developing a guinea pig model to investigate the benefits of immediate and delayed stand-alone therapy on the development of clinical signs, EEG, heart rate, respiration and AChE activity in blood and brain after soman poisoning. The model allowed the determination of the therapeutic effects at the short-term of obidoxime, atropine and physostigmine. Obidoxime exerted the highest therapeutic efficacy at administration of the lowest dose (3.1 mg/kg i.m.), whereas two higher doses (9 and 18 mg/kg) were less effective on most parameters. Addition of atropine at 0.03 and 3 mg/kg (i.m.) to the treatment did not improve the therapeutic effects of obidoxime alone. Physostigmine (0.8 mg/kg im) at 1 min after poisoning increased mortality. Two lower doses (0.1 and 0.3 mg/kg i.m.) showed improvements on all parameters but respiration. The middle dose was most effective in preventing seizure development and therefore assessed as the most efficacious dose. Combined treatment of obidoxime and physostigmine shortened the duration of seizures, if present, from up to 80 min to ~10–15 min. In practice, treatment will be employed when toxic signs appear, with the presence of high levels of AChE inhibition in both blood and brain. Administration of physostigmine at that moment showed to be redundant or even harmful. Therefore, treatment of OP poisoning with a carbamate, such as physostigmine, should be carefully re-evaluated.

Introduction

Organophosphates (OPs) are highly toxic chemicals, which mainly exert their toxicity by irreversible inhibition of acetylcholinesterase (AChE) and the consequent excessive and deleterious buildup of acetylcholine (Maxwell et al. 2006). Nerve agents are the most toxic OPs and might be used in military conflicts or in terrorist attacks, such as those in the Tokyo subway station in 1995 (Suzuki et al. 1995). The buildup of acetylcholine (ACh) following OP exposure in central and peripheral synapses leads to typical cholinergic signs due to overstimulation of nicotinergic and muscarinergic synapses. This overstimulation evolves into hypersecretions, failure of neuromuscular transmission, seizures, convulsions and eventually death due to respiratory failure (Shih and McDonough 1997; van Helden and Bueters 1999). In preclinical studies, the currently available treatment regimens appeared to be ineffective in preventing this, giving way to seizures that might induce brain damage and concomitant deficits in cognition and attention even when they are short-lasting (Joosen et al. 2009; Raveh et al. 2003).

Treatment is generally aimed at three targets. The first one, achieved by oxime treatment, is breaking the covalent bond of the OP from the serine residue in the active site of AChE. However, no broad-spectrum oximes are available to manage the chemistry of all nerve agents (Aas 2003;

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Maxwell et al. 2008). Moreover, none of the oximes are capable to reactivate inhibited and "aged" AChE, i.e. when the alkyl tail of the OP molecule has gone, thereby rendering the remaining bond virtually irreversible (Berman and Decker 1986). The second target of treatment is directed towards blocking muscarinic overstimulation using compounds such as atropine sulphate. Although rather effective at very high doses, atropine rapidly looses efficacy when administered at low doses or after development of full-blown seizures (Shih and McDonough 2000). The final treatment is aimed at terminating seizures by benzodiazepines in order to prevent brain injury. Recent research suggested that seizures lasting no longer than 10 min did not induce visible brain damage but rather inhibited neurogenesis resulting in impaired cognitive performance (Joosen et al. 2009). These and other findings show the need to advance understanding of the toxic as well as the antidotal mechanisms in order to obtain more effective medical countermeasures.

Therefore, the present study was aimed at investigating several features of OP poisoning and treatment. The nerve agent soman was chosen as a model compound because it is expected to have the highest risk of inducing brain damage (McDonough et al. 1999, 2000), and after binding to AChE, it ages very rapidly. This makes poisoning with soman less prone to successful treatment with any of the existing oximes, such as 2-PAM, obidoxime and HI-6. It is known that preserving or restoring AChE pools is one of the most efficacious treatments in case of nerve agent poisoning and most effective when employed very rapidly after poisoning. Although the reactivating capacity of obidoxime towards soman-inhibited AChE activity is known to be very low, this oxime was used in the present experiments for two reasons. The first reason was purely practical in that obidoxime is currently in use as an auto-injector compound. Second, the use of obidoxime would enable the identification of other targets for obidoxime efficacy than AChE reactivation alone (Maxwell et al. 2006).

Recently, post-poisoning treatment with carbamates has been proposed. For example, physostigmine, previously shown to be highly effective as pretreatment against OP poisoning, was effective against 5 LD50 soman in guinea pigs, administered 1 min post-poisoning in combination with scopolamine and HI-6 (Wetherell et al. 2006, 2007). Also galantamine showed efficacy when administered shortly after poisoning with VX or soman (Albuquerque et al. 2006; Hilmas et al. 2009).

In the present study, a soman-poisoned guinea pig model was developed to enable the investigation of the impact of different doses of soman and subsequent relief of toxic signs by different treatment protocols. The guinea pig is the most widely accepted non-primate model for predicting

treatment efficacy for nerve agent poisoning in primate species (Dirnhuber et al. 1979; Gordon et al. 1978). Our approach enabled online monitoring of vital physiological signs and off-line determination of biochemistry in terms of AChE and butyrylcholinesterase (BuChE) activity. Both physiology and biochemistry were investigated for being suitable parameters predictive for clinical outcome. The therapeutic efficacy of several treatment combinations of obidoxime, atropine sulphate and physostigmine, administered at 1 or 10 min following poisoning, was tested. The research was focused on the identification of the contributing role of physostigmine in the treatment.

Methods

Animals

Male Dunkin Hartley guinea pigs (Crl:HA) obtained from Charles River (Maastricht, The Netherlands) were used in the present study. Prior to the experiments, they were housed with two animals per cage and allowed to get accustomed to standard conditions for 1 week. Room temperature was kept at 19–22°C, and relative humidity was maintained at 55–65%, and lights were on from 7 am to 7 pm. Acidified water and standard guinea pig chow (Teklad global diet 2040, Harlan, Horst, The Netherlands) were available ad libitum. The experiments described received prior approval from the TNO Animal Ethical Committee.

Chemicals

Hypnorm® (fentanyl/fluanisone) was purchased from Janssen Pharmaceutica (Beerse, Belgium), and Dormicum® (midazolam) was delivered by Roche Nederland BV (Mijdrecht, The Netherlands). Soman (O-Pinacolyl methylphosphonofluoridate) was obtained from the stocks of TNO Defence, Security and Safety and of purity ≥98% (GC). The other chemicals used were of standard purity and purchased from renowned companies.

Study design

The design of the study is presented in Fig. 1. After obtaining baseline values of the indicated parameters for 30 min, the guinea pigs were poisoned with soman (30; 37 or 44 µg/kg s.c.; corresponding to ∼1.2; 1.5 and 1.8 24 h LD50, respectively, (Gordon and Leadbeater 1977), followed by i.m. treatment 1 or 10 min later. The experiment was divided in four parts: establishment of a model dose for soman; efficacy of conventional treatment by obidoxime and atropine; efficacy of physostigmine; and the
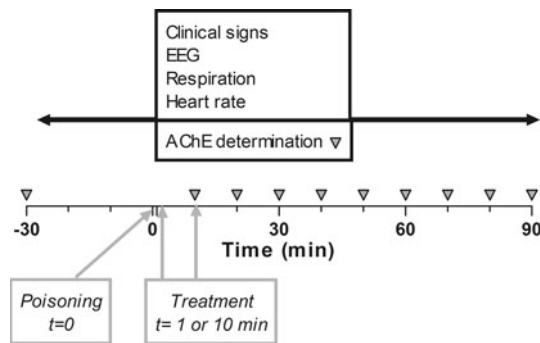
Fig. 1 Overview of the experimental design. Before and after poisoning, EEG, ECG, respiration and clinical signs were continuously monitored. Every 10 min after poisoning, blood was drawn from the ear vein to determine blood ChE activity

efficacy of postponed treatment (Table 1). A control group was injected with saline (s.c.) followed by an i.m. saline injection. At the indicated time points (∇), blood samples were drawn from the ear vein to assess AChE activity. Following poisoning, the guinea pigs were observed for toxic cholinergic signs such as chewing, tremor, salivation

and convulsions. Clear chewing movements involving the entire head were scored as chewing, often accompanied by extensive drooling (salivation) later on. Involuntary shaking of parts of the body or head was scored as tremor. Convulsions were defined as involuntary movements of the entire body, while the animal looks dissociated from its environment. At the end of the experiment, 90 min after poisoning, different brain parts (hippocampus, striatum and medulla oblongata) were isolated for AChE level determination.

Surgical procedures

Guinea pigs were anaesthetized with 6 ml/kg FFM-mix (1.25 mg/ml midazolam, 2.5 mg/ml fluanisone, 0.079 mg/ml fentanyl citrate) via a single i.p. injection. Premedication with 0.05 mg/kg atropine sulphate (s.c.) was administered to prevent respiratory arrest. Two stainless steel screws (A8.0 and P1.2 mm relative to bregma and 1 mm from the sagittal suture) placed at the dura mater, fixed to a plug, served as EEG electrodes. To obtain an ECG signal, two leads sutured in the superficial muscles under the skin just

Table 1 Overview of experimental groups

Experiment	Exposure	Treatment				Signs					
	Soman (µg/kg s.c.)	Obidoxime (mg/kg i.m.)	Physostigmine (mg/kg i.m.)	Atropine sulphate (mg/kg i.m.)	Time of treatment (min after soman)	Seizures		Convulsions		90-min survival	
						(n)	%	(n)	%	(n)	%
Model	0	–	–	–	–	0/4	0	0/4	0	4/4	100
	30	–	–	–	1	6/6	100	6/6	100	5/6	83
	37	–	–	–	1	6/6	100	6/6	100	3/6	50
	44	–	–	–	1	6/6	100	6/6	100	1/6	17
Conventional	30	3.1	–	–	1	4/6	67	2/6	33	6/6	100
	30	9	–	–	1	5/6	83	4/6	67	6/6	100
	30	18	–	–	1	5/6	83	4/6	67	6/6	100
	30	–	–	3	1	5/6	83	2/6	33	6/6	100
	30	3.1	–	0.03	1	5/5	100	4/5	80	5/5	100
	30	3.1	–	3	1	4/5	80	1/5	20	4/5	80
	44	3.1	–	3	1	6/6	100	6/6	100	5/6	83
Physostigmine	30	–	0.1	–	1	4/4	100	1/4	25	4/4	100
	30	–	0.3	–	1	2/6	33	2/6	33	6/6	100
	30	–	0.8	–	1	1/2	50	2/2	100	0/2	0
	30	3.1	0.1	–	1	3/5	60	1/5	20	5/5	100
	30	3.1	0.3	–	1	2/6	33	1/6	17	6/6	100
	30	3.1	0.8	–	1	4/4	100	4/4	100	2/4	50
	30	3.1	0.3	3	1	1/6	17	0/6	0	6/6	100
Postponed	30	3.1	0.3	–	10	4/6	67	3/6	50	2/6	33
	30	3.1	0.3	3	10	6/6	100	6/6	100	5/6	83
	30	3.1	–	3	10	6/7	86	5/7	71	7/7	100

The left column refers to the corresponding results section. Time and dose of treatments applied, occurrence of seizures and convulsions, and survival rate are shown from left to right

below the right collarbone and between the second and third rib (Lead II configuration) and also fixed to the plug. The plug and the electrodes were fixed to the skull with dental cement.

Acquisition and analysis of EEG and respiration

Before and after poisoning, the animals were placed in a whole-body plethysmograph (Buxco sytems XA, USA) cage to monitor their respiration characteristics. Among other parameters, P-enhanced (Penh), an arbitrary value for bronchoconstriction, and respiratory minute volume were calculated from the signal using IOX software (EMKA technologies, France).

EEG signals were obtained using the PhysioTel telemetry system from Data Sciences Inc. (DSI) using the ML2-11-EET-F40 transmitter. The transmitter was attached to the plug fixed on the guinea pig's skull. A receiver board measured the signal from the transmitter, which was consolidated and stored on an IBM-compatible personal computer via a Data Exchange matrix at 100-Hz sampling frequency. By visual inspection of the EEG, the starting and ending of seizures, defined as amplitudes increasing three times over baseline, were scored. Additionally, FFT analysis using 10-s epochs was performed to calculate the total power in the spectrum, thereby serving as a semi-quantitative measure of seizure activity. Animals were awake during the measurement.

Enzyme activity

The brain parts were homogenized (900 rpm, 10% w/v homogenate) in ice-cold TENT buffer, which consisted of 50 mM Tris, 5 mM EDTA, 1 M NaCl and 1% v/v Triton X-100, pH 7.4. The homogenates were centrifuged at $12,000g$ in an Eppendorf centrifuge at 4°C, and supernatants were immediately frozen in liquid nitrogen and stored at −20°C until analysis of enzyme activity. Blood samples drawn from the ear vein were diluted 10 times in 1% saponin in MQ, frozen in liquid nitrogen and stored at −20°C until analysis.

Tissue supernatants were analysed for AChE activity using the method described by Ellman et al. (1961), modified for analysis in a 96-well plate reader. Shortly, samples were diluted in 0.8 mM DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid)). To 100 μl of diluted sample, 100 μl of 0.8 mM β-methylacetylthiocholine iodide was added, in quadruple. For blood samples, 0.8 mM of butyrylthiocholine (BuSChI) was added to separate portions of 100 μl. The delta OD per min at 415 nm at ambient temperature served as measurement for ChE activity.

Guinea pig AChE does not react with BuSChI, rendering the BuChE activity determined an appropriate representation of BuChE activity. Brain samples showed no reactivity towards BuSChI. However, AChE activity in blood had to be corrected for cross-reactivity of β-methylacetylthiocholine with BuChE. The maximum velocity turnover of β-methylacetylthiocholine by BuChE was 47% of that of BuSChI, calculated from separate experiments in which isolated guinea pig AChE and plasma BuChE were incubated with both substrates. Therefore, 47% of the extinction measured with BuSChI as substrate was subtracted from the extinction measured with β-methylacetylthiocholine, which reacts at a similar rate with AChE and BuChE. A similar adaptation has been described by Bosgra et al. (2009). Enzyme activity in the tissue supernatants was normalized versus the average enzyme activity of six control animals. AChE and BuChE activities in blood were normalized versus the baseline sample.

Data presentation and statistical analysis

All data were analysed using one-way ANOVA or MANOVA followed by Dunnett's or Tukey's post hoc test using soman or saline as control animals using SPSS statistical software. Results were considered significant for $P < 0.05$.

For determination of continuous relationships between blood AChE activity and occurrence of clinical signs, logistic regression analysis was performed. AChE activity of similarly treated animals was coupled to presence of a sign (1) or absence of sign (0). To analyse the effects of soman, animals injected with soman only were included in the analysis ($n = 18$). To analyse the effect of separate treatments, animals were grouped into soman-poisoned animals treated with obidoxime alone, physostigmine alone, atropine alone or the combinations thereof administered at 1 min after poisoning. Using logistic regression analysis, probability for each level of AChE activity was calculated.

Results

Soman-poisoned guinea pig model

In the first set of experiments, various soman doses (30, 37 and 44 μg/kg) were tested for their induction of clinical signs and effects on physiology and biochemistry. A dose-dependent effect on survival was found during the 90-min experimental period (See Fig. 2; Table 1). Apart from survival rate, no differences regarding the appearance of other clinical signs were found between the different soman doses, albeit that the onset times of clinical signs, if present, were significantly shorter after poisoning with 44 μg/kg of soman (s.c.) compared to the lower doses (Fig. 2).
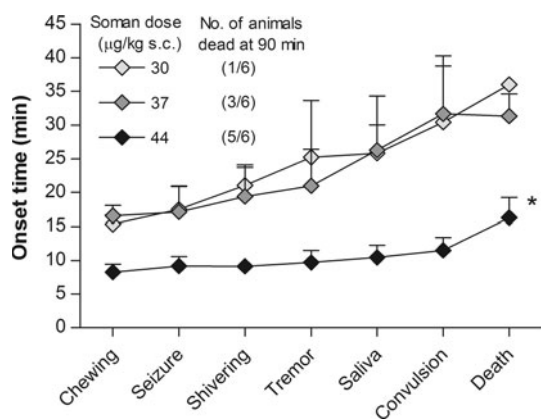
Fig. 2 Average onset times of clinical signs after different doses of soman. Results are presented as means ± SEM. Onset times of clinical signs in animals poisoned with 44 μg/kg soman were significantly shorter than in animals poisoned with 30 or 37 μg/kg (one-way ANOVA, within factor signs and between factor dose, $P < 0.05$ followed by Tukey's post hoc test,*, $P < 0.001$)

Cholinesterase activity in different brain regions (AChE) at 90 min and in blood (AChE and BuChE) at 10 min after poisoning was dose dependently inhibited by soman (MANOVA, $P < 0.001$). However, there were no significant differences in inhibition between the different doses [Tukey HSD post hoc test $P > 0.05$ (Table 2)].

Regardless the dose, all animals poisoned with soman developed seizures within 5–30 min, albeit that animals poisoned with the highest dose tended to develop full-blown seizures more rapidly (Fig. 3a). Seizure development is characterized by an increase in total power of the fast Fourier transformation of the spectrum of the EEG signal (FFT) compared to seizure-free EEG. In contrast to animals poisoned with 30 or 37 μg/kg, which merely survived the 90-min observation period, animals poisoned

Table 2 Relative residual ChE activity in blood at 10 min and in different brain areas at 90 min post-poisoning

% ChE activity in blood and brain parts	Soman dose (μg/kg s.c.)		
	30	37	44
Blood			
AChE	0.9 ± 1.2	3.9 ± 2.8	11.0 ± 16.1
BuChe	3.5 ± 0.6	8.4 ± 1.5	28.6 ± 7.4
Brain			
Medulla oblongata	23.9 ± 3.3	19.7 ± 6.0	11.2 ± 5.2
Striatum	13.1 ± 2.6	12.8 ± 5.7	9.1 ± 1.5
Hippocampus	10.0 ± 3.4	4.7 ± 2.4	2.0 ± 1.1

High levels of inhibition of AChE and BuChE are achieved in blood and in brain. Enzyme activity was significantly lower than that of *unpoisoned* control animals (100% activity), and no difference between soman doses was found (MANOVA followed by Tukey HSD post hoc test)

with 44 μg/kg died rapidly after onset of seizures. In surviving animals, seizures continued with similar intensity over the entire 90-min observation period (Fig. 3a).

Additionally, heart rate and respiratory parameters were measured as main vital parameters. There were no obvious and consistent effects of soman on heart rate (not shown), whereas effects on respiration parameters were clearly present. After soman poisoning, a clear and significant increase in respiratory minute volume (RMV) compared to control animals was present (Fig. 3b). Soman poisoning also resulted in an increase in bronchoconstriction, which was, however, not significant (not shown). For these physiological parameters, no dose–response effect of soman was observed.

Efficacy of conventional therapy

In animals poisoned with 30 μg/kg s.c. soman, components of the treatment currently in use were tested. The effects of different doses and combinations of obidoxime (3.1, 9 and 18 mg/kg) and atropine (0.03 and 3 mg/kg) were tested. None of the obidoxime doses tested induced a significant reactivation of AChE or BuChE in blood at any time point (not shown). Only a dose of 3.1 mg/kg obidoxime had induced a significant increase in AChE activity in the medulla oblongata at 90 min after poisoning (Fig. 4a).

Analysis of the occurrence of clinical signs (Table 1) showed that only the lowest dose of obidoxime (3.1 mg/kg) prevented the development of convulsions in 67% of animals. Although convulsions were prevented in a high number of animals, seizures were prevented in a low number of animals (33%). Addition of either 0.03 or 3 mg/kg atropine to the treatment did not further improve this. None of these treatments had a significant effect on the onset time of clinical signs, although the increase in EEG power over time (Fig. 5a) showed some delay in seizure onset. Only the highest dose of atropine (3 mg/kg), either with or without obidoxime, prevented the development of salivation, in contrast to the autoinjector-equivalent dose of 0.03 mg/kg.

Regarding the respiration parameters, the lowest dose of obidoxime (3.1 mg/kg) showed a slight and statistically insignificant improvement of respiratory minute volume (Fig. 6a). Addition of atropine to the treatment with obidoxime showed some positive but insignificant effect on RMV (Fig. 6a), whereas atropine alone did not show any improvement at all. In contrast to no treatment, treatment with the highest dose of obidoxime induced a clear effect on heart rate. A pressor effect directly after injection of obidoxime (i.m.) was followed by a depressant effect at ~45 min after injection (not shown).

Fig. 3 **a** Effects of different doses of soman on total power in EEG. **b** respiratory minute volume (RMV). Results are presented as mean ± SEM. No significant differences were observed between the different soman doses
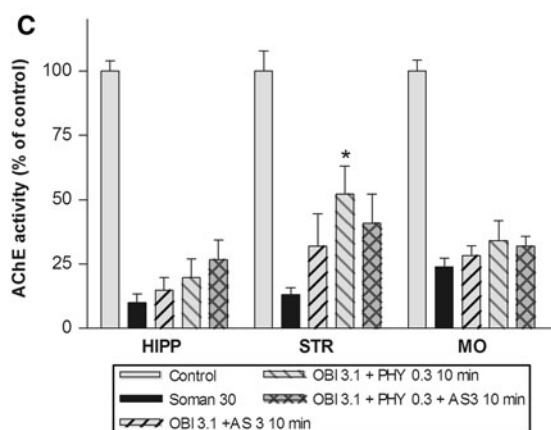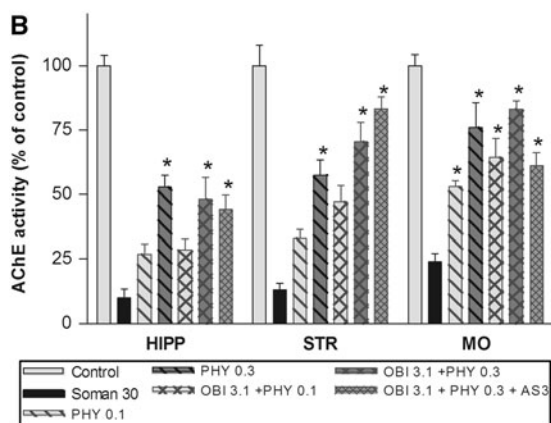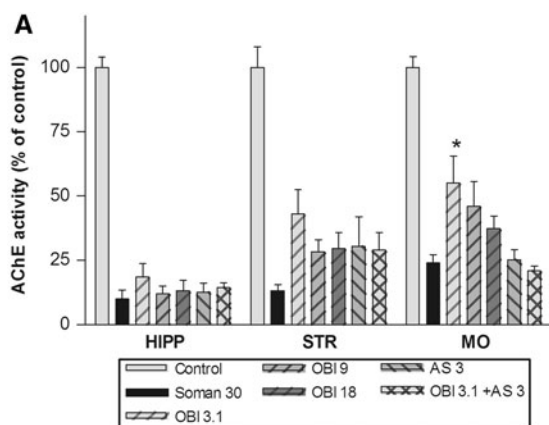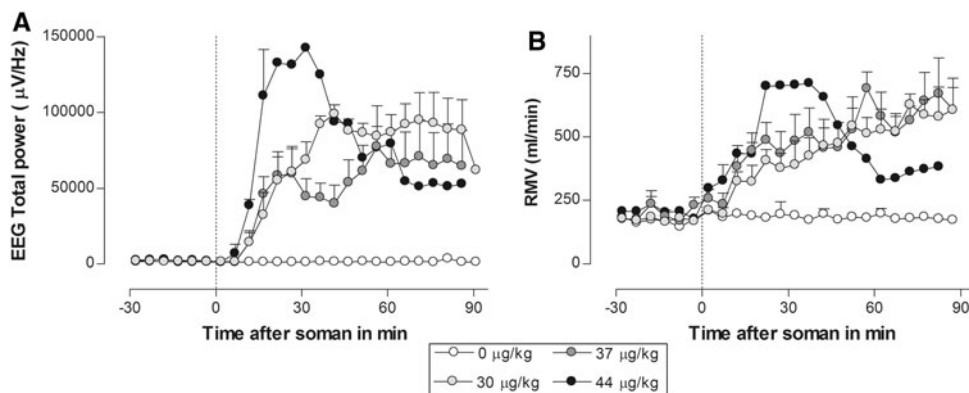

◀ Fig. 4 **a** AChE activity in hippocampus, striatum and medulla oblongata (HIPP, STR and MO) 90 min after poisoning with soman and subsequent treatment. The effect of obidoxime (3.1, 9 and 18 mg/kg) and atropine (3 mg/kg) 1 min after poisoning with 30 μg/kg soman. **b** Efficacy of addition of physostigmine (0.1 and 0.8 mg/kg). **c** the effects of treatments postponed to 10 min. Results are presented as mean ± SEM and analysed by MANOVA followed by Tukey's HSD post hoc test. *indicates significant ($P < 0.05$) compared to soman alone

To investigate the extent of efficacy against a higher dose of soman, the optimal obidoxime treatment (3.1 mg/kg) combined with atropine sulphate was tested against 44 μg/kg of soman s.c. Although this treatment promoted survival, none of the other physiological effects, such as convulsions or seizure development and respiratory effects, were prevented (data not shown).

Efficacy of physostigmine

The therapeutic efficacy of physostigmine as immediate treatment in case of organophosphate poisoning was tested in three different doses: 0.1; 0.3 and 0.8 mg/kg i.m., administered at 1 min after soman poisoning. In particular, the middle dose (0.3 mg/kg) showed a major improvement on most parameters. Only a small proportion of the animals treated with this dose of physostigmine showed the development of seizures and convulsions (2/6 and 1/6, respectively, see Table 1). Additionally, soman-induced effects on respiration were prevented in animals treated with 0.3 mg/kg of physostigmine, but not by the other doses (Fig. 6b). This was accompanied by high residual AChE activity in blood and brain regions (Fig. 4b). Treatment with the highest dose of physostigmine, 0.8 mg/kg, accelerated the development of clinical signs. In addition to an increase in RMV, these animals showed high levels of bronchoconstriction. In contrast to untreated animals, these animals died within 30–45 min after poisoning, in spite of residual AChE activity similar to that of animals treated with 0.3 mg/kg. The lowest dose of physostigmine, 0.1 mg/kg, prevented the development of convulsions in
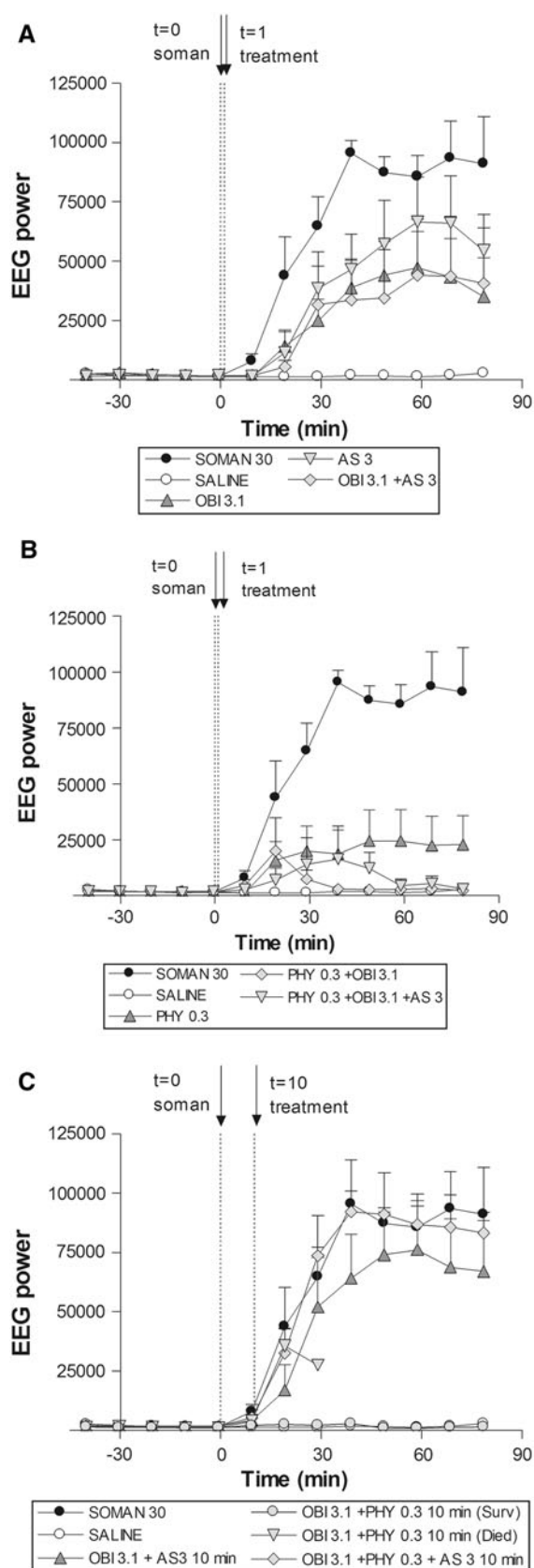
Fig. 5 **a** Treatment effect on total power in EEG before and after soman poisoning. Results are presented as 10-min averages ± SEM. The effect of obidoxime (3.1 mg/kg) and atropine (3 mg/kg) administered at 1 min after soman poisoning. **b** The effect of physostigmine (0.3 mg/kg) alone or as an addition to obidoxime and/or atropine. **c** The effect of similar treatments administered at 10 min after poisoning

five out of six animals, but not seizures. Residual AChE activity in the brains of these animals showed to be lower than in the animals treated with other physostigmine doses (Fig. 4b). In particular, AChE activity showed to be preserved by physostigmine, and no residual BuChE activity was detected in blood.

When different physostigmine doses were combined with the most efficacious dose of obidoxime (3.1 mg/kg), again the dose of 0.3 mg/kg physostigmine showed the highest efficacy. Seizure development was prevented in most animals, and if present, they only lasted for a maximum of 15 min (Fig. 5b). The highest dose of physostigmine (0.8 mg/kg) combined with obidoxime also aggravated signs, including survival (3/6 animals died within 90 min), but the effects were less deleterious compared to physostigmine (0.8 mg/kg) treatment alone. Physostigmine 0.1 mg/kg combined with obidoxime showed some efficacy on residual enzyme activity and accordingly on convulsions, seizures and respiration. In contrast to physostigmine alone, seizures were prevented in two out of six animals. Residual enzyme activity was not different between animals treated with physostigmine alone and physostigmine combined with obidoxime (Fig. 4b).

The addition of atropine (3 mg/kg) to the most efficacious physostigmine/obidoxime combination showed no improvements compared to the combination without atropine (Fig. 5b). One animal developed seizures for a short period of ∼30 min, which was similar to the result shown in the group of animals treated with physostigmine and obidoxime. In all animals, RMV increase and bronchoconstriction were prevented, but to a similar extent as in animals treated with the combination of physostigmine and obidoxime (Fig. 6b).

Efficacy of postponed treatment

As in practice, treatment will be postponed until overt signs are present; we also administered the most efficacious combination of obidoxime (3.1 mg/kg) and atropine (3 mg/kg) at 10 min after poisoning with 30 μg/kg soman, when toxic signs were already visible. These experiments showed that in that case, the therapeutic efficacy of the combination nearly completely lost its effectiveness on all
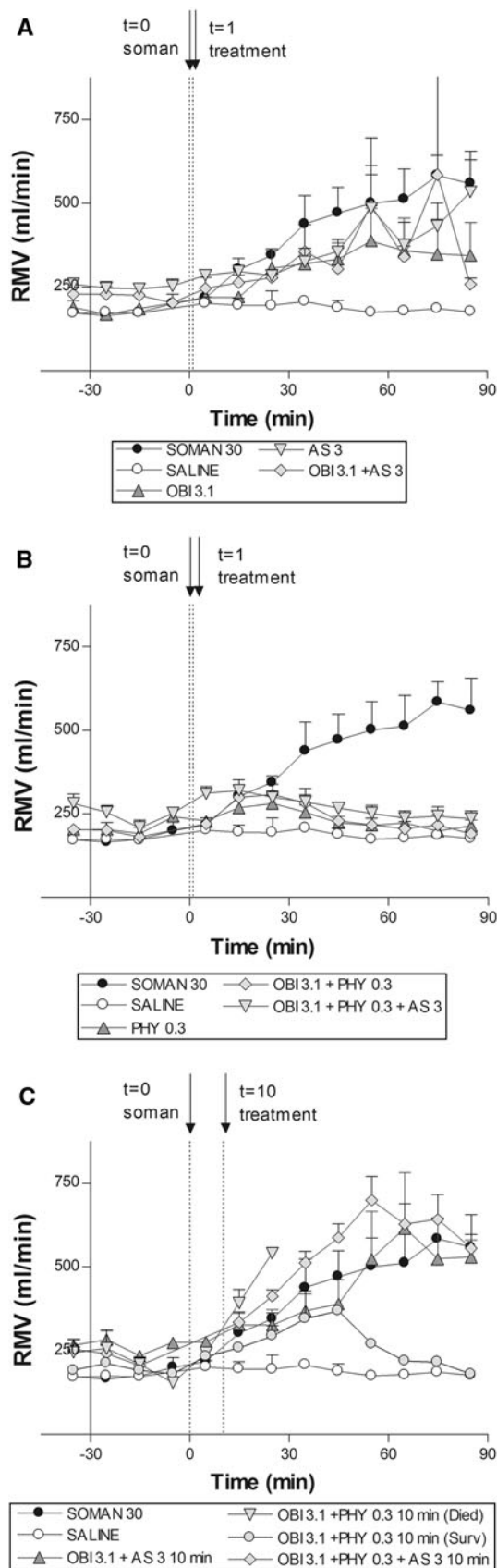
◀ **Fig. 6 a** Treatment effect on respiratory minute volume (RMV) before and after soman poisoning. Results are presented as 10-min averages ± SEM. The effect of obidoxime (3.1 mg/kg) and atropine (3 mg/kg) administered at 1 min after soman poisoning. **b** The effect of physostigmine (0.3 mg/kg) alone or as an addition to obidoxime and/or atropine. **c** The effect of similar treatments administered at 10 min after poisoning

parameters. Salivation was prevented in all animals, but RMV increase (Fig. 6c) and seizure development were not affected (Fig. 5c). In two out of six animals, convulsions were prevented (Table 1), whereas the onset time of convulsions in the remaining animals tended to be later ($P > 0.05$).

The combination physostigmine (0.3 mg/kg) and obidoxime (3.1 mg/kg), showing outstanding efficacy when administered at 1 min after poisoning, showed full efficacy in two out of six animals, but in four out of six animals, lethality was accelerated when the combination was administered at 10 min.

The full combination of obidoxime, atropine and physostigmine also completely lost efficacy, in that seizure development (Fig. 5c) and increase in RMV (Fig. 6c) were similar to that of untreated animals. Accordingly, no improvements in residual AChE activity were found in the different brain regions, except the physostigmine–obidoxime combination, which showed some improvement in the striatum (Fig. 4c).

Discussion

In the present experiments, a guinea pig model was developed to study soman poisoning and therapeutic efficacy of immediate and postponed treatment. The guinea pig soman model doses showed to be suitable for investigating different levels of therapeutic efficacy. First of all, the lowest dose, 30 μg/kg, showed to be most suitable to investigate therapeutic effects on physiology, whereas the highest dose allowed the assessment of efficacy regarding survival. Seizure development, accompanied by increased RMV and to a lesser extent to an increase of bronchoconstriction, was present in all animals at all soman doses. The increase in RMV was primarily mediated by an increase in tidal volume, rather than by increased respiratory frequency. Although this finding was unexpected, a pronounced involvement of, in particular, the M1 and M3 mAChRs in the control of respiration might explain the increased tidal volume and RMV observed after soman exposure (Boudinot et al. 2008). In addition, bronchorrhea and bronchoconstriction mediate lower pO_2 levels in blood, thereby stimulating chemoreceptors in the respiratory centres (Wiener and Hoffman 2004). Bradycardia expected

to be present due to cholinergic overstimulation was absent, probably due to a sympathetic response. In contrast with AChE activity in blood at 10 min post-poisoning, a soman dose–response effect on AChE activity was found in all brain regions analysed, resulting in 80–90% inhibition at 90 min post-poisoning. Similar results were reported by Shih et al. (2005), who investigated the differential inhibition of various nerve agent types in several organs.

AChE activity in blood mainly serves as biomarker for exposure and not for toxicity (Lotti 1995). It was shown previously that AChE activity in blood is a bad predictor for cholinergic clinical signs due to acute OP poisoning (Bueters et al. 2003). Logistic regression analysis of the present results, using lower doses of OP compared to that study, revealed the inhibition of AChE in blood to be a good predictor for the probability of chewing, shivering, seizures, convulsions and tremor, but not for respiratory distress and death (Fig. 7a). This shows that seizures are accompanied and not necessarily preceded by mild clinical signs. It also shows that poisoning with lower doses does not ameliorate the chance of development of severe clinical signs. The most severe sign well predicted by blood AChE
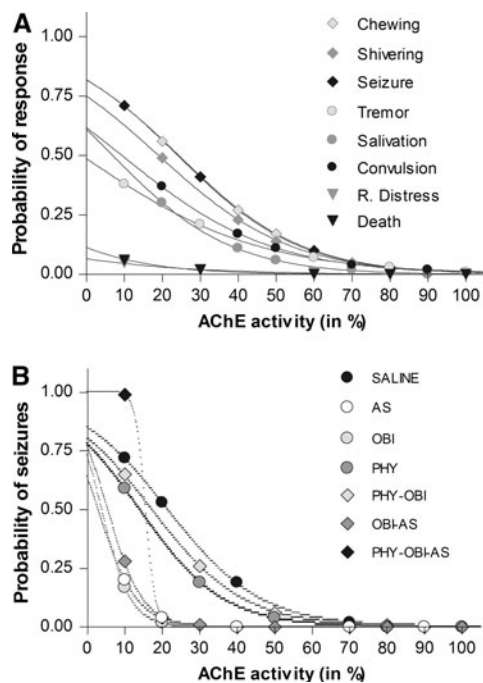

Fig. 7 Continuous relationships of the occurrence of soman-induced signs obtained with logistic regression analysis. **a** The relationship between AChE activity in blood and clinical signs of animals poisoned with three doses of soman but no treatment ($n = 18$). **b** The relationship between the probability of seizures and the residual AChE of animals poisoned with 30 µg/kg soman treated at 1 min post-poisoning with single drugs or their combinations ($n = 6$–18 per treatment)

activity was seizure development, mostly held responsible for adverse effects at the long term. The toxicokinetics of soman are not linear, higher doses lead to relatively much higher AUC and less rapid clearance than following lower doses (Van der Schans et al. 2008). This implicates that at higher doses, binding sites will more rapidly be saturated, leading to a more rapid development of clinical signs. In contrast, at lower doses, as used in the present study, clearance is higher, leading to a more gradual and slower pattern of poisoning, and AChE activity in blood might more accurately reflect the levels of neuronal AChE activity, resulting in a better prediction of development of clinical signs.

These findings underscore that protection of AChE activity is a primary target for treatment in case of OP exposure. Only at the lowest dose of 3.1 mg/kg and not at both higher doses of 9 and 18 mg/kg, obidoxime showed significant reactivation in the medulla oblongata, but not in blood or other brain parts. The efficacy of the lowest dose of obidoxime on the development of convulsions, seizures and respiratory deterioration might be explained by the higher level of AChE activity found in the medulla oblongata. It might be speculated that some reactivation of soman-inhibited AChE occurs in the first minutes after the administration of obidoxime, before our first measuring point and ageing of soman-inhibited AChE. The higher number of binding sites for soman in blood probably reduced the soman load that entered the brain, causing less inhibition in the medulla oblongata. The decline of therapeutic efficacy at higher doses of obidoxime might be ascribed to the inhibitory actions that phosphonylated obidoxime exerts on AChE (Harvey et al. 1986; van Helden et al. 1994). The slight delay of complete inhibition of AChE in the blood and probably also in the brain by obidoxime might explain its therapeutic efficacy, as only little amounts of active AChE in the brain are sufficient to improve the aforementioned parameters (Bueters et al. 2003). In addition, suspected direct pharmacological actions of obidoxime might facilitate adaptive processes (van Helden et al. 1994, 1996). Obidoxime is capable of increasing ACh release two- to threefold in the absence of AChE inhibitors, which can contribute to accelerated adaptation of cholinergic synapses to increased ACh levels and thereby to protection against the toxic load of soman (Oydvin et al. 2005). Second, obidoxime is known to allosterically modulate the mAChR2 receptor (Gnagey and Ellis 1996; Grossmuller et al. 2006; Kloog et al. 1985), which is involved in cholinergic feedback mechanisms (Segal 1989).

Addition of atropine sulphate to obidoxime in exactly one autoinjector equivalent (0.03 mg/kg) had no added value at all. A higher dose of atropine, 3 mg/kg, a dose similar to that often used in OP-research, revealed some protection, in

particular against extensive salivation and convulsions, and to a lesser extent to seizure development and respiration. In contrast to low autoinjector-equivalent doses, only such higher doses showed to be an effective support for anti-convulsant treatments (Shih et al. 2007). In addition, we show that the combined treatment with atropine and obidoxime enhances survival in case of poisoning with 44 µg/kg of soman. Other researchers have showed higher efficacy using relatively high doses of the much more potent mAChR antagonist scopolamine. It might therefore be advisable to replace atropine by scopolamine, although extrapolation of the dose from animal research to the human situation remains difficult (Wetherell et al. 2007).

Physostigmine was previously proposed as alternative immediate treatment against 5 LD50 of soman (Wetherell et al. 2006, 2007). In case of poisoning with soman, physostigmine, at a similar dose as used in our study, was rather effective, and its efficacy increased when high doses of HI-6 were used, which was ascribed to the competition with incoming soman for binding sites (Wetherell et al. 2007). In the present study, the therapeutic window of physostigmine appeared to be very small. We showed that 0.1 mg/kg physostigmine provided some protection when administered at 1 min after poisoning and that 0.3 mg/kg showed to be most effective, but 0.8 mg/kg appeared to aggravate clinical signs and to diminish survival. Residual AChE activity was measured using a modified Ellman reaction, revealing the AChE fraction that was inhibited by physostigmine in vivo. During sample preparation, physostigmine-inhibited AChE was likely to fully decarbamylate because of its short half-life of approximately 32 min in vitro (Wetherell and French 1991). Therefore, the inhibited fraction most likely reflects the portion of AChE inhibited by soman, because without treatment, the dose of soman used was sufficient to fully inhibit AChE activity in blood. Measurements of residual AChE activity showed that the lowest dose (0.1 mg/kg) provided the lowest protection of AChE by physostigmine, whereas the higher doses 0.3 and 0.8 mg/kg inhibited a similar fraction of AChE (∼50% of activity). These findings indicate that the small therapeutic window is probably due to the carbamylation rate of physostigmine, being too low at a dose of 0.1 mg/kg, whereas the carbamylation rate of physostigmine exceeded decarbamylation at a dose of 0.8 mg/kg. Only at the middle dose tested, 0.3 mg/kg, carbamylation and decarbamylation were balanced leading to protection of sufficient amounts of AChE to exert protection. Although the measurements of residual AChE activity in animals receiving postponed treatment with physostigmine did not reveal a significant decline or protection of AChE activity, our results show that postponed treatment with a dose of 0.3 mg/kg physostigmine clearly led to a lower survival rate and loss of therapeutic efficacy in these animals. These results indicate that postponed treatment with physostigmine induces an additional increase in AChE inhibition in blood and brain to that already brought about by soman. This implies that administration of physostigmine can only be protective when a considerable fraction of AChE is not yet occupied by soman, which was accomplished by the 1-min treatment.

To compare all treatments for their mechanism of action, logistic regression analysis was performed to estimate the probability of seizure development at certain levels of blood AChE activity in animals receiving different treatments after soman poisoning (30 µg/kg) (Fig. 7b). This analysis showed that when atropine or obidoxime or their combination was used as a treatment, the seizure probability dramatically decreased at similar AChE activity, confirming the mechanism of atropine and obidoxime in this study to be largely independent from AChE reactivation. However, when physostigmine treatment without atropine was employed, seizure probability at similar AChE activity remained comparable to that in untreated animals. This indicates that the efficacy of physostigmine is probably only due to its ability to prevent soman from binding to AChE, thereby reserving a decarbamylating pool of AChE while the OP is degraded, and is not due to suspected effects on nicotinic receptors (Harris et al. 1990).

In conclusion, the present results show that the combination atropine and obidoxime is hardly effective against soman poisoning, even at a fairly low toxic load as used in the present study. However, for nerve agents that are more prone to reactivation than soman, this regimen is expected to show higher efficacy. Although physostigmine significantly improved therapeutic outcomes, there might be some drawbacks to adding physostigmine to the current autoinjector components. In practice, treatment will only be administered at appearance of toxic signs, indicating inhibition of the major fraction AChE by nerve agent. In particular, at high levels of nerve agent poisoning, carbamates will fully inhibit the small remaining pool of AChE, thereby further pushing cholinergic overstimulation and worsen the clinical condition. Treatment of OP poisoning with a carbamate, such as physostigmine, should therefore carefully be re-evaluated.

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