Towards improved models for treatment of organophosphate poisoning

Joosen, M.J.A.

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Chapter 4

Percutaneous exposure to the nerve agent VX: efficacy of combined atropine, obidoxime and diazepam treatment

Marloes J.A. Joosen, Marcel J. van der Schans and Herman P.M. van Helden

TNO Defence, Security and Safety, BU CBRN Protection, Rijswijk, The Netherlands

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Abstract

The nerve agent VX is most likely to enter the body via liquid contamination of the skin. After percutaneous exposure, the slow uptake into the blood, and its slow elimination result in toxic levels in plasma for a period of several hours. Consequently, this has implications for the development of toxic signs and for treatment onset. In the present study, clinical signs, toxicokinetics and effects on respiration, electroencephalogram and heart rate were investigated in hairless guinea pigs after percutaneous exposure to 500 µg/kg VX.

We found that full inhibition of AChE and partial inhibition of BuChE in blood were accompanied by the onset of clinical signs, reflected by a decline in respiratory minute volume, bronchoconstriction and a decrease in heart rate. Furthermore, we investigated the therapeutic efficacy of a single dose of atropine, obidoxime and diazepam, administered at appearance of first clinical signs, versus that of repetitive dosing of these drugs on the reappearance of signs. A single shot treatment extended the period to detrimental physiological decline and death for several hours, whereas repetitive administration remained effective as long as treatment was continued. In conclusion, percutaneous VX poisoning showed to be effectively treatable when diagnosed on time and when continued over the entire period of time during which VX, in case of ineffective decontamination, penetrates the skin.

1 Introduction

Organophosphorous compounds (OPs) are potent irreversible acetylcholinesterase (AChE) inhibitors and widely used as insecticides. The most toxic OPs might be used as chemical warfare agents. Low volatility nerve agents like VX (O-ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate) are likely to enter the body via the skin rather than via the respiratory route. Effective treatment of skin intoxications is difficult because of unpredictable toxicokinetics (van der Schans et al. 2003; van der Schans et al. 2008).

Previous studies demonstrated that after percutaneous exposure of anesthetized hairless guinea pigs to VX, maximum blood levels of VX were not reached until several hours after exposure, followed by a slow elimination (van der Schans et al. 2003). The variability in toxicokinetics has shown to lead to a delayed and variable onset of toxic signs (Joosen et al. 2008; Wetherell et al. 2007). In addition, effective decontamination is hampered by the unawareness of time of intoxication and skin location of exposure, causing a long duration of the toxic effects. This has important implications for military or civilian first responders with respect to triage, decontamination approach and therapeutic drug regimens (Dalton et al. 2006; Hamilton et al. 2004). The slow absorption of VX by the skin into the circulation does not parallel the short biological half lives of the current medical countermeasures, likely making a single injection treatment insufficient.

Relatively little is known about the physiological consequences of the persistent action of VX. To investigate toxicity mechanisms following percutaneous exposure to VX, we developed a hairless guinea pig model, in which toxicokinetics of VX, acetylcholinesterase and butyrylcholinesterase inhibition, and a broad range of physiological parameters can be measured. The hairless guinea pig skin closely reflects that of human skin in vitro, which justifies the use of this animal model to study toxicokinetics after VX exposure (Frasch and Barbero 2009). In this model, three characteristic target organs can be evaluated: the brain by measuring seizure activity by electroencephalogram (EEG) and acetylcholine (ACh) levels using microdialysis, the lungs by monitoring respiratory function using whole body plethysmography, and autonomic control of the heart by telemetric recording of ECG. Measuring physiological consequences is of importance as they might be independent from AChE inhibition and therefore may need separate treatment.

In the present study we investigated the toxicokinetics of percutaneously administered VX and the development of typical signs of poisoning at different physiological levels after exposure to dose levels considered relevant for human poisoning. In previous experiments, a percutaneous dose of approximately 4 LD50 (500 µg/kg), yielded a reproducible development of signs of toxicity in hairless guinea pigs (Joosen et al. 2008). The human equivalent dose, calculated by extrapolation of this dose using basal metabolic rates (BMR) or surface area corrections, is approximately 120 µg/kg, which is 1.5 times the human LDL0 (Maynard and Beswick 1992). After a thorough examination of all model parameters affected during VX poisoning, the effect of the treatment with the anticholinergic atropine, the AChE reactivator obidoxime, and the anticonvulsant diazepam, was investigated following single treatment or after repeated administration.

2 Methods

2.1 Experimental design

Five groups of hairless guinea pigs were challenged percutaneously with 500 µg/kg of VX. Levels of unbound VX and obidoxime in treated animals were determined in plasma. AChE and BuChE was determined in blood. Simultaneously, the physiological parameters heart rate and respiration were registered in these animals. The guinea pigs were either untreated, treated with atropine, obidoxime and diazepam doses at the guidance of appearance and recurrence of clinical signs or treated at first clinical signs with one single dose. Groups 1 and 2 served as control groups for the effects of percutaneous VX intoxication. Treatment efficacy was tested in groups 3, 4 and 5. The atropine dose used was 10 mg/kg, to yield a maximal anticholinergic effect in guinea pigs. The obidoxime dose (8.2 mg/kg) was corrected for its proven lower efficacy in VX reactivating potency in guinea pigs compared to humans (Worek et al. 2002). The diazepam dose used was 0.5 mg/kg, determined as ED50 value for terminating ongoing seizures independent of the atropine dose used according to the model of Shih et al. (2007). A summary of the parameters measured and treatments applied are shown in Table 1.

***Table 1**. Overview of experimental groups, number of animals per group and experimental parameters measured. Hairless guinea pigs were challenged with VX (500 µg/kg pc) and the treatment regimens applied are indicated in the table. For each group, the experimental parameters measured are indicated with X.*

Group	Number of animals	Treatment					Parameters measured per experimental group								
		Drug (mg/kg i.m.)			Nr. of injections										
		Atropine	Obidoxime	Diazepam	Single	Repetitive	Clinical signs	VX levels	AChE Activity (blood)	BuChE Activity (blood)	Obidoxime levels	Respiration	Heart rate (ECG)	EEG	ACh levels (Striatum)
1	9	0	0	0	-	-	X	X	X	X		X			
2	6	0	0	0	-	-	X						X	X	
3	6	30	24.6	1.5	X		X	X	X	X	X	X	X	X	
4	8	10	8.2	0.5		X	X	X	X	X	X	X			
5	6	10	8.2	0.5		X	X						X	X	X

To obtain EEG and ECG signals, animals were equipped with a combined set of electrodes on the skull and thorax at 5-7 days before VX exposure (groups 2, 4 and 5). For ACh determination, a microdialysis probe guide combined with an ECG/EEG electrode was attached to the skull 5-7 days before VX exposure (group 4). For analysis of VX and obidoxime levels in plasma, and cholinesterase activity in blood , animals were equipped with a carotid cannula 12- 16 hours before exposure. In these animals, respiration was measured non-invasively in a whole body plethysmograph. The procedures are described in detail below.

2.2 Animals

Male hairless guinea pigs [Crl:IAF(HA)BR] (375 ± 10 g), obtained from Charles River (Maastricht, The Netherlands) were used in the present study. Prior to the experiments they were housed with 2 animals per cage, and allowed to acclimate to standard conditions for two weeks. 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.

2.3 Chemicals

Acetylcholinesterase, choline oxidase, acetylcholine, choline, atropine sulphate and Triton-X100 were obtained from Sigma Chemical Co. (Zwijndrecht, The Netherlands). Kathon CG (1.5% 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) was provided by Rohm and Haas (Croyden, UK). LiChrosorb® NH2 was purchased from E. Merck (Amsterdam, The Netherlands). Hypnorm® (fentanyl/fluanisone) was purchased from Janssen Pharmaceutica (Beerse, Belgium) and Dormicum®(midazolam) was delivered by Roche Nederland BV (Mijdrecht, The Netherlands). Obidoxime, pralidoxime, (±) VX (O-ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate), the n-propyl analogue of (±) VX, O-n-propyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate and soman (O-Pinacolyl methylphosphonofluoridate) were 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. For HPLC analysis the highest purity grade was used. All solutions were prepared with water tapped from a Milli-Q system (Millipore SA, Molsheim, France).

2.4 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 below the right collarbone and between the second and third rib (Lead II configuration) and were also fixed to the plug. The plug and the electrodes were fixed to the skull with dental cement. In a separate group of animals a concentric microdialysis probe guide (CMA12, CMA, Sweden) was stereotaxically (KOPF Instruments, Tujunga, CA) inserted into the ventral striatum (P1.8, L 2.8, V 8.3 mm relative to bregma and the dura mater) next to the plug. Animals received peri-operative pain medication and antibiotics by sc injections (1 ml/kg) with Rimadyl (Carprofen, 5 mg/kg) and Borgal (1:10 dilution in PBS) before and at 24 and 48 hours after surgery. Animals were allowed to recover for 5-7 days from the surgical procedure.

For toxicokinetic experiments, naive guinea pigs or guinea pigs equipped with an EEG/ECG electrode 5 days earlier, were anesthetized with isoflurane (2-4%). A cannula was installed into the carotid artery and was guided under the skin to exit the body in the neck. The cannula was filled with heparinized PBS (20 IU/ml) and closed with a plug and loosely attached to the skin. Next, the animals were allowed to recover for 12-16 hours before application of VX.

2.5 VX application, observation and treatment

After obtaining baseline data from the parameters, the guinea pigs were gently fixated by hand, and a solution of VX in Isopropylalcohol (32 mg/ml) was applied to a well-defined spot on the belly of approximately 2 cm2 at a dose of 500 µg/kg (16 µl/kg). After the fluid was absorbed(approximately after 20 seconds), the spot was covered with a stainless-steel cup, which was strapped to the skin with surgical tape and removed after one hour. Upon exposure, the guinea pigs were monitored and the onset of the following cholinergic symptoms was registered: 1) Chewing: A clear chewing-like movement of the guinea pig in which the entire head is involved as a consequence of increasing saliva production. 2) Shivering: Continuous shivering of the entire body. The animal is capable to move around. 3) Salivation: Extensive drooling and tears fluid. 4) Tremor: Extensive shivering combined with involuntary movements in which the entire body is involved. The guinea pig looks mentally dissociated from the environment and has lost control over its movements. 5) Respiratory Distress: Low respiratory rate and heavy breathing.

In groups 3-5 initial clinical signs served as indicators for treatment. The presence of two signs for a period of several minutes was chosen as an indication for treatment injection. Treatment consisted of either a combined single im injection containing atropine, obidoxime and diazepam (30, 24.6 and 1.5 mg/kg respectively, group 3) or repetitive treatment lower doses of these drugs (10, 8.2 and 0.5 mg/kg im respectively, groups 4 and 5). The repetitive treatments were injected at the first presence of signs as previously indicated, and repeated after clear reappearance of clinical signs, mostly within 1-2 hours. Animals received 7-10 single shot injections over a time frame of 8-13 hours. When the heart rate of guinea pigs dropped below 150 BPM the animals were humanely euthanized to minimize unnecessary suffering.

2.6 EEG/ECG acquisition and analysis

In groups 2, 4 and 5, EEG and ECG of the animals challenged with VX were registered. The signals were obtained using PhysioTel telemetry system from Data Sciences Inc. (DSI) using the ML2-11-EET-F40 transmitter body. The transmitter was attached to the plug fixed on the guinea pigs 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 250 Hz (EEG) and 500 Hz (ECG) sampling frequency. Heart rate was exported from the Data Science ART analysis program. After storage, the EEG data were converted into EDF format. Every 10 seconds power spectra were calculated using Fast Fourier Transformation (FFT).

A low cut off filter at 1.6 Hz was used to filter eye movement artefacts and high digital filter at 50 Hz was used. Relative powers of EEG bands were calculated by dividing the sum of the absolute power in the frequency range by the total power of the spectrum. The results of relative power in the Delta (0.5-3.99 Hz) band is shown as representative power band.

2.7 Microdialysis procedure for acetylcholine determination

In group 2, ACh levels in the striatum of animals exposed to VX and repetitively treated with atropine, obidoxime and diazepam was registered in combination with EEG and ECG. Five days after surgery, the guinea pigs were individually placed in a perspex cage (25 x 25 x 40 cm) with free access to food and water. The following day, experiments were started between 9 and 10 AM. The microdialysis probes used were CMA12 probes with 4 mm of exposed PAES membranes. The probes were inserted one hour before the start of the experiment. Baseline values were measured in the following hour, followed by application of VX as previously described and intra-muscular injection of treatment upon clinical signs. The microdialysis probe was perfused with a Ringer solution at 2.0 µl/min delivered by a microinjection pump (CMA 100, CMA microdialysis AB, Solna, Sweden). To obtain detectable quantities of acetylcholine in the dialysate, 100 nM neostigmine was added to the perfusate. The guinea pig was connected directly to the sample loop, allowing on-line analysis of the microdialysates. The injection valve was automatically activated every 10 min, resulting in an injection volume of 20 µl.

2.8 HPLC Instrumentation and acetylcholine analysis

Microdialysate samples were assayed for acetylcholine and choline using a HPLC system according to Damsma et al. (1987) with some modifications, as described previously (Joosen et al. 2008). Briefly, ACh was separated on a EC 125/2 Nucleosil 100-5 C18 AB analytical column (Aurora Borealis Control, Schoonebeek, The Netherlands), preloaded with 0.5% sodium lauryl sulphate, and converted into choline and hydrogen peroxide on a post-column enzyme reactor containing immobilized acetylcholinesterase (80 U) and choline oxidase (40 U). Hydrogen peroxide was electrochemically detected at + 450 mV using a VT03 flow cell with Pt work electrode (Antec Leyden BV, Hazerswoude, The Netherlands) The mobile phase consisted of a 166 mM potassium phosphate buffer (pH 8.5) containing 1 mM tetramethylammonium chloride, 0.79 mM EDTA and 250 µl/l Kathon CG. The system was run at 0.35 ml/min and temperature was maintained at 30 ^{o}C. Before the beginning and at the end of each experiment a 10 nM acetylcholine calibration standard was injected in duplicate, and if necessary corrections for reduced sensitivity during the experiment were made. Limit of quantification was 1 pmol/ml. Linear calibration curves were obtained in the range from 5 – 1000 pmol/ml ($r > 0.990$). The intra-assay coefficients of variation for 10 and 100 pmol/ml were 2.9 % and 4.1 %, respectively. Inter-assay variability could not be determined due to variable enzyme activities in the post-column reactor between days.

2.9 Toxicokinetic experiments

In experimental groups 1, 3 and 5 toxicokinetics of VX, pharmacokinetics of obidoxime and ChE activity in blood were determined upon VX exposure either with or without treatment. One hour before exposure, the cannulas were opened and clamped to have free access to blood samples without handling the animal during the measurement and 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 (RMV) were calculated from the signal using IOX software (EMKA technologies, France). After obtaining baseline values, VX dissolved in isopropylalcohol solution was applied on the belly of the animal as described previously (Joosen et al. 2008). Blood samples were taken at least every two hours, or every hour when clinical signs progressed rapidly. Blood samples (200 µl) were immediately processed to determine VX levels. For the obidoxime determination plasma was prepared starting with a 100 µl blood sample by centrifugation (15s, 11,000 rpm). Small blood samples (20 µl) for AChE and BuChE were drawn every hour.

2.10 Sample preparation for determination of VX

Aliquots of 20 µl blood were diluted 10 times in 1% saponin solution and frozen immediately in liquid nitrogen. These samples were used for determination of AChE and BuChE activity. Aliquots of 200 µl blood were mixed with 10 ng of O-n-propyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate as internal standard and soman (100 ng) to occupy available binding sites. Next, the sample was made alkaline with 30 µl 1M sodium hydroxide and extracted twice with two volumes of a mixture of 5% methanol in hexane. The extract was evaporated and the residue was dissolved in 100 µl n-hexane and analyzed with GC using nitrogen phosphorous detection.

2.11 Gas chromatography for VX analysis

VX levels in plasma were determined using gas chromatography as described previously (van der Schans et al. 2003). The analyses were performed on a HRGC Mega II 8560 instrument (Fisons Instruments, Milan, Italy) equipped with a nitrogen-phosphorus detector (300 °C), a split-splitless injector and an AS 800 auto sampler. The chromatographic conditions comprised a CPSil8 column length, 25 m, i.d. 0.25 mm and film thickness 1.2 µm (Varian, Middelburg, The Netherlands). Injections (8 µl) were performed using the splitless mode at an injector temperature of 260 °C. At the start, the oven temperature was maintained at 100 °C for 2 min, increased to 200 °C at a rate of 25 °C/min for 7 minutes, and finally elevated to 300 °C at a rate of 25 °C/min. The carrier gas (helium) flow was 2.5 ml/min. Detector gas flows were 35, 350, and 37 ml/min for hydrogen, air and helium, respectively.

2.12 Obidoxime analysis

Obidoxime in plasma was determined with capillary electrophoresis. Plasma samples were diluted 1:1 with a mixture of 60 U/ml heparin and internal standard (pralidoxime, 10 µg/ml). Electrophoretic analyses were performed on a P/ACE MDQ from Beckman Coulter (Fullerton, CA, USA). Running buffer consisted of 200 mM 6-aminohexanoic acid/acetate, pH 4.5. Samples were introduced by pressure injection for 5 s at 1 psi. Length of the fused silica capillary (i.d. 75 µm, PolyMicro, Phoenix, AZ, USA) was 35 cm, the effective length to the detector was 25 cm. Separation voltage was 15kV. The detection wavelength was 290 nm.

2.13 Enzyme activity

Samples were analyzed for AChE and BuChE activity using a modification of the method by Ellman et al. (1961). The assay was performed in 96 well plates, in which AChE and BuChE activity were determined simultaneously on the same plate. In short, samples were diluted in 0.8 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (Sigma Aldrich B.V.). To 10 µl of diluted sample, 100 µl of 0.8 mM β-methylacetylthiocholine iodide was added, in quadruple, for determination of AChE activity. For assessment of BuChE activity in the samples, 100 µl of 0.8 mM of butyrylthiocholine was added. The delta OD per min at 412 nm at ambient temperature served as measurement for ChE activity. Guinea pig AChE does not react with butyrylthiocholine, rendering the BuChE activity determined an appropriate representation of BuChE activity. AChE activity in blood was corrected for cross reactivity of BuChE with β-methylacetylthiocholine by subtracting of 47% of the extinction of butyrylthiocholine developed by BuChE. This was calculated from separate experiments in which isolated guinea pig AChE and plasma BuChE were incubated with β-methylacetylthiocholine (Bosgra et al. 2009). AChE and BuChE activity in blood were normalized versus the baseline sample.

2.14 Data presentation and statistical analysis

All data were analyzed using one-way ANOVA or repeated measures ANOVA followed by Dunnett's post-hoc test using the VX group or baseline values as control. Results were considered significant for $p<0.05$.

For determination of continuous relationships between blood AChE and BuChE activity and occurrence of clinical signs logistic regression analysis was performed. AChE or BuChE activity of animals pc exposed to VX was coupled to presence of a sign (1) or absence of sign (0). Using binary logistic regression analysis with SPSS statistical software, the logits (l) of the probability of response for clinical signs (p) at each level of ChE activity (j) were determined. The probability of response between 0 (no response) and 1 (response) for each clinical sign was calculated from the logits of the probability p using the equation:

$$p = \frac{e^{l_j}}{1 + e^{l_j}}$$

3 Results

3.1 Toxicokinetics and clinical signs

The toxicokinetics of VX after percutaneous application was studied in hairless guinea pigs that either received no treatment, a single shot treatment or repetitive treatment (atropine 10 mg/kg; obidoxime 8.2 mg/kg and diazepam 0.5 mg/kg im per injection). During the experiment the animals were scored for clinical signs. The average onset times of these signs are shown in figure 1A. The first clinical signs appeared in a gradual fashion, starting with chewing and shivering at 15 to 220 minutes and 55 to 374 minutes, respectively. This was followed mostly by more heavy tremors at 75 to 122 minutes. In most animals this was followed by respiratory distress shown by heavy breathing. Only few animals developed convulsions. Animals died on average at 237 ±37 minutes, with a minimum of 96 minutes and a maximum of 560 minutes, indicating a large variation in the development of toxic signs.

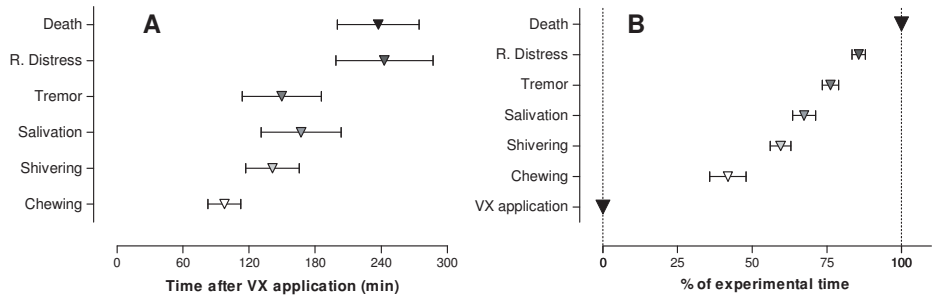

***Figure 1**. A. First appearance of cholinergic signs in individual animals (vertical axis) over experimental time (horizontal axis) between VX application (0h) and death. B. First appearance of cholinergic signs in individual animals (vertical axis) at percentages of total experimental time (horizontal axis) between VX application (0%) and death (100%). The average first appearances of signs ±SEM have been plotted (n=15).*

We scored the first appearance of the cholinergic signs versus the % of time elapsed between VX application (0%) and death (100%) (Fig. 1B). On average, the onset time of the first occurrence of each sign was predictive for the time of the occurrence of more severe signs, and signs occurred always in a similar sequence. Chewing occurs at 41±6 % of the total survival time, followed by shivering and salivation at 59±3% and 67±4% of survival time, respectively. On average, animals started showing tremor at 76±3% and the most severe symptom, respiratory distress, was only seen shortly before death at 85±2% of total time.

We measured the average concentration of VX in blood of 9 animals that were exposed percutaneously to 500 µg VX/kg (Fig. 2). In most animals, VX levels raised to more than 1 ng/ml within 1-2 hours. The maximum VX levels ranged from 0.3 to 5.5 ng/ml, which were reached after 5 hours or 90 minutes, respectively. On average, plasma levels reached maximum levels of 3-4 ng/ml at 2 hours after the intoxication. AChE was completely inhibited at that time (Fig. 2C). BuChE activity showed a similar pattern, however with a slight delay in inhibition compared to

AChE activity (Fig. 2D). In particular during the first 90 minutes after exposure a clear difference between AChE and BuChE inhibition was observed.

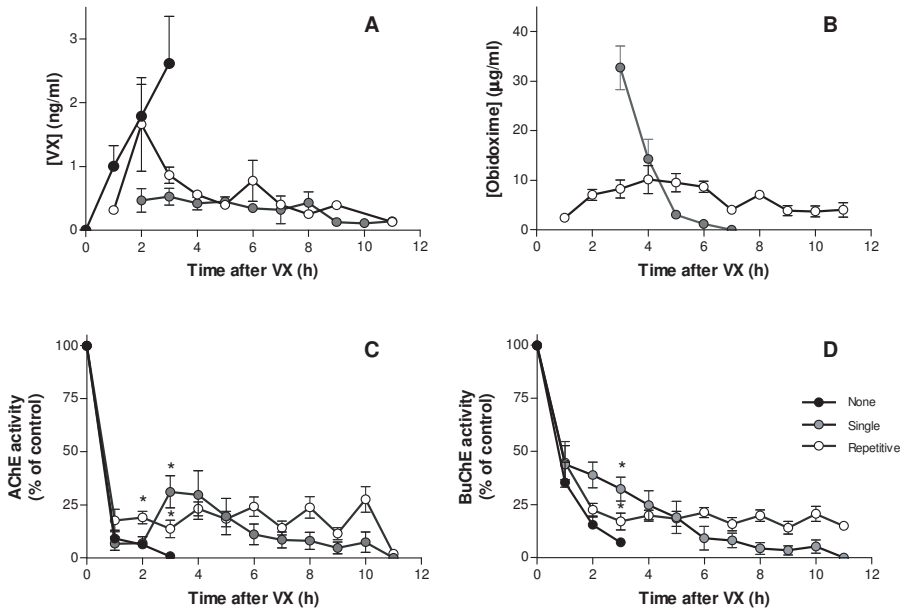

***Figure 2**. A. Toxicokinetics of VX in animals pc exposed to VX (500 µg/kg pc). B. Obidoxime plasma concentrations in animals exposed to VX (500 µg/kg pc). C. AChE and D. BuChE activity in blood of animals exposed to VX (500 µg/kg pc). Open upward triangles represent values obtained from untreated animals. Open squares represent animals treated with a single shot treatment at appearance of first signs, in general after approximately 1 hour, downward closed triangles represent animals receiving repeated injections after skin VX poisoning, in genereal every 60-90 minutes after VX poisoning. Values are represented as means ±SEM. *: p <0.05; significantly different from untreated animals, One way ANOVA followed by Dunnett's post-hoc test.*

The fraction of animals showing cholinergic clinical signs, at each level of blood ChE activity, is shown in figure 3 A-C. Analysis of the relationship between the occurrence of clinical signs and the enzyme activity of AChE and BuChE activity in blood of untreated animals by logistic regression revealed that over 80% of AChE had to be inhibited before initial clinical signs emerged (Fig. 3). The first mild signs of poisoning, chewing movements, shivering and slight tremor, generally did not occur before over 80% of AChE activity was inhibited. At that time, only 40-60% of BuChE appeared to be inhibited, resulting from a much slower inhibition rate of the BuChE activity. Full inhibition of BuChE showed to be a much better predictor of death compared to AChE inhibition. Full inhibition of AChE in blood predicted death in only 50% of the cases, whereas full BuChE inhibition was correlated with death in 80% of cases.

Animals in the group that received a single shot treatment showed similar toxicity signs. Treatment on indication of the first toxicity signs, i.e. chewing and

shivering, delayed the progression of the signs. However, they reappeared after approximately 1 hour in most animals. Three out of 6 animals died within 10-12 hours after poisoning, whereas 1 animal survived for 22 hours. Two other animals were euthanized at 10 hours because of their poor condition. Blood samples were drawn from these animals to determine AChE, BuChE, VX and obidoxime. A single shot of three auto injector equivalents led to maximum obidoxime levels of 30 µg/ml at one hour after the injection and an elimination half-life time of 1 h (Fig. 2B). Upon administration of the therapeutics, AChE and BuChE were reactivated to 40% of the control value but were completely re-inhibited within 9 hours by remaining VX (Fig. 2C,D). The VX levels in these animals ranged from 0.5 to 1 ng/ml, which was lower than in the non-treated animals (Fig. 2A).

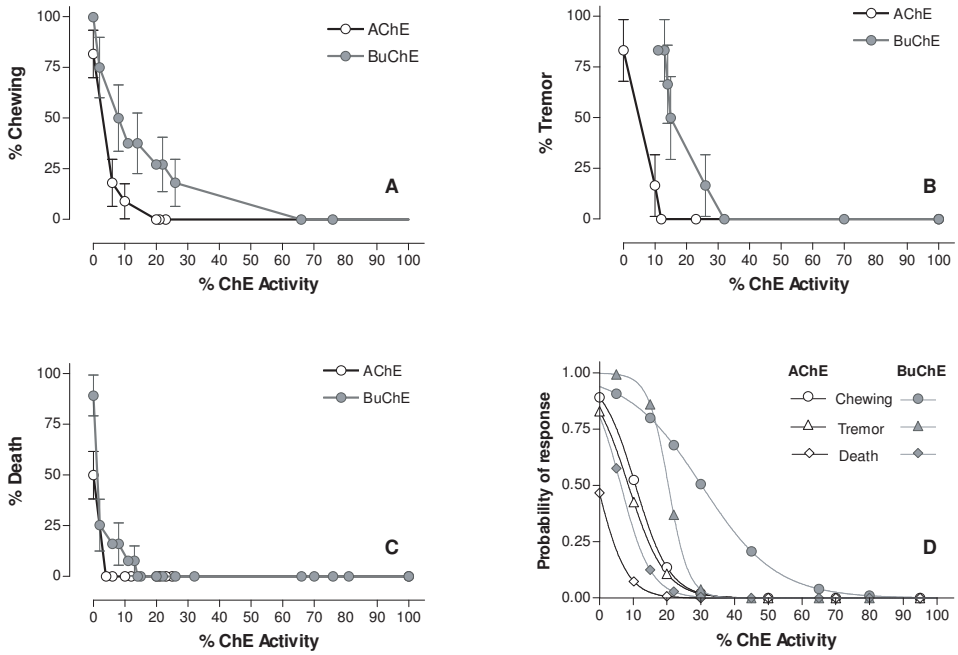

***Figure 3**. Toxicity signs in hairless guinea pigs after percutaneous exposure to 500 µg/kg of VX at a certain AChE or BuChE activity in blood. A, B and C. The percentage (mean ±SEM) of animals showing Chewing, Tremor, and Death respectively (n=9). D. The probability of response at each ChE level calculated from logistic regression analysis of the appearance of different toxicity signs following skin VX exposure in hairless guinea pigs.*

In another experimental group, treatment was given repetitively on the guidance of clinical signs. In case one injection was given after appearance of the first toxicity signs, the signs worsened again after approximately one hour. Additional treatment injections postponed further progression of signs to respiratory distress and death, but less severe signs such as shivering and tremor could not be prevented. After repetitive treatment with 6-8 auto-injector equivalents over 10-14 hours, all animals

but one survived the observational period of 24 hours. However, the animals surviving this period showed severe signs of poisoning, such as severe tremors and respiratory distress at 24 h after application of VX.

Upon repetitive injections, obidoxime levels in plasma were approximately 10 µg/ml and were stable during the period that treatment was provided (Fig. 2B). AChE and BuChE were reactivated and were steady at a level of 25% of control values during that time (Fig. 2C,D). VX levels in blood were approximately 0.8 ng/ml throughout the time of the experiment, which was again lower than in the non-treated animals (Fig. 2A). At 24 h after the application of VX, AChE and BuChE were completely inhibited, VX levels in blood were lower than 0.1 ng/ml and the obidoxime concentration in plasma was below 2 µg/ml (not shown).

3.2 Physiology

Respiration dynamics of the most representative parameters Penh (a measure for bronchoconstriction) and respiratory minute volume (RMV), were registered during the toxicokinetic experiments (Fig. 4). Bronchoconstriction showed a significant increase, together with a significant decline in RMV as the intoxication became more severe. Both the single shot treatment and repetitive treatment delayed the increase of Penh. After washout of drugs, Penh increased further (Fig. 4A). The decline in RMV was only prevented by the high dose single shot treatment (Fig. 4B). This treatment induced a short period of RMV increase, which declined again at approximately 6 hours after poisoning and 3 hours after treatment. Repetitive treatment with atropine, obidoxime and diazepam (10, 8.2 and 0.5 mg/kg im respectively) did not prevent a significant RMV decrease, and RMV further declined after cessation of treatment at 12 hours after poisoning.

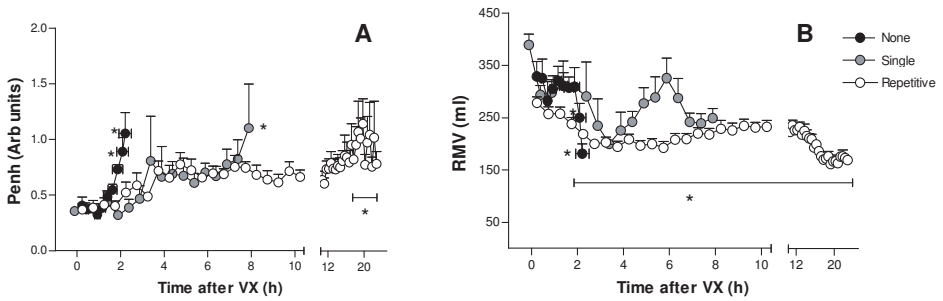

***Figure 4**. Respiration parameters after pc VX exposure (500 µg/kg). A. Averaged Penh (bronchoconstriction) after pc VX exposure. B. Averaged Respiratory minute volume. The graphs show averages ± SEM of untreated animals (black dots, n=9). Grey dots represent animals treated with one single injection of atropine, obidoxime and diazepam (10, 24.6 and 1.5 mg/kg i.m respectively,, n=6), and animals repetitively treated with injections are shown by open dots (n= 6).*: p<0.05, Repeated measures ANOVA followed by Dunnet's posthoc test.*

Animals exposed to VX (500 µg/kg pc) without treatment showed a significant decline in average heart rate at 90 minutes after poisoning (Fig. 5). A single im injection with atropine, obidoxime and diazepam (30, 24.6 and 1.5 mg/kg im respectively) induced an obvious delay in the heart rate decline (Fig. 5). Repeated treatment preserved the heart rate for a longer time, but after cessation of treatment the decline progressed.

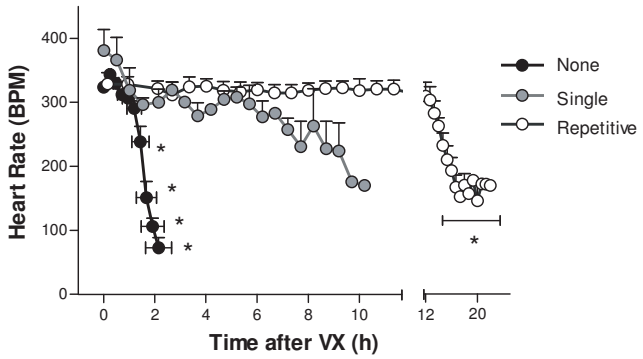

***Figure 5**. Averaged ± SEM heart rate after pc VX exposure (500 µg/kg) in untreated animals (black dots, n=6), animals treated with atropine, obidoxime and diazepam (30, 24.6 and 1.5 mg/kg im respectively) (grey dots, n=6), and animals repetitively treated with single injections of atropine, obidoxime and diazepam (10, 8.2 and 0.5 mg/kg im respectively) (open dots, n= 6). *: p<0.05, Repeated measures ANOVA followed by Dunnet's posthoc test.*

Animals repetitively treated with im injections of atropine, obidoxime and diazepam (10, 8.2 and 0.5 mg/kg respectively) eventually showed increases in extracellular ACh levels (Fig. 6). The effect of a single high dose injection on extracellular ACh levels was not tested.

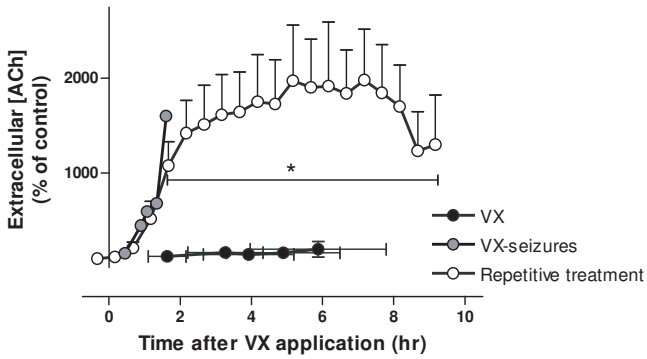

***Figure 6**. Extracellular ACh concentrations in striatum of VX exposed guinea pigs. Black dots represent untreated animals without seizures and grey dots the ACh levels of one untreated animal displaying seizures on EEG (data from Joosen et al. 2008). The open dots represent the ACh levels in the striate body of animals repetitively treated with injections of atropine, obidoxime and diazepam (10, 8.2 and 0.5 mg/kg im respectively) at appearance and reappearance of clinical signs, The average time between injections was 60-90 minutes. *: p<0.05, Repeated measures ANOVA followed by Dunnet's posthoc test.*

For comparison, ACh levels of untreated animals are also presented in Figure 6. In these animals, the accumulation of ACh was not accompanied by an increase in total EEG power, and not by the appearance of seizures. The treatment applied thus prevented the occurrence of seizures induced by the increase in ACh concentration.

The EEG was also analyzed for energy shifts in power bands. The shifts in the Delta band are shown in Figure 7. The power in the Delta band of the EEG showed a clear initial increase in the first 2 hours after exposure, and then became significant. Both treatment regimens applied showed a reverse of these effects. The single shot treatment induced a shorter and steeper reversal than the repetitive treatment, and prevented statistically significant changes. The repetitive treatment induced a significant decline in the power in the delta band, which persisted for several hours.

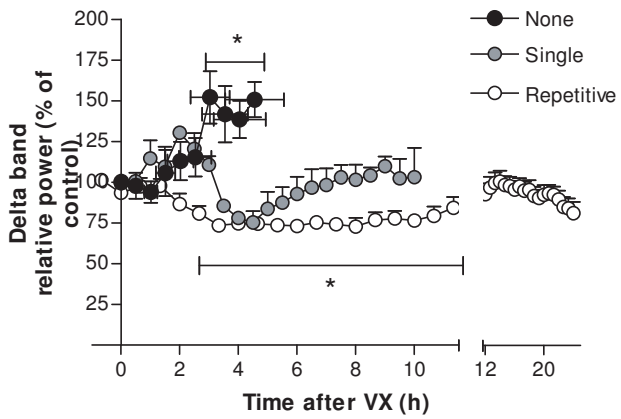

***Figure 7**. EEG effects after pc exposure to VX (500 µg/kg) in guinea pigs. The averaged normalized relative power in Delta band of the EEG is shown. Grey dots represent the animals receiving a single injection of atropine, obidoxime and diazepam (30, 24.6 and 1.5 mg/kg im respectively). Open dots represent the animals that received repetitive treatment with atropine, obidoxime and diazepam (10, 8.2 and 0.5 mg/kg im respectively). *: p<0.05, Repeated measures ANOVA followed by Dunnet's posthoc test.*

4 Discussion

The toxicokinetics of VX after percutaneous exposure to 500 µg/kg and the consequent physiological effects were investigated in hairless guinea pigs. The guinea pigs were either untreated, treated with atropine, obidoxime and diazepam doses at the guidance of appearance and recurrence of clinical signs or treated at first clinical signs with one single dose. The use of a small animal, such as the hairless guinea pig, requires the use of diluted VX to obtain a relevant level of exposure. It might be expected that both the body surface area exposed and the solvent might influence toxicokinetics. However, an effect on the toxicokinetics related to the solvent and the resulting increased surface area is not implicated (Hamilton et al. 2004). Similar to a study in the domestic swine, we found variable toxicokinetics

in untreated animals. The levels of VX in blood of animals that received treatment appeared to be lower than the VX levels in untreated animals. A possible explanation is that BuChE was also reactivated by obidoxime, thereby releasing binding sites for penetrating VX. Another possibility is that untreated animals showed to have a much lower heart rate than animals receiving treatment, implicating that the distribution and therefore the elimination rate of VX was lowered.

In line with the variability of VX toxicokinetics and AChE and BuChE inhibition, the time of onset and development of toxic signs showed a similar variability in time when left untreated. In one of our previous studies (Joosen et al. 2008), we showed that the onset time of clinical signs following percutaneous VX application showed to be a reliable predictor for the time course and presence of more severe signs and death. In this study, onset times of clinical signs were expressed as a % of the total time period between VX application and death. Using this approach in the present study, we showed again that compensation of the variable and delayed absorption through the skin by normalising the time factor, minimized inter-individual variability in the first occurrence of clinical signs (Fig. 1).

In contrast to the subcutaneous exposure route reported in the literature (Hilmas et al. 2009; Shih et al. 2007), full blown seizures were only apparent in 33% of animals skin exposed to VX. The fact that less seizures were found compared to previous work may be explained by the much slower absorbance of VX from a skin depot into the circulation than that following subcutaneous injection. The appearance of the most severe signs and death, implicates that penetration of VX into critical organs such as the brain and diaphragm will not occur until the entire scavenging pool for VX in blood is inhibited (Sidell 1997). By entering the circulation very slowly, the protonated amine in VX may be more hampering its entrance into the brain (Epstein et al. 1974; Maxwell et al. 1997), than at higher free concentrations of VX. However, at several hours after skin exposure with VX, we found substantial AChE inhibition in the brain. On the other hand, very low levels of VX have shown to lower the firing rate and amplitude of action potentials in hippocampal neurons in vitro (Rocha et al. 1999). Translated to the in vivo situation, this may cause a more generalized reduction of action potentials in the brain and a shift of EEG power to lower frequency ranges opposed to the expected increase induced by ACh accumulation. When seizures were absent, we found an increase in delta and a decrease in theta power of the EEG. Either ischemia or direct effects of VX, for example by lowering the frequency of theta power generated by hippocampal neurons, may have caused this effect (Chang et al. 1998; Cohen et al. 1994; Joosen et al. 2008).

The absence of profound central effects after percutaneous VX exposure in the majority of animals prompted us to put focus on physiological peripheral effects such as respiration and heart rate in addition to toxicokinetics and EEG. A significant life threatening decrease in heart rate and respiratory minute volume in combination with bronchoconstriction developed at approximately 60-70 % of survival time, while AChE and BuChE in blood were inhibited for 90% and 80%, respectively. These physiological changes paralleled the alterations found by EEG. In contrast, obvious less severe clinical signs, such as chewing occurred as early as

at 40% of survival time, at lower levels of BuChE and AChE inhibition of over 80 and 60%, respectively. These findings showed that mild clinical signs are good predictors for the toxicological status, and that measurement of blood cholinesterase is a proper indicator for initial severity of poisoning and treatment efficacy in case of skin exposure involving VX.

The treatment employed improved the clinical outcome on all parameters as long as treatment was continued on worsening of clinical signs. Discontinuation of treatment led to worsening of signs of poisoning after some time, resulting from VX slowly diffusing from the skin into the blood. Treatment with an intramuscular bolus of atropine, obidoxime and diazepam (30, 24.6 and 1.5 mg/kg i.m. respectively)at first signs of poisoning led to a delay of progression of life-threatening signs and prolonged survival time. This increase in treatment efficacy was probably for a great deal due to reactivation of AChE and only to some extent BuChE activity. Although critical physiological parameters were mainly preserved, the animals receiving either single or repetitive treatment kept showing mild to moderate clinical signs such as shivering and slight tremor, rendering the animals most likely incapable of performing any tasks. A single high dose treatment broadened the time window needed to receive additional intensive medical care, which was prolonged when the repetitive treatment scenario was employed.

Continuous treatment with single injections of atropine, obidoxime and diazepam (10, 8.2 and 0.5 mg/kg i.m. respectively) led to a substantial level of approximately 10 µg/ml of obidoxime in blood, which continuously reactivated a small amount of AChE and a very small fraction of BuChE. The single shot high treatment, leading to 3 times higher levels of obidoxime, induced a larger initial reactivating effect, after which the reactivated enzymes gradually were re-inhibited within the following hours by continuing VX release from the skin, again leading to toxicologically relevant levels.

In addition to oxime treatment, atropine served as a major component for treatment of peripheral signs. First of all, atropine is effective in counteracting salivation and respiratory deterioration and prevents vagally induced bradycardia by blocking mAChRs. Clinical findings suggest that atropine demand strongly depends on the level of AChE inhibition. In patients showing less than 90% of AChE inhibition, only very low doses of atropine were necessary (less than 10 µg/kg/h), whereas in patients showing over 90% of RBC AChE inhibition, very high levels of atropine were needed to treat mAChR mediated cholinergic transmission (Thiermann et al. 2009).

Changes in relative power in frequency bands of the EEG, probably induced by a severe impairment of respiration and decline in heart rate, were also reversed by repetitive treatment. In these treated animals, there was a huge accumulation of ACh in the brain, pointing to the entrance of VX into the brain and insufficient restoration of AChE activity to prevent ACh accumulation. Similar accumulation of ACh levels caused by OP poisoning in general, leads to development of seizures (Joosen et al. 2008; Joosen et al. 2009; Lallement et al. 1992). The treatment with the combination of atropine and diazepam probably prevented the development of seizures in spite of high levels of ACh in these animals. In addition, the toxicological process induced by skin applied VX is relatively slow, enabling synaptic adaptation

of the brain to the excess of ACh. The present findings imply that in spite of the initial absence of seizures in percutaneous VX exposure, diazepam or an alternative anticonvulsant is a necessity in the treatment of VX poisoning through the skin.

In conclusion, parameters such as EEG, heart rate and respiration showed to be less sensitive than typical cholinergic clinical signs for determining the toxicological status of the animal, but showed to be major and objective read-out parameters for treatment efficacy. The use of clinical signs as early indicator for starting and continuation of treatment with atropine, obidoxime and diazepam showed to be highly effective in preventing life-threatening effects on heart rate and respiration in the guinea pig. Percutaneous VX exposure appeared to be effectively treatable when diagnosed on time and if an appropriate timing of treatment is employed. Moreover treatment needs to be continued over the period of time during which all VX, that cannot longer be removed by skin decontamination, has entered the body and has been eliminated.

Although extrapolation of results from small animal models to man is difficult due to dose calculations, we predict that our findings in the guinea pig with clear consequences for treatment in this model, will hold predictive value for the human situation.

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