



Non-cholinergic intervention of sarin nerve agent poisoning

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ABSTRACT

The protective effects of selected anesthetic regimens on sarin (GB) were investigated in domestic swine. At 30% oxygen, the toxicity of this agent in isoflurane anesthetized animals ($LD_{50} = 10.1 \mu\text{g/kg}$) was similar to literature cited values in awake swine ($LD_{50} = 11.8 \mu\text{g/kg}$) and slightly higher than that of both ketamine ($LD_{50} = 15.6 \mu\text{g/kg}$) and propofol ($LD_{50} = 15.3 \mu\text{g/kg}$) anesthetized swine. Use of 100% oxygen in ketamine anesthetized animals resulted in three-fold protective effects compared to 30% oxygen. Use of 100% oxygen in both isoflurane and propofol anesthetized animals, compared to 30% resulted in profound protection against GB poisoning ($>33\times$). There were no differences in the severity of the poisoning or recovery time in animals treated over dose ranges of 10–350 $\mu\text{g/kg}$ (isoflurane) or 15–500 $\mu\text{g/kg}$ GB (propofol). Survivors of high GB challenges that were revived from propofol anesthetic exhibited no signs of cognitive impairment seven days later. Protective treatments did not attenuate cholinesterase (ChE) inhibition; survivors of otherwise supralethal GB concentrations exhibited very low blood ChE activities. This work indicates that propofol has protective effects against GB, and that oxygen tension may have an important role in treating nerve agent casualties. More importantly, it demonstrates that non-cholinergic protective mechanisms exist that may be exploited in the future development of medical countermeasures against organophosphorous nerve agents.

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1. Introduction

Organophosphorous (OP) compounds have found multiple uses due to their high toxicity, notably in both insects, and in humans. They have been extensively used as a first line defense against insect pests and although utilized with decreasing frequency in recent years due to toxicity in non-target species, including humans, many different OP insecticides are still in use around the world. The OP nerve agents are the most toxic members of the chemical warfare agents and were first developed immediately prior to and during World War II. These chemical weapons are relatively easy to synthesize and disperse, have sufficient toxicity to ensure lethality in a significant portion of exposed individuals, and also have the added attribute of creating psychological terror in those in close proximity to an attack. They have been documented as being used in terrorist attacks in Japan by the Aum Shinrikyo cult (Nagao et al., 1997; Nozaki et al., 1995a,b; Okudera et al., 1997), as well as by the Iraqi forces of Saddam Hussein against their Iranian opponents, and their own Kurdish civilian population (Macilwain, 1993; United Nations, 1986, 1987, 1988). The development of medical countermeasures against OP compounds has been predicated on their perceived mechanism of action, which is accepted

as being primarily the inhibition of acetylcholinesterase (AChE). This enzyme inhibition prevents the degradation of the neurotransmitter acetylcholine and results in its build-up at the synaptic or neuromuscular cleft. The resultant symptoms of intoxication include miosis, blurred vision, headache, respiratory secretions, nausea, vomiting, diarrhoea and fasciculations. In severe cases of exposure, these symptoms progress to paralysis, loss of consciousness, apnea, seizures and death (Cannard, 2006; Newmark, 2004; Sidell, 1997). Treatment of individuals exposed to OP nerve agents include decontamination, anticholinergics such as atropine to counteract the muscarinic effects, oxime reactivation of inhibited AChE and an anticonvulsant. Other than work investigating the efficacy of OP scavenging enzymes such as butyrylcholinesterase (Doctor and Saxena, 2005), very little effort has been expended on the development of medical countermeasures that do not target AChE.

The G series of OP nerve agents are inhalation hazards and exposure results in a rapid progression of signs of poisoning. During research activities involving a variety of different anesthetic regimens, it became apparent that the choice of anesthetic played a role in the resultant toxicity of this agent. Unexpectedly, it also appeared that inhaled oxygen tension was a significant determinant in the poisoning of anesthetized animals. In this paper we report work that systematically evaluated the effects of isoflurane, ketamine, propofol and oxygen on the toxicity of intravenously injected sarin (GB) in domestic swine. In addition, we also describe efforts to

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identify the mechanism through which selected anesthetic regimens exerted their dramatic protective effects against this agent.

2. Methods

2.1. Animal work

Castrated male York-Landrace cross pigs (~20 kg) were purchased from a local supplier and housed indoors. The animals were housed in groups of six per pen in a temperature controlled area with a 12 h/12 h light/dark cycle. The animals had free access to water and were fed twice per day. Animals were allowed to acclimatize for at least one week prior to experimental use. At the end of each treatment regimen, the animals were euthanized by intravenous injection of 10 mL (540 mg/mL) of sodium pentobarbital (Bimeda-MTC, Cambridge, Ontario, Canada). In conducting this research the authors adhered to the "Guide to the Care and Use of Experimental Animals" and "The Ethics of Animal Experimentation" published by the Canadian Council on Animal Care. Domestic swine have been shown to be a good surrogate for human physiological studies (Weiskopf et al., 1992) and this laboratory has a great deal of experience with this animal model.

2.2. Instrumentation

After weighing, all animals underwent an inhalation induction in the transport sling. Isoflurane 5% (Abbott Laboratories, Montreal, Quebec, Canada) in a carrier gas of 100% oxygen (O_2) at a flow rate of 8 L min⁻¹ was administered utilizing a Boyle anesthesia machine with a circle patient anesthesia circuit. Post-induction, the animal was placed in the dorsal recumbent position on a heated operating table. Animals were intubated with a 6.5 mm internal diameter (ID) cuffed oral endotracheal tube (Ruschelitz, Willy Rusch AG, Kernen, Germany) and the cuff inflated with room air to achieve a seal. The isoflurane concentration was reduced to 3% in 100% O_2 , or 30% O_2 in air at a flow rate of 1 L min⁻¹. The animals received intravenous fluids via a 22 gauge catheter. Core body temperature was maintained at approximately 38.5 °C with a Sage-London Industries Inc. Therm-o-matic™ heated operating table (Sage-London Industries Inc., Cambridge, Ontario, Canada).

2.3. Anesthetic regimens

Once the animals were fully instrumented, maintenance anesthesia was started. For animals receiving isoflurane, this was administered at a rate of 2% in medical grade air supplemented with oxygen to a FiO_2 of 0.3 or 1.0. When ketamine or propofol were the maintenance anesthetics, a second iv was established and the maintenance dose of the respective anesthetic started (ketamine; 30 mg/kg/h, propofol; 10–16 mg/kg/h after an initial bolus of 5–10 mg). The animals were breathing medical grade air supplemented with oxygen to a FiO_2 of 0.3 or 1.0. Concomitantly with the anesthetic, all animals received maintenance iv normal saline (sodium chloride 0.9%, Abbott Laboratories Ltd.) at a rate of 9.5 mL/kg/h via a volumetric infusion pump (Travenol FloGard 8000, Travenol Laboratories).

2.4. Monitors

End-tidal partial pressure of carbon dioxide in mm Hg, percentage oxygen saturation of arterial blood, heart rate and rhythm (ECG) and direct arterial blood pressure were monitored with a Siemens SC 7000 patient monitor. Data was continuously downloaded and stored on a Windows-based computer using customized programming (Infinity Computer Services, Medicine Hat, Alberta, Canada). A VarFlex flow transducer (700–2–300, Allied Healthcare Products, Inc., Riverside, CA) was attached to the proximal end of the endotracheal tube. An 8 FR Smart-Cath oesophageal catheter (700–3–100, Allied Healthcare Products, Inc., Riverside, CA) and extension (700–3–200, Allied Healthcare Products, Inc., Riverside, CA) was positioned in the oesophagus. These were connected to a Bicare CP 100 pulmonary monitor (Bear Medical Systems, Inc., Riverside, CA) that was, in turn, connected to a Dell Optiplex 590 IBM compatible PC. Data was displayed and stored using customized software (Pulmonary Monitor 1.01, Black Cat Software, Calgary, Alberta, Canada). After the monitors were placed, the animals were allowed to stabilize for at least 30 min, during which time steady-state anesthesia (SSA) was established and baseline physiological measurements were obtained.

2.5. Biochemistry and cholinesterase (ChE) activity

Samples for blood gas analysis and cholinesterase levels were obtained prior to induction of general anesthesia, after the equilibration period of 30 min on 2% isoflurane, ketamine or propofol, and throughout the experimental period. Blood gas analysis was performed using an ABL5 Blood gas analyzer (Radiometer Copenhagen, Denmark). Whole blood cholinesterase (ChE) activity was determined by the radiometric method of Johnson and Russell (1975) and was a measure of the combined activities of acetylcholinesterase and butyrylcholinesterase.

2.6. LD₅₀ determination

Sarin (>98%, isopropyl methyl phosphonofluoridate; GB) was synthesized in the Canadian Single Small Scale Facility at DRDC Suffield. After SSA was obtained, GB diluted in 3 mL of isotonic saline was administered via an iv injection over a 5 min period into an ear vein. The animals were followed for 6 h post-agent treatment. Animals not undergoing behavioural testing were euthanized by injection of a lethal dose of euthanyl at the termination of the 6 h exposure period.

An up-and-down method for small samples (Dixon, 1965) was used to determine the 6 h LD₅₀s of the animals whilst they were under anesthesia. The minimal number of animals per group was 4. If all 5 animals in a group yielded the same outcome, additional animals were used until at least one outcome was different. Cholinesterase activity within anesthetic groups was statistically analyzed using an unpaired *t*-test with *p* < 0.05 (GraphPad Prism, version 5 for Windows, GraphPad Software, San Diego, CA).

2.7. Behavioural task

The training task was carried out as described by Alam et al. (2005), where the swine rely on visual cues such as color, as well as their strong "rooting behaviour". Animals were obtained at approximately 15 kg of weight for these studies. The test/training animal was placed in a square training arena with three square plastic bowls containing food that were placed on the floor in three of the four corners. Each box had a different colored lid, with only one lid color signifying a container that could be opened and the food accessible. The animal was expected to recognize the container with accessible food, and progress directly towards it to obtain the food, without attempting to open the other two containers. The containers were placed in three of the four corners of the training arena and at each session were rotated into different positions. The time to complete the task during each session was monitored for each animal (maximum time was 300 s) and a composite performance score was calculated, as follows:

- +2 = opens blue container
- +1 = touches blue container but does not open it
- +0 = does not approach the other two containers
- +0 = smells either of the other two containers
- 1 = tries to open either of the other two containers.

The maximum score possible was +3 (opening of blue container without moving towards the other two containers). Each false attempt was scored as a negative mark, of which there was no maximum. The session ended as soon as the blue box was opened or at 300 s. Each animal was given one trial per day for 5 consecutive days to reach a criterion score of at least +2 within 60 s.

One day after the last training session, the animals were anesthetized using the propofol/100% oxygen regimen. Test animals were exposed to 100 µg/kg GB (representing 10× LD₅₀ of sarin under the isoflurane/30% oxygen regimen) and then closely observed for 6 h post-exposure. Following the 6 h period, the anesthetic infusion was stopped and a 10 mg dose of diazepam (im) was given. The animals were kept sedated for most of the first night and monitored closely until they appeared to be physiologically stable. They were then returned to the housing area to fully recover. On the second, third, fourth and seventh day after exposure, the animals were retested on the previously learnt task. The animals were sacrificed after testing on the seventh day.

2.8. Isolated diaphragm-phrenic nerve preparation

This neuromuscular junction preparation was set up in a manner similar to that described by Bulbring (1946). The left hemi-diaphragm with the attached phrenic nerve was isolated from male Sprague Dawley rats (150–175 g, Charles River, St. Constant, Quebec, Canada) and mounted on a special tissue carrier. The tissue holder was immersed in an organ bath containing Krebs-Henseleit solution maintained at 37 °C and bubbled with 95%/5% O_2 /CO₂ gas. The tissue preparation was attached via a thread suture to an isometric transducer for recording contractions on a Biopac® systems MP150. The diaphragm contracts in response to "direct" stimulation using a set of parallel electrodes which serve to anchor the diaphragm, or for "indirect" stimulation via a second set of electrodes which stimulates the phrenic nerve. Using a Grass S88 stimulator, the phrenic nerve was stimulated continuously with supra-maximal square wave pulses (0.25 ms) at a frequency of 0.1 Hz followed by three 3 s periods at 30 s intervals at several different frequencies (25 Hz, 50 Hz and 100 Hz) to evoke twitch and tetanic responses, respectively. The procedure was repeated every 15 min. Stimulated contractions were assessed in the presence and absence of sarin and/or propofol added to the bath as follows: (i) the contraction of the tissue preparation prior to being treated with any of the drugs was first recorded, prior to the tissue being exposed to 0.1 µM of GB for 15 min, followed by a 60 min washout period of the muscle bath buffer; (ii) propofol (0.5 mM) was administered for 15 min followed by a 60 min washout period; (iii) propofol and GB were given simultaneously for 15 min followed by the washout period, and (iv) the phrenic nerve preparation was pre-treated with propofol for 15 min. Then, in the continuous presence of propofol, the phrenic nerve was exposed to GB for 15 min, followed by the washout period.

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