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The dose–response effects of repeated subacute sarin exposure on guinea pigs☆

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Abstract

The present study assessed the effects of repeated subacute exposure to the organophosphorous nerve agent, sarin. Guinea pigs were injected five times per week (Monday–Friday) for 2 weeks with fractions of the established LD_{50} dose of sarin (42 μg/kg sc). The animals were assessed for the development of cortical EEG seizures. Changes in body weight, red blood cell (RBC) acetylcholinesterase (AChE) levels and neurobehavioral reactions to a functional observational battery were monitored over the 2 weeks of sarin exposure and for an extended postinjection period. There were dose-related changes in body weight and RBC AChE levels. No guinea pigs receiving 0.3, 0.4 or $0.5 \times LD_{50}$ of sarin showed signs of cortical EEG seizures despite decreases in RBC AChE levels to as low as 10% of baseline. Seizures were evident in animals receiving $0.6 \times LD_{50}$ of sarin as early as the second day, and subsequent injections led to incapacitation and death. Animals receiving $0.5 \times LD_{50}$ sarin showed obvious signs of cholinergic toxicity, which included a significant increase in their angle of gait. Overall, 2/13 animals receiving $0.5 \times LD_{50}$ sarin died before all 10 injections were given. By the 10th day of injections, the animals receiving saline were significantly easier to remove from their cages and handle as compared to the first day of injections. They were also significantly less responsive to an approaching pencil and touch on the rump in comparison to the first day of testing. In contrast, the animals receiving $0.4 \times LD_{50}$ sarin failed to show any significant reductions in their responses to an approaching pencil and a touch on the rump as compared to the first day. The $0.5 \times LD_{50}$ sarin animals failed to show any significant changes to the approach response and touch response and did not adjust to handling or cage removal from the first day of injections to the last day of handling. In summary, the guinea pigs receiving the $0.4 \times LD_{50}$ and $0.5 \times LD_{50}$ doses of sarin failed to habituate to some aspects of the functional observational battery testing.

Keywords: Organophosphate; Sarin; Functional observational battery; Acetylcholinesterase inhibitors; Acetylcholinesterase; Nerve agents

1. Introduction

Chemical warfare nerve agents, such as sarin, soman and VX, disrupt normal nervous system transmissions through the irreversible inhibition of acetylcholinesterase (AChE), the enzyme that breaks down the neurotransmitter acetylcholine. The buildup of acetylcholine in response to a large exposure to nerve agents can lead to muscle weakness, increased secretions, respiratory depression, seizures, coma and death, unless promptly treated. The progression of symptoms and their neuropharmacological basis elicited from acute high-dose exposures has been well characterized (McDonough and Shih, 1997). However, much less is known about long-term effects of repeated low-dose nerve agent exposure. For a comprehensive review of the available literature on low-level chemical warfare agent toxicity, see the text by Romano et al. (2001).

There have been previous animal studies investigating the neurobehavioral effects of repeated subacute exposure to organophosphate AChE inhibitors. For example, Prendergast et al. (1998) demonstrated that long-term organophosphate exposure (>5 days) leads to memory deficits in the

☆ Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals, and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

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rat. These animals were exposed to diisopropylfluorophosphate (DFP; 0.25 mg/kg/day) for 14 days. The DFP-treated rats showed significant declines, as compared with controls, in their abilities to initially learn a spatial recognition task. In the great majority of the available literature on repeated low-dose exposure to chemical warfare nerve agents, soman is utilized as the organophosphate. Repeated low-dose soman exposure has been investigated in guinea pigs (Sterri et al., 1981, 1982), mice (Sterri et al., 1981), rats (Dulaney et al., 1985; Howerton et al., 1991; Hymowitz et al., 1985; Kerenyi et al., 1990; Shih et al., 1990; Sterri et al., 1980) and primates (Blick et al., 1991, 1994; Gause et al., 1985). The effects of the repeated soman exposures, cited above, ranged from performance decrements on a well-learned compensatory tracking task (Blick et al., 1994) to development of attention deficits (Gause et al., 1985) to hyperreactive responses to handling (Shih et al., 1990).

Unlike soman, the nerve agent sarin has been used previously by extreme terrorist groups (e.g., 1995 Tokyo subway incidence; Nozaki et al., 1995) and on the battlefield (Brown and Brix, 1998; Macilwain, 1993). Additionally >98,000 Gulf War soldiers have been notified that they potentially were exposed to a plume of sarin when American forces destroyed an ammunition depot shortly after the end of the Gulf War (Enserink, 2001). While all these instances may be classified as acute or potentially acute exposures to sarin, the possibility of repeated low-dose sarin exposure to workers certainly exists somewhere along the way from manufacturing to transport to usage to detoxification of the nerve agent. Additionally, there is a worldwide movement toward destruction of chemical weapons stockpiles that are maintained by more countries than ever before (Marrs et al., 1996) and the possible long-term neurobehavioral effects of trace nerve agent exposure on the workers are virtually unknown. In general, exposure to low-level chemical warfare nerve agents is a potential emerging health hazard that requires further investigation.

Despite these facts, the amount of literature investigating the effects of repeated low-level exposure to sarin is rather sparse. Burchfiel et al. (1976) exposed rhesus monkeys to repeated low levels of sarin (1 μg/kg im) once per week for 10 weeks. Despite increases in high-frequency beta activity, upon EEG analysis, there were no signs of adverse health or long-term behavioral effects. In contrast, when sarin was administered (subcutaneously) to rats once per day for up to 85 days, doses less than $0.3 \times LD_{50}$ resulted in significant reductions in body weight gains by as early as the seventh day of injections (Dulaney et al., 1985). In the same study, a dose approximately $0.36 \times LD_{50}$ resulted in the death of 4 of 11 animals by the 10th day of injections. Husain et al. (1993), using a repeated inhalation protocol (5 mg/min/m^3 for 20 min/day for 10 days) in mice, showed that sarin exposure amounting to less than $0.2 \times LC_{50}$ per day resulted in delayed (14th day after exposure) muscle twitching and weakness in the extremities and slight ataxia.

Ongoing medical research against the toxic effects of nerve agents in our laboratory has been focused on a model using the guinea pig. Guinea pigs are considered a more valid rodent animal model for the toxicological effects of nerve agents such as sarin or soman than are mice or rats. Rats and mice possess large amounts of carboxyesterase enzyme that nonspecifically binds nerve agents such as sarin, and they require substantially higher acute doses of these agents to produce equivalent toxic effects than do guinea pigs or higher species such as nonhuman primates (Maxwell et al., 1987). The primary objective of the current study was to develop a model for repetitive subacute sarin exposure in the guinea pig that did not elicit severe symptoms of intoxication (e.g., tremors, epileptiform seizures or death) when injected once per day over a 2-week (Monday–Friday) period. This model was used to assess whether *subtle* neurobehavioral and/or physical deficits develop and persist in the animals exposed to the 2 weeks of sarin injections. As a positive control, a dose group that did develop overt signs of sarin intoxication was also included.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs (290–430 g starting weight), obtained from Charles River Laboratories (Kingston, NY), were used for these studies. Upon arrival, the animals were quarantined and tested for evidence of disease. They were individually housed in polycarbonate cages at controlled temperature (21 ± 2 °C) and humidity (50 ± 10%). They were maintained on a 12–h light/dark cycle with lights on at 0600 h. Laboratory chow and water were freely available at all times animals were in home cages.

2.2. Surgery

Guinea pigs were anesthesized with isoflurane and surgically implanted with cortical screw electrodes using standard small animal aseptic surgical techniques reported previously (Shih and McDonough, 1999). Stainless steel cortical EEG screws were placed approximately 3.0 mm lateral from midline and equidistant between bregma and lambda. The screws were attached to a miniature connector via stainless steel wires. The screws, wires and connector were then anchored to the skull with dental acrylic. The guinea pigs were allowed to recover for at least 6 days before experiments began.

2.3. Nerve agent

Sarin was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). It was diluted in sterile saline (0.9% NaCl, USP) in concentrations to deliver injection volumes equal to 0.5 ml/kg and

maintained on ice. An institute historic database indicated that the subcutaneous LD_{50} for sarin is in the range of 38–46 μg/kg. An initial experiment was conducted to verify the LD_{50} dose of sarin in guinea pigs, which was determined to be 42 μg/kg sc. Subsequent dosing was based on 42 μg/kg sc as the LD_{50}.

2.4. Experimental protocol

Animals were injected with selected doses of sarin or saline 5 days/week (Monday–Friday) for 2 weeks. The injections were administered (subcutaneously) under the skin of the back in a volume equal to half of the animal's body weight. The subcutaneous route of administration was chosen based on its extensive use by other investigators on the effects of chronic nerve agent exposure and the fact that there is minimal first-pass detoxification of the agent by the liver (Dulaney et al., 1985; Shih et al., 1990; Sterri et al., 1980, 1981, 1982). A pilot study was first performed to determine what dose of sarin could be repeatedly injected over the 2-week period. The doses of sarin tested in this pilot study were $0.3 \times LD_{50}$, $0.4 \times LD_{50}$, $0.5 \times LD_{50}$ and $0.6 \times LD_{50}$ ($LD_{50} = 42$ μg/kg; $n = 4$ animals at each dose). Baseline EEG recordings were taken for 10 min prior to all sarin injections, and EEG recordings were continued for an additional 40 min following sarin injections. In the pilot study, the guinea pigs were monitored for epileptiform seizure activity, changes in body weight and red blood cell (RBC) AChE levels. A predetermined criterion for the main experiment was to determine the maximum allowable doses of sarin that did and did not elicit symptoms of nerve agent intoxication. From the results obtained in the pilot study, the $0.4 \times LD_{50}$ and $0.5 \times LD_{50}$ doses of sarin were chosen as the doses to be injected in the full experiment, along with saline controls. In the full study, animals were randomly assigned to treatment groups (saline, $0.4 \times LD_{50}$ or $0.5 \times LD_{50}$ sarin). The experiment was run in a series of replications, with each replication consisting of 8–10 guinea pigs randomly distributed among the three treatment conditions. In the full experiment, the same regimen of sarin dosing and monitoring of EEG, body weight and RBC AChE was used. In addition, the animals were evaluated with a functional observational battery to determine such neurobehavioral functions as righting response, movement, sensory and physical deficits, etc. Functional observational battery evaluation was performed on the animals prior to the first sarin or saline injection (baseline) and then within 1 h after the sarin or saline injections on the 3rd, 5th, 6th, 8th and 10th days. Functional observational battery evaluation was also performed on the fourth and sixth days following termination (recovery period) of injections. When functional observational batteries were given following a sarin or saline injection, the animals were allowed to recover for at least 60 min from the time of injection before the functional observational battery was begun. Blood was drawn for RBC AChE levels prior to the first and sixth (weekend recovery) sarin or saline injections and 2–3 h after the 2nd, 5th, 7th and 10th injections. The animals were allowed to recover for up to 21 days with RBC AChE measurements taken on the 4th, 7th, 14th and 21st days of recovery. To reduce animal discomfort, the blood draws in the guinea pigs were staggered such that blood was not drawn from every animal on every day of blood collection.

2.5. AChE assay

RBC AChE activity was assessed by extracting approximately 0.5 ml of blood via toe-nail clip (Vallejo-Freire, 1951). Whole blood was prevented from clotting by the addition of a small amount (15μl) of EDTA (4 g/l). Whole blood was separated into plasma and RBC by centrifugation (11 min; $14{,}000 \times g$). RBC AChE activity was determined, using acetylthiocholine iodide as a substrate, by an automated method using a COBAS/FARA clinical chemistry analyzer (Roche Diagnostics, Nutley, NJ). The analytical procedure was based on the manual method of Ellman et al. (1961) and modified for the COBAS/FARA by Hobson et al. (1988).

2.6. Neurobehavioral testing

The functional observational battery is a sequence of rapid tests (completed in 6–8 min) used to assess neurological functions. It allows for the qualitative and quantitative evaluation of the behavioral and physiological effects of neurotoxicants (Bowen and Balster, 1997; Moser et al., 1988; Tegeris and Balster, 1994; Youssef and Santi, 1997). Two technicians, who were unaware of the treatment of the animals, performed all functional observational battery scorings. The order of animal selection, for the neurobehavioral testing, was performed randomly by the scorer. The scorers tended to test the animals in numerical order, from lowest to highest, based on their identification numbers. The scoring sheet (see Appendix A) was adapted from those previously published (Moser et al., 1988; Youssef and Santi, 1997), with slight modifications for guinea pigs. The specific sequence of testing was as follows.

2.6.1. Home cage

While in their home cages, the guinea pigs were scored positive or negative for the presence of agitation, chewing, tremors, facial dysmorphia and vocalizations. They were graded on a scale for ease of removal, ease of handling and presentation of physical symptoms, such as fur appearance (piloerection), emaciation, lacrimation and salivation.

2.6.2. Open field

The animals were placed on top of a laboratory cart and latency to first movement was timed. The animals were then allowed to move freely for 2 min. During this time, the animals were scored on their gait description and their level

of arousal. The number of grooms, urine spots, fecal matter and rears was counted and recorded.

2.6.3. Reflexes

The guinea pig's responses to an approaching pencil, a tap on the rear and a loud click behind the head were graded. Righting reflex was then measured by placing the animal on its back and recording the time it took for the guinea pig to get to his feet. The guinea pig was then dropped, from a supine position, from a height of 30 cm onto a soft landing area. The ease of landing was scored.

2.6.4. Splay and gait

The guinea pig's hindlimbs were painted with water-based tempura paint. Hindlimb foot splay was obtained by dropping the guinea pig, from a prone position, from approximately 30 cm high onto a sheet of paper placed on the countertop. The distance between the middle toes of each footpad was measured. The footpads were then repainted and the guinea pigs were placed on a new sheet of paper and allowed to walk freely. The testing was concluded when the guinea pigs maintained forward movement for a minimum of three successive steps in a straight path. The angle from the first footstep to the second and third steps was measured.

2.7. Pathology evaluation

Within 1 month of the animals' last sarin or saline injection, the guinea pigs were sacrificed by deep anesthesia (75 mg/kg ip pentobarbital) followed by intracardiac perfusion and fixation. The fixed brains and hearts were then removed, sectioned and stained for hematoxylin and eosin (H&E) to assess tissue damage. Brain and heart pathology was analyzed as previously published (McDonough et al., 1995). Animal tissues were evaluated by a board-certified veterinary pathologist who was unaware of the experimental history of a given subject.

2.8. Data analysis

For RBC AChE data and numerical data in the functional observational battery, one-way analysis of variance (ANOVA) was used to determine whether significant differences existed. For body weight change data, gait angle data and foot splay data, a two-way (Treatment × Day) repeated-measures ANOVA was used. Post-hoc Tukey tests were then further used to identify significant effects. For the results of the scored parts of the functional observational battery, Kruskal–Wallis ANOVA on ranks (Hollander and Wolfe, 1973) was used to detect whether there were significant differences between baseline group scores. A Wilcoxon signed-rank test was then used to detect statistical differences between the same individual animals before and after nerve agent treatment. A difference of $P < .05$ was considered significant.

3. Results

3.1. Pilot study

None of the lower-level doses of sarin ($0.3 \times LD_{50}$, $0.4 \times LD_{50}$ and $0.5 \times LD_{50}$) elicited epileptiform seizure activity during periods of EEG recordings immediately following the sarin injections ($n = 4$ for each dose of sarin tested). However, there were noticeable symptoms (hyperexcitable, muscle tremors, piloerection, chewing) of nerve agent intoxication in animals receiving $0.6 \times LD_{50}$ sarin dose ($n = 4$) by the second day of injections. The $0.6 \times LD_{50}$ sarin dose caused seizures in two of four guinea pigs by the fourth day of injections and death in three of four guinea pigs before the final day of injections. Thus, the $0.6 \times LD_{50}$ dose of sarin did not meet the predetermined criteria and was disqualified for use in any further studies. The $0.4 \times LD_{50}$ and $0.5 \times LD_{50}$ sarin doses were chosen for full experimentation because they were the highest possible doses that did not elicit cortical EEG seizures nor produced significant toxicity when given over the 2 weeks of daily injections. The weight gain and RBC AChE data from the four animals in each preliminary treatment group were added to the data obtained from the animals in the full experiment. This was done to increase the overall number of animals available for statistical analysis of weight gain and RBC AChE data only. In the full experiment, there were 24 animals that received saline or $0.4 \times LD_{50}$ sarin (final total $n = 28$ for saline and $n = 28$ for $0.4 \times LD_{50}$) and 9 animals that received $0.5 \times LD_{50}$ sarin in the full experiment (final total $n = 13$). The reason for the lower number of guinea pigs receiving $0.5 \times LD_{50}$ sarin is explained below.

3.2. Neurotoxicity

While 4/4 guinea pigs receiving $0.5 \times LD_{50}$ sarin survived without seizures for the 10 injections given during the pilot study, this was not the case in the full experiment, where two of nine guinea pigs receiving $0.5 \times LD_{50}$ sarin died before the 2 weeks of injections were completed. These two animals never showed signs of EEG epileptiform seizures. Therefore, while the $0.5 \times LD_{50}$ sarin dose given in the pilot study met predetermined criteria, the same dose in the full experiment did not. For this reason, we terminated further use of the $0.5 \times LD_{50}$ dose of sarin, and the total number of animals given this dose was 13 (four from the pilot study plus nine from full experiment).

3.3. Weight changes

The guinea pigs showed dose–response changes in body weight over the 2 weeks of injections (Fig. 1). The overall average weight gains (calculated as the animal's weight on the 10th day of injections minus the animal's weight prior to the first injection) for the animals receiving saline, $0.4 \times LD_{50}$ and $0.5 \times LD_{50}$ sarin were 56.89 ± 2.36,

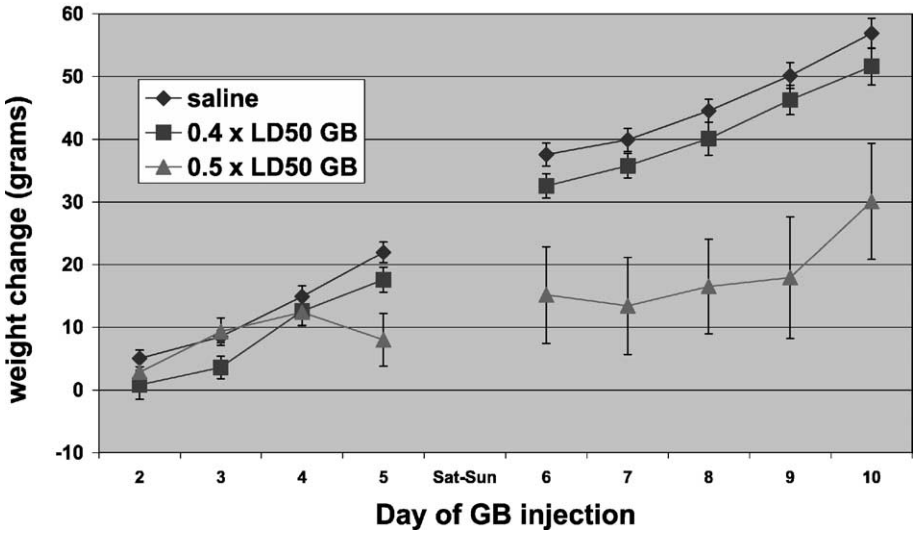

Fig. 1. Weight change vs. day of sarin injection. The animals were weighed prior to the first sarin injection and then before each subsequent day's injection. Data are graphed as the change between the initial weight and the weight prior to each day's sarin injections. Values are expressed as mean ± S.E.M. $n = 28$ for saline group, $n = 28$ for $0.4 \times LD_{50}$ sarin group, $n = 11$ for $0.5 \times LD_{50}$ sarin group (there were 13 guinea pigs in total, but two guinea pigs died before the eighth day of injections and thus were not included in statistical analysis using a two-way repeated-measures ANOVA). There is a dose–response change in weight in relation to the dose of sarin given. The animals receiving the $0.5 \times LD_{50}$ dose of sarin showed significantly less overall weight gain ($P < .005$) than either the animals receiving saline or $0.4 \times LD_{50}$ sarin.

51.6 ± 2.81 and 30.09 ± 5.25 (mean ± S.E.M.) g, respectively. The two-way ANOVA, with repeated measures on days, showed significant main effects for Treatment [$F(2,64) = 8.18$, $P < .001$], Days [$F(8,512) = 263.39$, $P < .001$] and the Treatment × Days interaction [$F(8,512) = 8.81$, $P < .001$]. Both the saline control and $0.4 \times LD_{50}$ sarin groups gained significantly greater amounts of weight than did the $0.5 \times LD_{50}$ sarin group throughout the 10-day exposure period. The weight differences first became significant on Day 5, with the $0.5 \times LD_{50}$ sarin group weighing significantly less than the saline control group. Over the next week, this growth difference became even more notable, with the $0.5 \times LD_{50}$ sarin group gaining significantly less weight than either the saline controls or the $0.4 \times LD_{50}$ sarin group on injection Days 6–10. There were no significant differences between the weight gains

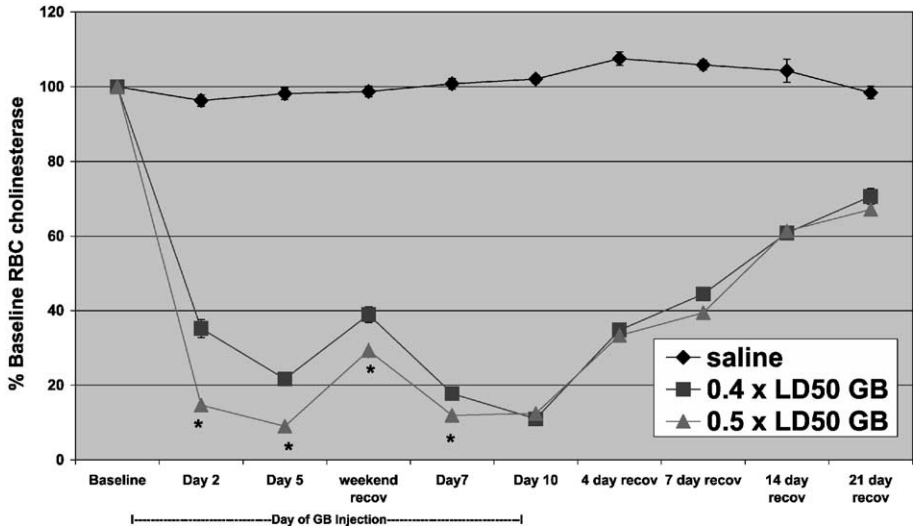

Fig. 2. RBC AChE levels vs. day of sarin injection. Blood was taken via toe-nail clip at different times during the sarin injections and RBC AChE levels were determined by standard methods. To reduce animal discomfort, blood was not drawn from every animal on every occasion except for baseline values. Values are expressed as mean ± S.E.M. with a minimum of $n = 7$ RBC samples (each run in triplicate) for each data point. By the second day of sarin injections, RBC AChE in the animals receiving $0.5 \times LD_{50}$ sarin injections dropped to significantly ($P < .001$) lower levels than in the $0.4 \times LD_{50}$ sarin animals. However, by the 10th day of sarin injections, RBC AChE levels were nearly identical (11% vs. 12% of baseline values) in the $0.4 \times LD_{50}$ and $0.5 \times LD_{50}$ sarin groups. At 21 days of recovery, RBC AChE levels in the animals receiving $0.4 \times LD_{50}$ and $0.5 \times LD_{50}$ sarin had returned to 68% and 67% of baseline values, respectively. * $P < .01$ vs. $0.4 \times LD_{50}$ sarin group.

of the saline controls or the $0.4 \times LD_{50}$ sarin group on any day during the dosing period.

3.4. RBC AChE changes

By the second day of sarin injections, RBC AChE in the animals receiving $0.5 \times LD_{50}$ sarin had dropped to less than 15% of baseline values, which was significantly ($P < .001$) lower than the 35% of baseline RBC AChE levels in animals receiving $0.4 \times LD_{50}$ sarin (Fig. 2). However, by the 10th day of sarin injections, RBC AChE levels had dropped to near identical levels (11% vs. 12% of baseline values, respectively) in the $0.4 \times LD_{50}$ and $0.5 \times LD_{50}$ animals. The average 2-day weekend (Saturday–Sunday) RBC AChE recovery between the 2 weeks of sarin injections was 17% and 20%, respectively, for the $0.4 \times LD_{50}$ and $0.5 \times LD_{50}$ sarin groups. Even 21 days after termination of the injections, the RBC AChE levels of the animals receiving $0.4 \times LD_{50}$ and $0.5 \times LD_{50}$ sarin remained at 68% and 67% of control values, respectively, which were still significantly lower than the control AChE levels.

3.5. Functional observational battery

Both the guinea pigs receiving saline and those receiving $0.4 \times LD_{50}$ sarin became significantly ($P < .05$) easier to remove from their cages when comparing functional observational battery scores after the 10th injection, with those scores obtained as baseline (Table 1). The average "cage removal" score (mean ± S.E.M. on a scored scale from 1 to 3) went from 1.50 ± 0.13 to 1.04 ± 0.04 and from 1.83 ± 0.2 to 1.0 ± 0.0 for the saline and $0.4 \times LD_{50}$ sarin animals, respectively (see Appendix A for the functional observational battery score sheet used). The guinea pigs receiving saline and $0.4 \times LD_{50}$ sarin also became significantly ($P < .05$) easier to handle over the same time period. The average "handling" score (mean ± S.E.M. on a scored scale from 1 to 4) went from 2.54 ± 0.15 to 2.04 ± 0.04 for the animals receiving saline and from 2.50 ± 0.12 to 1.96 ± 0.04 for the animals receiving $0.4 \times LD_{50}$ sarin. The guinea pigs receiving saline also developed significantly ($P < .05$) decreased approach (from 2.04 ± 0.19 to 1.29 ± 0.14 on a scored scale from 1 to 6) and touch (from 2.21 ± 0.2 to 1.46 ± 0.2 on a scored scale from 1 to 6) responses over the same period. In contrast, the animals receiving $0.4 \times LD_{50}$ sarin failed to show any significant decreases in the approach and touch responses when comparing their baseline scores with the score after the 10th sarin injection. The $0.5 \times LD_{50}$ sarin animals failed to show significant changes in cage removal, touch response and approach response, and they did not adjust to handling. Additionally, no significant changes in functional observational battery scoring were observed for any of the other measurements (lacrimation, salivation, fur appearance, latency to move, number of grooms, number of rears, arousal, gait, fecal boluses, urine spots, click response, righting reflex or drop reflex) in animals treated with saline, $0.4 \times LD_{50}$ or $0.5 \times LD_{50}$ sarin (Table 1).

Table 1
Summary of the observed changes in functional observational battery scoring after 2 weeks of sarin dosing

	Saline	0.4 × LD50	0.5 × LD50
Cage removal[r]	↓ (easier to remove) *	↓ (easier to remove) *	No significant change
Handling[r]	↓ (easier to handle) *	↓ (easier to handle) *	No significant change
Lacrimation[r]	No significant change	No significant change	No significant change
Salivation[r]	No significant change	No significant change	No significant change
Fur appearance[r]	No significant change	No significant change	No significant change
Latency to move[n]	No significant change	No significant change	No significant change
Number of grooms[n]	No significant change	No significant change	No significant change
Number of rears[n]	No significant change	No significant change	No significant change
Arousal[r]	No significant change	No significant change	No significant change
Gait[r]	No significant change	No significant change	No significant change
Fecal boluses[n]	No significant change	No significant change	No significant change
Urine spots[n]	No significant change	No significant change	No significant change
Click response[r]	No significant change	No significant change	No significant change
Approach response[r]	↓ (less reactive)*	No significant change	No significant change
Touch response[r]	↓ (less reactive)*	No significant change	No significant change
Righting reflex[r]	No significant change	No significant change	No significant change
Drop reflex[r]	No significant change	No significant change	No significant change

Summary of the observed changes in functional observational battery scoring after the last day of sarin injections as compared with those scores obtained before the first injection (baseline scores). For the ranked data (r), Kruskal–Wallis ANOVA on ranks was used to detect that there were no significant differences between baseline group scores. A Wilcoxon signed-rank test was then used to detect statistical differences between the same individual animals before and after nerve agent treatment. One-way ANOVA was used to analyze numerical data (n) for significance. Guinea pigs that received saline ($n = 24$) became significantly easier to remove from their cages and handle. They also became significantly less reactive to an approaching pencil and to a touch on the rear. In short, they became "habituated" to some aspects of the testing. The average (mean ± S.E.M.) baseline scores and scores after the 10th day of injections obtained for each significant score change are referenced within the Results section of the text. The guinea pigs receiving $0.4 \times LD_{50}$ sarin ($n = 24$) failed to show any significant decreases in the approach response and touch response. The guinea pigs receiving $0.5 \times LD_{50}$ sarin ($n = 9$ for baseline but two animals died before injections were complete) failed to show any significant changes in cage removal, approach response and touch response, and did not adjust to handling.

* $P < .05$.

A two-way repeated-measures ANOVA of gait angles revealed significant main effects for Treatment Group

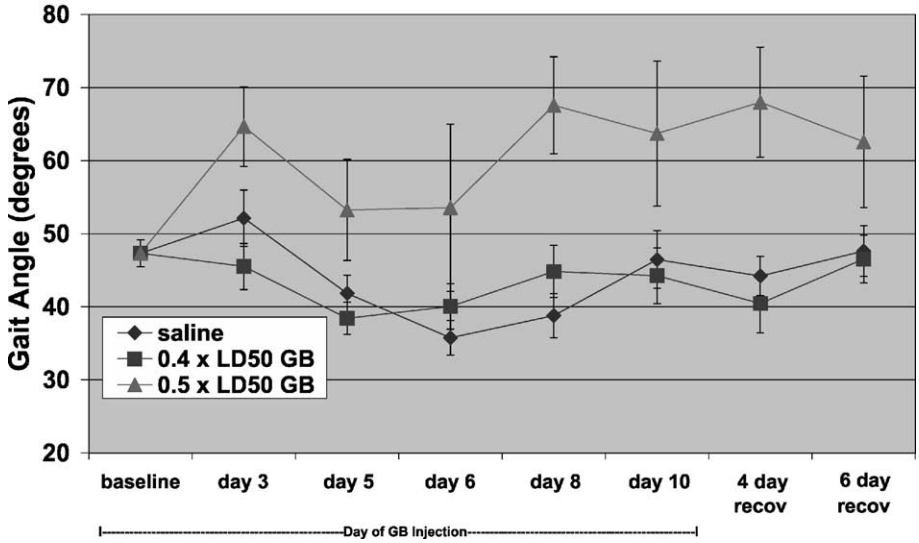

Fig. 3. Gait angle vs. day of sarin injection. This figure shows the changes in the guinea pig's angle of gait vs. the day of injections for saline and the different concentrations of sarin ($0.4 \times LD_{50}$ and $0.5 \times LD_{50}$). The gait angle is measured as described in the Materials and Methods section. Values are expressed as mean ± S.E.M. $n = 24$ for saline group, $n = 24$ for $0.4 \times LD_{50}$ sarin group, $n = 7$ for $0.5 \times LD_{50}$ sarin group (there were nine guinea pigs in total, but two guinea pigs died before the eighth day of sarin injections and thus were not included in statistical analysis using a two-way repeated-measures ANOVA). A repeated-measures ANOVA showed that, overall (main effect), the $0.5 \times LD_{50}$ sarin group had significantly greater gait angle than the saline controls or the $0.4 \times LD_{50}$ sarin group, which did not differ from one another. The average angle between hindlimb steps is exaggerated in the $0.5 \times LD_{50}$ sarin group because they tended to hop rather than walk.

[$F(2,52) = 16.05$, $P < .001$] and Days [$F(7,364) = 2.04$, $P = .05$]; the Treatment Group × Days interaction was not significant. These data are displayed in Fig. 3. The $0.5 \times LD_{50}$ sarin group had significantly greater gait angles than did the saline controls or the $0.4 \times LD_{50}$ sarin group; there was no difference between the gait angles of the animals receiving saline and those receiving $0.4 \times LD_{50}$ sarin. In general, it was observed that the guinea pigs injected with $0.5 \times LD_{50}$ sarin tended to keep their hindfeet parallel to each other and hop rather than alter steps as did the saline control and $0.4 \times LD_{50}$ sarin guinea pigs. This is illustrated by measuring the angle between consecutive footprints. Additionally, the drop reflex was impaired in 2/9 guinea pigs treated with $0.5 \times LD_{50}$ sarin; this was never observed in the saline controls or animals receiving $0.4 \times LD_{50}$ sarin. It was also observed that the animals receiving $0.5 \times LD_{50}$ sarin showed obvious muscle tremors upon attempts to move. In contrast, muscle tremors were never observed in the animals receiving the $0.4 \times LD_{50}$ dose of sarin. Hindlimb foot splay measurements were nearly identical for the saline, $0.4 \times LD_{50}$ and $0.5 \times LD_{50}$ sarin guinea pigs (data not shown) throughout the 10 days of injections and during recovery.

3.6. Pathology

No evidence of brain or heart pathology was found in any guinea pig that survived all 10 sarin injections. Of the animals that died during experimentation, no brain or heart tissue was submitted for postmortem examination because the animals died overnight and were not found until the following morning.

4. Discussion

The main objective of the current set of experiments was to establish a dose of sarin to be utilized in a model for low-dose repeated exposure to sarin in the guinea pig. A primary requirement of the model was to obtain a dose of sarin that could be given over a 2-week period of daily (Monday–Friday) exposures without causing severe, easily identifiable cholinergic symptoms such as tremors, EEG epileptiform seizures or death. Although no guinea pigs in the $0.5 \times LD_{50}$ sarin group showed signs of epileptiform seizures, 2/13 of the animals died before all 10 sarin injections were given. This dose also caused a rapid decrease in RBC AChE levels to approximately 10% of baseline values by the second day of sarin injections. Additionally, there were noticeable symptoms of sarin intoxication (chewing, hyperactivity, muscle tremor), alterations in angle of gait (Fig. 3) and impaired drop reflexes in some of the guinea pigs receiving $0.5 \times LD_{50}$ sarin. Youssef and Santi (1997) showed that multiple low-dose injections of either acrylamide or methanol, both known neurotoxicants, resulted in changes in the angle of gait similar to those observed in the study presented here. Moser (1995) used similar neurobehavioral screening batteries after a single acute dose of seven different AChE inhibitors to conclude that altered gait could be considered a "cardinal sign of toxicity for cholineste-

rase inhibitors." Because there was observatory evidence (e.g., observed tremors when attempting to move) that gait abnormalities developed after the first $0.5 \times LD_{50}$ sarin injection, it is most likely that this dose was more indicative of acute nerve agent poisoning. However, the gait problems identified in the animals receiving $0.5 \times LD_{50}$ sarin were accompanied by significant reductions in body weight gains over the same periods. Since our experiments did not include a group solely to look at the effect of weight loss on gait, we cannot completely rule out its' effect. In contrast to the signs of acute nerve agent toxicity witnessed in guinea pigs that received $0.5 \times LD_{50}$ sarin, the animals receiving $0.4 \times LD_{50}$ sarin showed no signs of acute toxicity. The fact that we found no pathological evidence in any of the animals that received all 10 sarin ($0.3 \times LD_{50}$, $0.4 \times LD_{50}$ or $0.5 \times LD_{50}$) injections supports prior studies showing that nerve agent-induced brain pathology is due to seizures (McDonough et al., 1995).

There were no statistical differences found for many of the functional observational battery scoring criteria (lacrimation, salivation, fur appearance, latency to move, number of grooms, number of rears, arousal, gait, fecal boluses, urine spots, click response, righting reflex or drop reflex) for animals receiving saline, $0.4 \times LD_{50}$ or $0.5 \times LD_{50}$ sarin (Table 1). However, the $0.4 \times LD_{50}$ and $0.5 \times LD_{50}$ sarin-treated animals showed decreased ability to habituate to certain aspects of the functional observational battery testing, unlike their saline control counterparts. One important point is that the guinea pigs used for the experiments were handled very minimally prior to the first day of injections. When comparing the functional observational battery scores obtained after the 10th day of saline injections with those obtained as baseline, it is most likely that the significant changes observed in cage removal and handling were due to the guinea pigs becoming acclimated to being handled by the technician scoring the functional observational battery. It is also most likely that the saline-treated guinea pigs became less reactive to an approaching pencil (approach response) and to a touch on the rump (touch response) because of this habituation. In contrast, the animals receiving $0.4 \times LD_{50}$ or $0.5 \times LD_{50}$ sarin failed to acclimate to some aspects of the functional observational battery testing (approach and touch responses for the $0.4 \times LD_{50}$ sarin dose; handling, cage removal and approach and touch responses for the $0.5 \times LD_{50}$ sarin dose). It is worth noting that the functional observational battery scores after the 10th day of sarin injections, for both the $0.4 \times LD_{50}$ and $0.5 \times LD_{50}$ sarin doses, were not "worse" than they were at baseline measurements. In short, the animals receiving sarin showed subtle neurobehavioral changes in that they demonstrated less ability to adapt to the functional observational battery than did the guinea pigs receiving saline. Our results are supported by a previous study (Shih et al., 1990) in which rats were injected (subcutaneously) with $0.4 \times LD_{50}$ of soman three times a week for up to 6 weeks. The animals became hyperreactive to normal handling procedures and demonstrated exaggerated startle responses to air puffs.

By the second day of sarin injections, RBC AChE levels in animals receiving the $0.5 \times LD_{50}$ dose had dropped significantly ($P < .001$) lower (14% of baseline values) than the RBC AChE levels in animals receiving $0.4 \times LD_{50}$ sarin (Fig. 2). The parallels between the onset of symptoms following the second $0.5 \times LD_{50}$ sarin injection and the rapid reduction of the RBC AChE levels to approximately 14% of control values after the second injection are consistent with the results of Grob, who found that the onset of symptoms upon human exposure to DFP (Grob et al., 1947) or sarin (Grob and Harvey, 1958) is correlated with rapid decreases of RBC AChE levels to 30% and 22% of baseline values, respectively. However, when the nerve agents were administered at lower doses over several days, there was no correlation between the onset of symptoms and RBC AChE levels. This is consistent with our studies in which RBC AChE in the animals receiving $0.4 \times LD_{50}$ sarin had dropped to 11% of baseline values by the 10th day of sarin injections. The animals receiving $0.4 \times LD_{50}$ sarin doses showed no obvious symptoms of nerve agent intoxication despite RBC AChE levels being nearly identical to those of the RBC AChE levels in the animals receiving $0.5 \times LD_{50}$ sarin after the 10th sarin injection. While the RBC AChE levels in the guinea pigs receiving $0.5 \times LD_{50}$ sarin dropped to approximately 10% of baseline values after the fifth day of injections, these levels were maintained between 9% and 12 % of baseline values despite five more injections of $0.5 \times LD_{50}$ sarin. Additionally, the RBC AChE levels in the guinea pigs receiving $0.4 \times LD_{50}$ sarin never fell below 10% of baseline values. It is commonly accepted that the only way that RBC AChE can be replaced once it is irreversibly inhibited by nerve agents is by de novo synthesis (Harris et al., 1971) of new red cells, which is thought to occur at the rate of 1–2% per day (Grob and Harvey, 1958). Therefore, we must rule out that daily replenishment alone of RBC AChE is accounting for the failure of RBC AChE levels in our animals to fall below 10% of baseline values. The failure of RBC AChE to fall below 10% of baseline levels is most likely a combination of de novo RBC AChE synthesis (Grob and Harvey, 1958; Harris et al., 1971) and an increasing rate of spontaneous reactivation of RBC AChE associated with repeated exposures to nerve agents (Lanks et al., 1977).

In summary, guinea pigs receiving $0.4 \times LD_{50}$ sarin for 2 weeks of repeated exposure were virtually indistinguishable in gross behavior and body weight changes from those that received saline over the same time period. These guinea pigs showed no obvious signs of nerve agent intoxication. In spite of these observations, animals that received $0.4 \times LD_{50}$ sarin showed a decrease of their RBC AChE to approximately 10% of baseline levels. The $0.4 \times LD_{50}$ sarin dose inhibited RBC AChE levels to approximately that of the $0.5 \times LD_{50}$ group without the subsequent symptoms of acute sarin exposure (hyperactivity, chewing, gait impairment, impaired reflexes, etc.). However, the functional observational battery

revealed that they showed very subtle neurobehavioral changes. The understanding of whether these subtle neurobehavioral changes can last for extended postexposure periods and whether these neurobehavioral changes progress in severity may be important for investigating the developing health hazards of long-term exposure to organophosphate inhibitors. Because of these reasons, the $0.4 \times LD_{50}$ dose of sarin is more suitable for utilization as a model for subacute repeated sarin exposure in the guinea pig. Studies are currently underway to utilize our model of repeated subacute sarin dosing in conjunction with in vivo microdialysis to assess neurotransmitter changes in brain, such as acetylcholine and choline, over a prolonged postinjection period.

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Appendix A. Functional observational battery score sheet

Date_____ Guinea pig number_____ Weight_____
Scoring code_____ Time started_____ Scorer_____

Home cage assessment

Agitated	___yes	_____no
Chewing	___yes	_____no
Facial dysmorphia	___yes	_____no
Tremors	___yes	_____no
Vocalizations	___yes	_____no

Animal handling

Ease of removal from cage (R) (choose one)
(1) Easy—little or no vocalization, without resistance
(2) Moderately easy—animal jumpy, initial movement followed by settling, with or without vocalizations
(3) Difficult—runs around cage, is hard to grab, with and without vocalizations

Ease of Handling Guinea Pig in hand (R) (choose one)
(1) Easy, but lethargic
(2) Easy, but alert—limbs may be pulled against body
(3) Moderately easy—vocalizations, little or no squirming
(4) Difficult—squirming, twisting, attempting to bite, with or without vocalizations

Lacrimation (R)
(1) None
(2) Slight
(3) Severe

Salivation (R)
(1) None
(2) Slight
(3) Severe

Fur Appearance (R) (choose one for each)
(1) Normal
(2) Slightly soiled/disheveled
(3) Very soiled/crusty
(4) Rough

Open field checklist (2 min)

Latency to first movement (s)_____ Total number of rears (C) _____
Total number of grooming episodes (C)_____

Arousal (R) (choose one)
(1) Very low (little or absent)
(2) Low (some head or body movement)
(3) Somewhat low (some exploratory movements with period of immobility)
(4) Normal (alert, exploratory movements)
(5) Somewhat high (slight excitement, sudden darting or freezing)
(6) Very high (hyperalert, excited, sudden bouts of running or body movements)

Gait description (D) (choose one)
(1) No movement
(2) Normal
(3) Impairment
(a) Uncoordinated movement (ataxia)
(b) Walking on toes
(c) Splayed hind limbs
(d) Exaggerated hind limb flexion
(e) Staggered gait
(f) Dragging hind limbs
(g) Unable to walk

Total number of fecal boluses (C)_____ Total number of urine spots (C)_____

Reflexes

Click response (R) (choose one)
(1) No reaction
(2) Slight reaction, ear flick or some evidence that sound was heard
(3) More energetic response than (2); may include vocalization
(4) Jumps, seems startled
(5) Freezes, actual muscle contraction
(6) Bizarre reaction: bites, attacks

Approach response (R) (choose one)
(1) No reaction
(2) Slow approach, sniffing or turning away
(3) More energetic response than (2); possible vocalizations
(4) Jumps, makes efforts to avoid object
(5) Freezes, actual muscle contraction
(6) Bizarre reaction: bites, attacks

Touch Response (R) (choose one)
(1) No reaction
(2) Slowly turns, walks away
(3) More energetic response than (2); possible vocalizations
(4) Jumps, makes efforts to avoid object
(5) Freezes, actual muscle contraction
(6) Bizarre reaction: bites, attacks

Gait scoring (C) (one trial)
Stride length (cm)_____
Stride width (cm)_____
Angle_____

Foot splay measurements (two trials) Trial 1(cm)_____ Trial 2 (cm)_____

Righting reflex (R)
(1) Normal (immediately rights itself)
(2) Slightly impaired (>1 s)
(3) Impaired (>2 s)
(4) Totally impaired (remains on back)

Drop reflex (R)
(1) Normal
(2) Slightly uncoordinated
(3) Lands on side
(4) Lands on back

Comments

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