An Animal Model to Study Health Effects During Long-Term Low Dose Exposure to Toxic Agents

Dr. Gudrun Cassel, Dr. David Rocksén, Dr. Ann Göransson Nyberg
Swedish Defence Research Agency, FOI CBRN Defence and Security
S-901 82 Umeå, Sweden

gudrun.cassel@foi.se

ABSTRACT

The immediate and long term consequences of acute intoxication with nerve agents and other toxic substances are well known. Potential harmful delayed effects of repeated exposure to low levels of these agents have attracted less attention. These effects may be of relevance to military personnel possibly exposed to non-symptomatic levels of toxic substances during international peace-keeping operations, to agricultural workers and to the general population exposed to toxic substances of widespread use. To verify or deny if exposure has occurred analyses of biological samples from human material are of importance. When it comes to pre-medication and adequate medical treatment, it is necessary to find specific markers for the toxic agent as early as possible after exposure. Typical biomarkers of exposure include measurements of the toxin or its specific metabolite, while changes in for examples physiological parameters are biomarkers of effect. The aim of this investigation was to develop an animal model to study the long-term health effects of chronic intoxication in awake, freely moving rats. To exclude the influence of stress, male Wistar rats were instrumented with telemetry devices for continuous (24 hours) measurement of mean arterial pressure, heart rate, body temperature, activity and respiratory rate. Sustained subcutaneous release of low doses of the organophosphate substance VX was administrated through an osmotic pump. Once a week blood samples for analysis of exposure biomarkers were taken. The result shows that this rat model is a useful tool to study long term effects of different doses of toxic substances in freely moving rats.

1.0 INTRODUCTION

Although the immediate and long term consequences of acute intoxication with organophosphorus (OP) AChE inhibitors are well known [1,2,3], potential harmful delayed effects of repeated exposure to low (non-symptomatic) levels of these agents have attracted less attention. These effects may be of relevance, if they exist, to military personnel possibly exposed to non-symptomatic levels of OP compounds [4], and to agricultural workers and the general population exposed to OP insecticides of widespread use. Administration of AChE inhibitors at low levels generates a number of physiological changes. Central ,AChE inhibition enhances arterial blood pressure [5,6], and decreases cerebrovascular resistance [7,8,9], 1991; Scremin et al., 1993; Scremin et al., 1988), decreases body temperature and elevates nociceptive threshold [10]. Previous work on delayed effects of low dose OP AChE inhibition has led to contradictory results. While some authors have reported some effects [11, 1], other studies have found no increase over the general population in the incidence of mental, neurological, hepatic, and reproductive pathology or cancer of subjects exposed in the work environment [12] or after accidental exposures [13, 14]. This project concerns the primary steps in a long-sighted investigation on health risks for people at exposure to toxic substances such as OP and related compounds. At present it is not known if and how these substances in low levels contribute to the incidence of diseases such as allergies, cancer, immunologic,

Cassel, G.; Rocksén, D.; Göransson Nyberg, A. (2007) An Animal Model to Study Health Effects During Long-Term Low Dose Exposure to Toxic Agents. In *Defence against the Effects of Chemical Hazards: Toxicology, Diagnosis and Medical Countermeasures* (pp. 3-1 – 3-10). Meeting Proceedings RTO-MP-HFM-149, Paper 3. Neuilly-sur-Seine, France: RTO. Available from: http://www.rto.nato.int.

hormonal neurological symptoms at long time exposure. The primary goal of this project are to develop an animal model to study the physiological effect of long time low level exposure and to search for early biomarkers to verify exposure and/or effect. As a model the nerve agent VX in sub-clinical doses was used. The substance of interest is introduced with a controlled dose and speed trough an osmotic pump that is placed beneath the skin of the rat. During the experiments various physiological parameters are registered such as blood pressure, breathing rhythms, temperature, pulse etc. with a newly developed telemetric equipment. We also want to study biomarkers for exposure such as the internal dose of VX in the animal during experiment.

2.0 MATERIALS AND METHODS

2.1 Animals

Adult male Wistar rats (Scanbur, Sweden) weighing 225–275 g at the beginning of the experiments were housed individually in plastic cages (43 cm×27 cm×18.5 cm) under a 12 h light–dark cycle (lights on 6 a.m.). As an environmental enrichment rats were given a red plastic block (18mm×18mm×100 mm) in *similar material to bedding*. Room temperature was maintained at 21±2 ˚C and relative humidity at 55 ±5%. Standard rodent pellet food and tap water were available ad libitum. The rats were acclimatised to these conditions for a minimum of 10 days prior to surgery. The animal experiments were approved by the Regional Research Ethical Committee in accordance with Swedish laws (SFS 1988:539, LSFS 1989:41).

2.2 Chemicals

The nerve agent VX (*O*-ethyl-*S*- [2(di-isopropylamino)ethyl] methyl phosphonothioate), >95% pure) was synthesised at the Department of Chemistry, FOI CBRN Defence and Security, Sweden, and diluted to its final concentration with the vehicle PPG (Polypropylene Glycol) 400 on the day of the experiment.

2.3. Surgery

All rats were given prophylactic antibiotic and peripheral acting analgesic treatment. 15 min prior to surgery injection of 0.1 ml Baytril® vet. (intramuscular (IM); 0 mg/ml enrofloxacin, Bayer, Germany) and 1.5 ml 1:50 dilution Rimadyl® vet. (subcutaneous (SC); 50 mg/ml carprofen, Pfizer, USA), respectively. Rats were anaesthetised with a 0.2 ml/100 g (SC) injection of, one part Hypnorm® (0.315 mg/ml fentanyl + 10 mg/ml fluanisone, Jassen Inc., USA), one part Dormicom® (5 mg/ml midazolam, F. Hoffmann-La Roche AG, Switzerland) in two parts sterilised water.

2.3.1 Implantation of radio-telemetric device

Telemetry transmitters (C50-PXT, Data sciences, USA) were implanted according to manufacturer's specifications into male Wistar rats while under anaesthesia. In brief, a midline incision was used to expose the abdominal aorta that was briefly occluded to allow insertion of the transmitter catheter. The catheter was secured in place with tissue glue. The transmitter body was sutured to the abdominal wall along the incision line as the incision was closed. The skin was closed with staples that were removed 7–10 days after the incision healed. Rats were allowed to recover from surgery and were returned to individual housing for at least 1 week before initiation of data acquisition. The individual rat cages were placed on top of the telemetry receivers, and heart rate, respiratory rate, body temperature, activity and arterial pressure waveforms were continuously recorded throughout the study.

2.3.2 Implantation of osmotic mini-pumps

Alzet® Osmotic Mini-pumps with a constant delivery rate of 0.55 µl/hr (Model 2002, Alza Corp., Palo Alto, USA) were used to deliver either the vehicle (PPG 400) or VX (solved in the vehicle) in different doses. After 1 week of telemetric baseline registration, the osmotic pumps were implanted subcutaneously on the back of the rats under anaesthesia.

2.3.2.1 Verification of the osmotic pump

Control of the delivery rate of the osmotic pump was performed according to manufacturer's specification. In short, the pumps where filled with a solution coloured by Comassie-blue and primed in physiological NaCl solution at 37 °C for 40 hours. The absorbance of a given volume of the physiological solution was then measured continuously for 4 weeks to estimate the delivery rate.

2.3.2.2 Stability of VX in the vehicle PPG 400

A solution of 1000 ppm (volume/volume) of VX was dissolved in PPG 400, diluted and kept in suitable containers in water bath at 37 °C. Samples was analyzed once a week for 4 weeks by GC/NPD.

2.4 Experimental design

2.4.1 Dose response measurement

To estimate an optimal dose of VX, that significantly inhibited AChE activity in blood without the animal showing clinical symptoms, a dose-related body weight gain and AChE inhibition were obtained by continuous infusion with different doses of VX. Osmotic mini pumps containing 0, 0.5, 1, 2.5, 5, 10, 15, 20, 30, and 40 µg/kg/24hr of VX were implanted subcutaneously on the back of the rats (n=4 for each dose).The rats were weighed before the surgery, and then once a week. This study was performed during two weeks. Because the animals gain weight during the acclimatisation period, the VX concentrations were based on the estimated weight of the animals one week after implantation based on the normal growth curve for rats in our laboratory.

2.4.2 Comparison between subcutaneous injection and administration by osmotic pump

To compare subcutaneously injection once a day, 5 days a week, with continuously administration by the osmotic pump a study was performed. AChE inhibition were followed after continuous infusion with VX; by implantation of minipumps containing 0, 0.5, 1, 2.5, 5, 10, 15, 20, 30, and 40 µg/kg/24hr of VX or after subcutaneous injection of 1/10 of the concentration of VX in the osmotic pump. The toxic agent was then administrated with a constant delivery rate of 0.55 µl/hr in different doses This study was performed during two weeks. Subcutaneous injections of VX in doses comparable with the pumps administration were given every day during 2 weeks. After 2 weeks the animals were blood samples were taken for analyse of AChE activity.

2.4.3 Telemetric registration during continuous administration of VX

Subsequently, based on the results obtained, matched subgroups of 4 animals each were formed. These groups were acclimatized for one week with implanted radio-telemetry devices. The baseline values of the different read-out systems were collected. After an additional week of base-line measurements the rats were implanted with osmotic pumps. The body weight, plasma corticosterone, free T4, S-100 levels, and blood-AChE activity were registrated once a week, for 8 weeks. We registried the telemetric parameters blood pressure, heart rate, temperature, respiratory rate and activity continuously during the experiment.

2.5 Measurement of blood AChE activity

Blood samples were collected once a week from the rat tail vein by syringe and needle. The acetylcholinesterase activity was measured in the blood using a modified method of Augustinsson *et al.,* [15], which has been described in detail in [16].

2.6 Assays

Enzyme immunoassay were used for the quantitative determination of corticosterone (Corticosterone EIA), Free T4 (Free Thyroxine (T4) ELISA), and S-100 (Sangtec® 100 ELISA) in serum or plasma.

3.0 RESULTS

3.1 Verification of the Alzet® osmotic pump

Our results confirm that the osmotic pump under our conditions function as declared by the company.

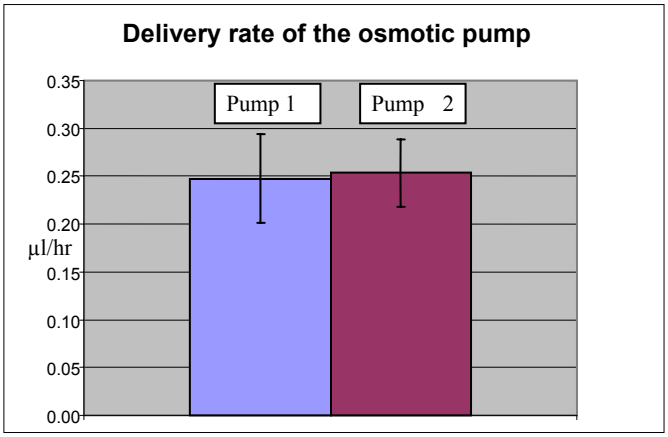

Figure 1: This figure shows no differences in the delivery rate of two different osmotic pumps.

3.2 Stability of VX in the vehicle PPG 400

Figure 1 depict the remaining quantity of VX (%) after 4 weeks in water bath at 37 °C. The results show that 80% remains active in PPG 400.

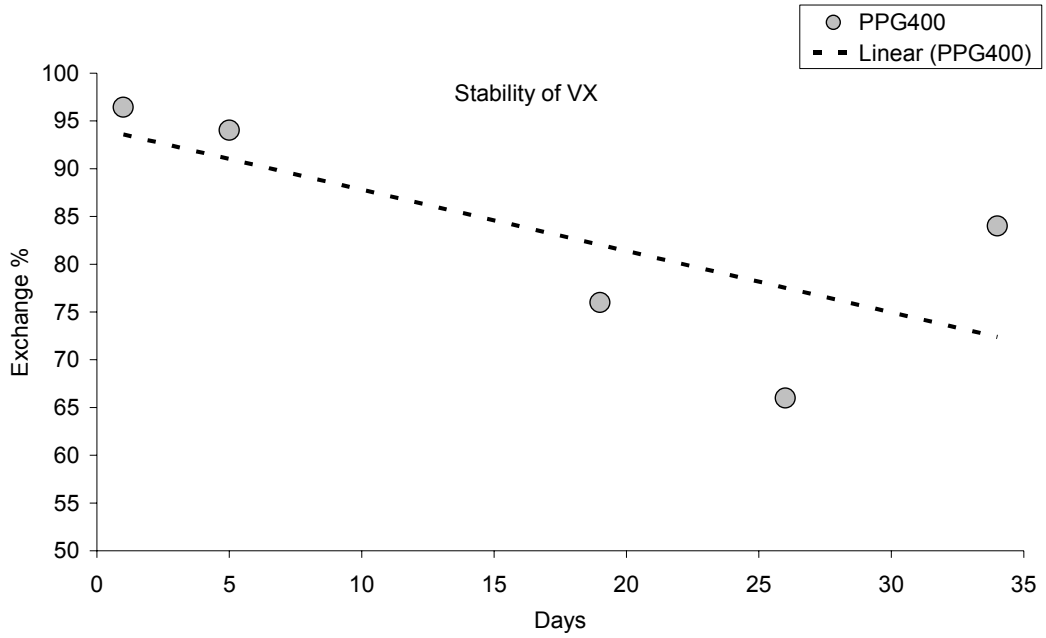

Figure 2: Stability of VX in PPG 400

3.3 Dose-responses

The current investigation started with a dose-response study to determine a dose of VX in the osmotic pump that produced an inhibition of AChE activity in blood without clinical symptoms (Figure 3). As can be seen in the figure, a breakpoint in response was observed at a dose of 2.5 µg/kg/24hr. From these results four subgroups were selected; 0, 2.5, 3.5, and 5.0 µg/kg/24hr to be further studied.

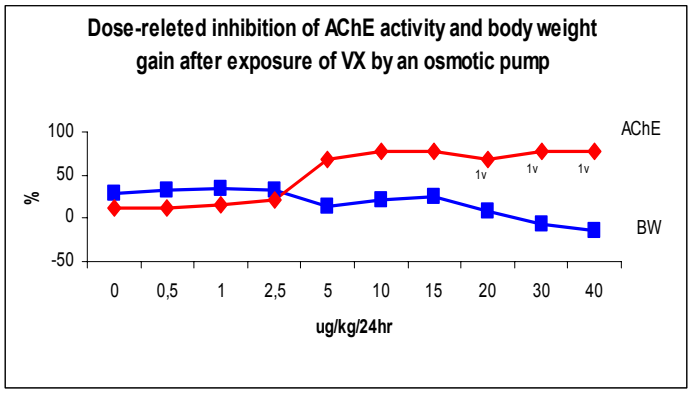

Figure 3. This figure shows AChE and body weight for the subgroups of rats.

3.4 Comparison between subcutaneous injection and administration by osmotic pump

As the figure below shows the rats given daily subcutaneously injection were more sensitive to VX and only needed 1/10 of the daily concentration to reach the same level of AChE inhibition as the animals that were administrate by the osmotic pump. There were no signs of clinical symptoms neither of the groups studied.

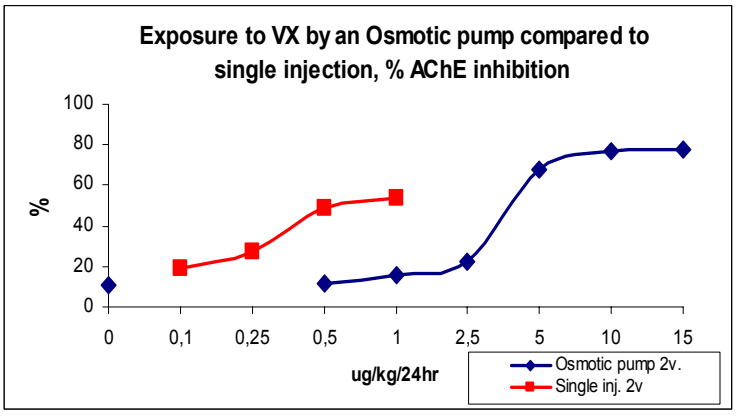

Figure 4: This figure shows comparison between subcutaneous administration and administration by osmotic pump.

3.5 Telemetric registration during continuous administration of VX

3.5.1 Telemetric registration

There were no changes in telemetric observations in either of the groups receiving administration through the osmotic pump (Figure 5).

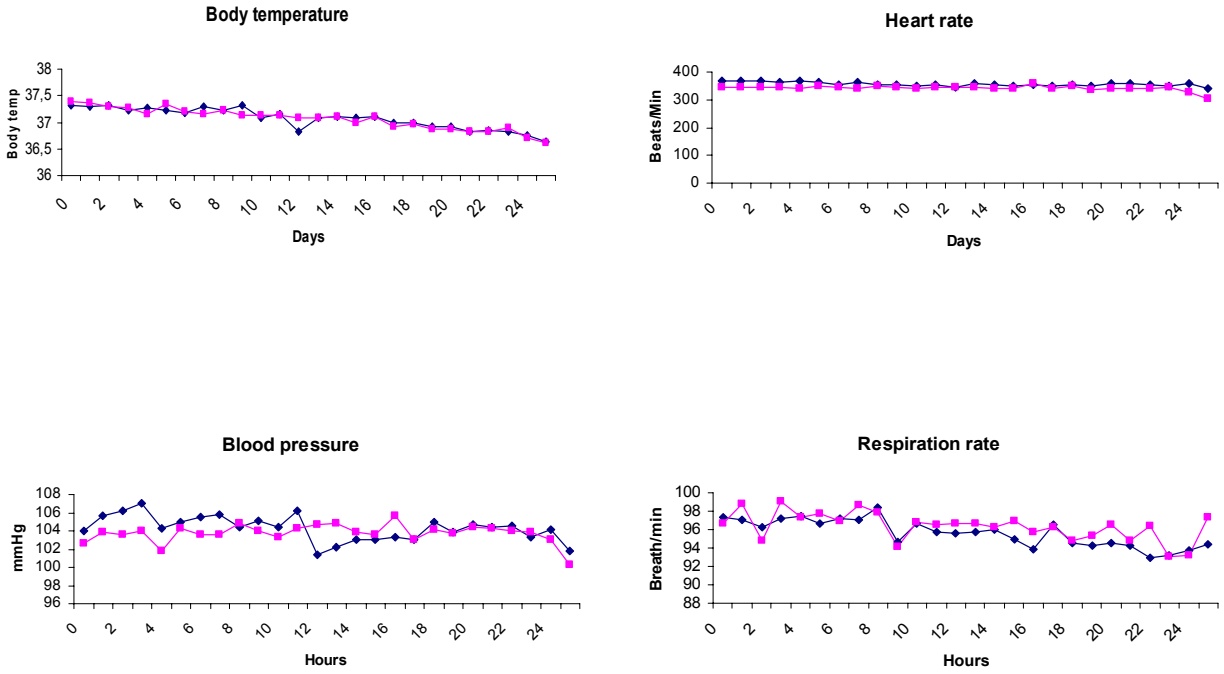

Figure 5: This figure shows the physiological parameters measured by telemetric transmitters. ■ Animal received VX by osmotic pump. ♦ Control animals.

3.5.2 Body weight

There is a small but not significant reduction of bodyweight after administration with 5 µg/kg/24 hours with the osmotic pump (Figure 6).

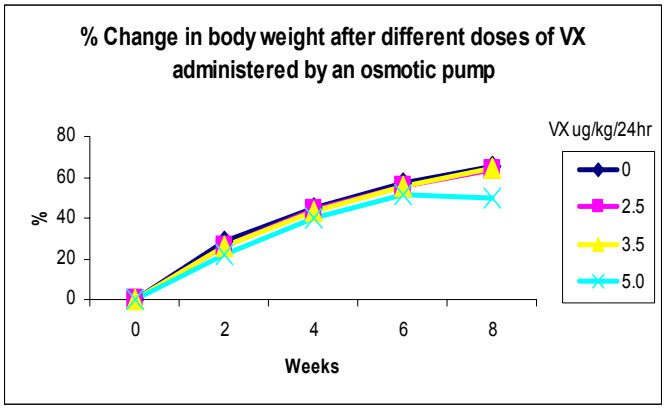

Figure 6: This figure shows the rat body weight in animals administrated with VX in different concentrations by an osmotic pump. Each point represents mean value for 4 rats.

3.5.3 Analysis of biomarkers

Already after 2 weeks, a reduction of AChE activity was analysed in all exposed animals without observation of any clinical symptoms. This reduction was sustained during the 8 week study. The other biomarkers T4, S-100 and corticosterone did not significantly differ from the control groups of rats.

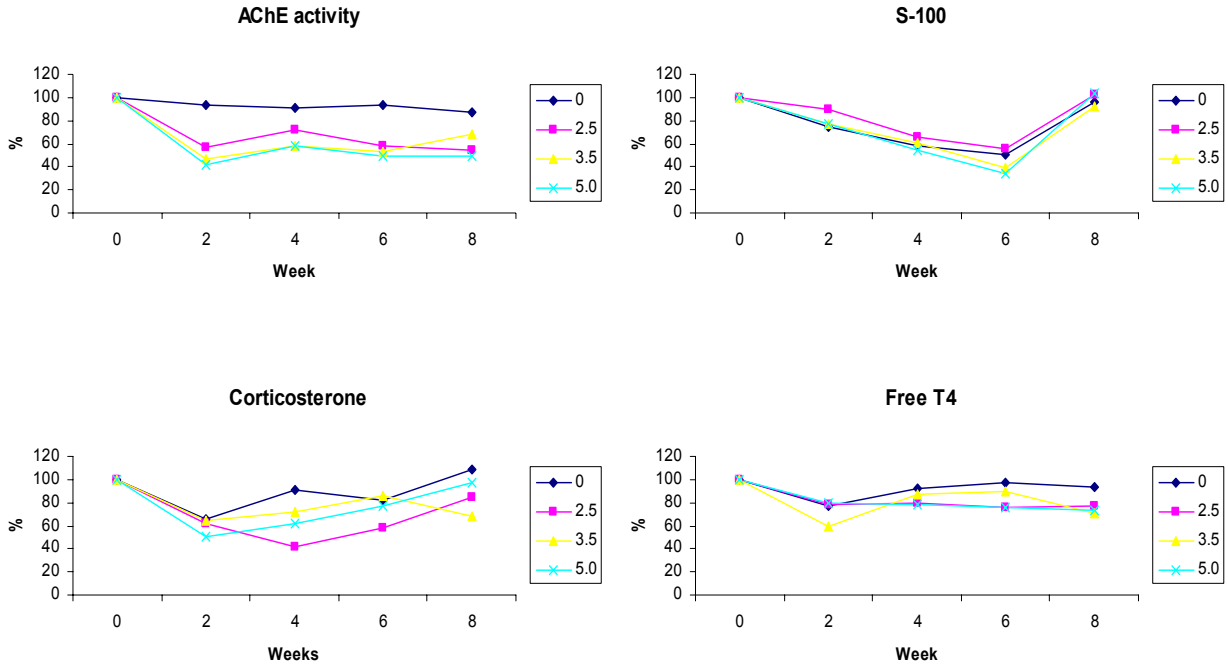

Figure 7: This figure shows the change in inhibition of AChE, the serum levels of free T4, S-100 and corticosterone in animals exposed for different doses of VX. Each point represents mean value for 4 rats.

4.0 DISCUSSION

These treatments induced no clinical signs of intoxication, even though red blood cell AChE was inhibited. The level of VX in blood was under detection limits in our experimental design. However we intend to further evaluate if it can be possible to detect VX metabolites in urine, which can be used as biomarkers for exposure. Our result shows a good correlation between the response in body weight gain and AChE inhibition. These results demonstrate the importance to pay attention to these parameters when determine a suitable dose for long- term and low dose experiment.

This project concerns the primary steps in a long-sighted investigation on health risks for people at exposure to toxic substances such as OP and related compounds. At present it is not known if and how these substances in low levels contribute to the incidence of diseases such as allergies, cancer, immunologic, hormonal neurological symptoms at long time exposure.

Specific goals in this project were to

- develop an animal model to determine the physiological effect of long time low level exposure
- find out what the target organs are from the animal model
- search for early biomarkers to verify exposure and/or effect

Our results demonstrate that different physiological parameters and biomarkers for exposure and effect can be studied in awake freely moving rats during a long period of time. This animal model can be exposed to separate chemicals or a mixture of exposures. With further development the model can be useful to study susceptibility between individuals. Moreover, the animal model used in this project is shown to give the animal a low level of stress and will not cause unnecessary pain to the animals [17].

5.0 REFERENCES

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