Younger rats are more susceptible to the lethal effects of sarin than adult rats: 24 h LC50 for whole-body (10 and 60 min) exposures

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Abstract

Chemical warfare nerve agents (CWNA) inhibit acetylcholinesterase and are among the most lethal chemicals known to man. Children are predicted to be vulnerable to CWNA exposure because of their smaller body masses, higher ventilation rates and immature central nervous systems. While a handful of studies on the effects of CWNA in younger animals have been published, exposure routes relevant to battlefield or terrorist situations (i.e. inhalation for sarin) were not used. Thus, we estimated the 24 h LC50 for whole-body (10 and 60 min) exposure to sarin using a stagewise, adaptive dose design. Specifically, male and female Sprague-Dawley rats were exposed to a range of sarin concentrations (6.2–44.0 or 1.6–12.5 mg/m^3) for either 10 or 60 min, respectively, at six different times during their development (postnatal day [PND] 7, 14, 21, 28, 42 and 70). For male and female rats, the lowest LC50 values were observed for PND 14 and the highest LC50 values for PND 28. Sex differences were observed only for PND 42 for the 10 min exposures and PND 21 and 70 for the 60 min exposures. Thus, younger rats (PND 14) were more susceptible than older rats (PND 70) to the lethal effects of whole-body exposure to sarin, while adolescent (PND 28) rats were the least susceptible and sex differences were minimal. These results underscore the importance of controlling for the age of the animal in research on the toxic effects associated with CWNA exposure.

Keywords

Chemical warfare nerve agent, inhalation, lethality, development

Introduction

Chemical warfare nerve agents (CWNA) such as sarin, soman and VX inhibit the enzyme acetylcholinesterase, which hydrolyzes the neurotransmitter acetylcholine in the central and peripheral nervous systems. Extensive inhibition of acetylcholinesterase activity leads to an acute cholinergic crisis characterized by glandular secretions, miosis, muscle fasciculations, seizures, tremors and ultimately death from respiratory failure (Pereira et al., 2014). If therapeutic intervention prevents death but does not control CWNA-induced seizures, then widespread brain damage and associated behavioral and cognitive deficits may occur (de Araujo Furtado et al., 2012). As tragically observed in Syria (Rosman et al., 2014; United Nations General Assembly, 2013), children and adolescents will be exposed in a mass casualty situation involving the dissemination of CWNA amongst a civilian population. In fact, these subgroups of the population may be at greater risk for whole-body exposures to CWNA than adults because of higher ventilation rates (Mortola, 1984), larger surface area to body mass ratios (Guzelian et al., 1992) and immature central nervous systems that are prone to seizures (Ben-Ari & Holmes, 2006).

Age-related differences associated with the toxicity of organophosphorus pesticides, which inhibits acetylcholinesterase activity similar to CWNA but with less potency, have been extensively investigated. Pope et al. (1991) reported that postnatal day (PND) seven rats are more susceptible than adult rats (PND 80–100) to the lethal effects of parathion, methyl parathion and chlorpyrifos when subcutaneously administered. Moser et al. (1998) found PND 17 rats orally exposed to chlorpyrifos are more likely to exhibit behavioral changes and cholinesterase inhibition than PND 70 rats, which may be attributed to differences in the activities of various esterases (carboxylesterase and A-esterase) responsible for detoxification. PND 17 rats have less esterase activities than the older age group of rats. Karanth & Pope (2000) also found that esterase activities are well correlated with age and toxicity to organophosphorus pesticides. They reported that PND 7 and 21 rats exhibit greater sensitivity to the lethal effects of subcutaneous exposure to chlorpyrifos and parathion when compared to three-month-old rats and that these younger age groups have lower esterase activities. Timchalk et al. (2006, 2007) used these studies and others to formulate a physiologically-based pharmacokinetic/pharmacodynamic (PBPK/PD)
model for developmental exposure to chlorpyrifos in rats, which supports the hypothesis that sensitivity to organophosphorus pesticides decreases with age as detoxification pathways mature. Later, Smith et al. (2014) developed a PBPK/PD model for human exposure to toxic levels of chlorpyrifos (oral doses greater than 0.6 mg/kg) and concluded that six-month-old children would have higher levels of cholinesterase inhibition in their blood compared to adults.

Unfortunately, few studies have examined the effects of CWNA in animal models of pediatric and adolescent populations. Stierri et al. (1985) reported that PND 5 rats are more susceptible than PND 30 rats to the lethal effects associated with subcutaneous exposure to soman, attributing this difference to increased levels of carboxylesterase as the rat ages. Shih et al. (1990) reported PND 30 rats are less sensitive than PND 60, 120 and 240 rats to the lethal effects associated with intramuscular exposure to soman. PND 30 rats also exhibit less weight loss and more rapid growth recovery following subcutaneous exposure than the older age groups. In contrast, Fawcett et al. (2009) showed that the lethal effects of subcutaneous exposure to soman in guinea pigs are not influenced by the animal’s age or sex. However, the LD₅₀ values for adult (PND 120–150), male guinea pigs subcutaneously exposed to sarin or VX are lower than age-matched females or younger age groups (PND 5–10 or 35–45). Exposure routes relevant to battlefield or domestic terrorist situations (inhalation for sarin or VX and dermal for VX) were not tested in any of these studies.

Consequently, we investigated the lethal effects of whole-body (primarily inhalation) exposure to a range of sarin vapor concentrations (6.2–44.0 or 1.6–12.5 mg/m³) for 10 or 60 min, respectively, in male and female rats at different times during their development (PND 7, 14, 21, 28, 42 and 70). These age groups were selected to span the brain growth-spurt period (PND 7, 14 and 21; Dobbing & Sands, 1971), as well as adolescence (PND 28 and 42; Spear, 2000), and to confirm previously published data for adult rats (PND 70; Mioduszewski et al., 2002). A stagewise, adaptive dose design was used to estimate the 24 h LC₅₀ values for each age group. These data allowed for comparisons of both age- and sex-related effects of whole-body exposure to sarin on lethality. We hypothesized that susceptibility to the lethal effects of sarin would decrease with age and no sex differences would be observed.

### Methods

#### Animals

Sprague–Dawley rats (CD IGS; Charles River Laboratories, Kingston, NY) were used to study the effects of age and sex on the toxicity of sarin after whole-body exposure. Male and female rats were age-matched into six groups for both the 10 and 60 min exposures. Each age-matched group consisted of males and females at PND 7, 14, 21, 28, 42 or 70. The pre-exposure weights of each age group and sex are listed in Table 1. Animals were housed on a 12:12 h normal light–dark cycle (lights on at 0600) with room temperature of 21 ± 2°C and humidity of 60 ± 20%. Lactating dams and litters (five males and five females in each litter) remained together until weaning on PND 21. Food and water were available *ad libitum* except for the exposure and off-gas periods. The experimental protocol was approved by the Animal Care and Use Committees at the US Army Medical Research Institute of Chemical Defense (USAMRICD) and the US Army Edgewood Chemical Biological Center (ECBC), and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89–544), as amended.

#### Chemicals

Sarin (isopropyl methyl phosphonofluoridate) was obtained from ECBC’s chemical agent standard analytical reagent material stock and verified as pure (97.2 ± 1.0 weight %) with acid–base titration. Sarin was stored in sealed ampules until vapor dissemination in the whole-body chamber or preparation of external standards. All external standards for sarin vapor quantification were prepared daily.

#### Whole-body exposure system

Whole-body exposures were conducted in a 1000 L dynamic airflow inhalation chamber as previously described in

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### Table 1. Body weight (mean ± SD) for male and female rats at various times in development.

| Group | Male | Female |
|-------|------|--------|
|       | Weight (g) | Ratio (cm²/g) | N | Weight (g) | Ratio (cm²/g) | N |
| PND 7 | 18 ± 2.6 | 3.23 ± 0.14<sup>a</sup><sup>b</sup><sup>c</sup><sup>d</sup><sup>e</sup> | 37 | 17 ± 2.1 | 2.36 ± 0.11<sup>a</sup><sup>b</sup><sup>c</sup><sup>d</sup><sup>e</sup> | 38 |
| PND 14 | 33 ± 2.4 | 2.73 ± 0.06<sup>a</sup><sup>b</sup><sup>c</sup><sup>d</sup><sup>e</sup> | 49 | 32 ± 2.3 | 2.75 ± 0.05<sup>a</sup><sup>b</sup><sup>c</sup><sup>d</sup><sup>e</sup> | 50 |
| PND 21 | 50 ± 4.0 | 2.45 ± 0.06<sup>a</sup><sup>d</sup><sup>e</sup> | 37 | 48 ± 4.0 | 2.48 ± 0.06<sup>d</sup><sup>e</sup> | 37 |
| PND 28 | 89 ± 7.6 | 2.11 ± 0.05<sup>d</sup><sup>e</sup> | 49 | 82 ± 8.9 | 2.16 ± 0.06<sup>d</sup><sup>e</sup> | 50 |
| PND 42 | 218 ± 14.0 | 1.66 ± 0.03<sup>c</sup> | 50 | 166 ± 2.3 | 1.79 ± 0.05<sup>c</sup> | 50 |
| PND 70 | 325 ± 20.5 | 1.50 ± 0.02<sup>c</sup> | 43 | 223 ± 15.8 | 1.65 ± 0.03<sup>c</sup> | 39 |

The body surface area of each animal was calculated using the equation of Spiers & Candas (1984), which takes into account the animal’s stage of development. This value was then divided by the animal’s body weight to give a body surface area to body weight ratio. A two-way analysis of variance (ANOVA) with age group and sex as factors was conducted to determine differences in these ratios. There was a significant interaction between the two factors (F (5, 518) = 16.961, p < 0.001); thus, a one-way ANOVA followed by a Bonferroni *post hoc* test was conducted for each sex to determine differences between age groups. The differences are noted as follows.

<sup>a</sup>Significantly different from PND 14, p < 0.05.
<sup>b</sup>Significantly different from PND 21, p < 0.05.
<sup>c</sup>Significantly different from PND 28, p < 0.05.
<sup>d</sup>Significantly different from PND 42, p < 0.05.
<sup>e</sup>Significantly different from PND 70, p < 0.05.
Muse et al. (2006). Briefly, the interior of the Rochester-style exposure chamber was maintained under negative pressure (0.5–0.8 cm of water) as monitored with a calibrated manehelic differential pressure gauge (Dwyer Instruments, Michigan City, IN). For sarin vapor generation, a Harvard Pump 11 Elite syringe pump (Harvard Apparatus, Holliston, MA) was used to deliver liquid sarin into a spray atomizer, where it was mixed with compressed air to form vapor. Chamber airflow and temperature were monitored continuously during the exposure period, and relative humidity was measured at the beginning and at the end of the exposure.

Two sampling methods were used to monitor and analyze sarin vapor concentration in the exposure chamber. The first method was quantitative and used solid sorbent tubes (Tenax/ Hayesep; CAMSCO, Houston, TX) to trap sarin, followed by thermal desorption and gas chromatography analysis (Agilent 6890; Agilent Technologies, Wilmington, DE). Samples were drawn from the chamber every 10 min, with each draw lasting 2 min. For the 10 min exposures, samples were collected the last 2 min of the exposure period. The second method was a continuous-monitoring technique using a real-time phosphorus monitor (HYFED, model PH262; Columbia Scientific Industries Corporation, Austin, TX). Output from the HYFED monitor provided a continuous strip chart record of the rise, equilibrium and decay of the chamber vapor concentration during an exposure.

Sarin exposures

Un-anesthetized rats were placed in compartmentalized cages (51 cm wide × 36 cm long × 5 cm high; 10 compartments/cage) and transported to a procedure room containing the whole-body exposure system. The cages were placed in the exposure chamber where they remained for the duration of the exposure. Exposures were conducted between 0900 and 1500. Ten minutes after the exposures were completed, the cages were moved to another chamber to off-gas for 20–30 min before the rats were returned to their colony room. Special care was taken with regard to hypothermia and dam rejection. For example, maternal separation times were kept to a minimum (exposure time plus time to off-gas the agent), and the compartmentalized cages containing PND 7 or 14 rats were placed on a heating pad for the off-gassing process.

LC50 estimations

The 24 h lethality for sarin was estimated by establishing concentration–response curves and estimating the LC50 for both the 10 and 60 min exposure periods. Each curve was generated using a stagewise, adaptive dose design (Feder et al., 1991a–c). Groups of animals received various concentrations (one concentration per animal) of sarin ranging from 6.2 to 44.0 mg/m3 for the 10 min exposures and 1.6–12.5 mg/m3 for the 60 min exposures. A split-litter design was utilized such that individual offspring of the same sex in each litter were exposed to different concentrations of sarin. In the first stage, five concentrations (n = two rats/concentration) were selected to span the predicted range of lethality from 0–100%. The lethality results from the first stage were used to select concentrations (n = 1–3 rats/concentration) for the second stage. In the subsequent stages, concentrations were selected to further focus on concentrations with a 50% lethality response and/or to better estimate the concentration–response curve. After each stage, probit dose–response models using maximum likelihood methods were fitted to the combined data for all stages (Feder et al., 1991b; Finney, 1971). The stage process continued until the half width of the 95% confidence interval (CI) defined as (Upper Bound–Lower Bound) / (2 × LC50) for the 24 h LC50 was less than 0.4 or a maximum of 40 animals was used for each exposure time, age group and sex.

Statistical analyses

The LC50 values were estimated by probit analysis using SAS NLIN (SAS Institute, Cary, NC) and the specialized programs of Feder et al. (1991a–c). The delta method was used to compute a 95% CI for each LC50 value. Comparisons between male and female LC50 values for each age group, as well as comparisons between age groups within each sex, were made by calculating a comparative ratio of the two LC50 values along with a 95% CI for the ratio (Supplemental Tables 1–3). This approach is a variation of the two-sided Z-test (α = 0.05). If the 95% CI for the comparative ratio did not encompass the value of 1.0, then the LC50 values of the age groups or sexes being compared were determined to be significantly (p < 0.05) different.

The slopes of the concentration–response curves were analyzed for significance using multiple comparison t-tests with Bonferroni corrections. Statistical significance was defined as p < 0.008 for the six sex comparisons and p < 0.003 for the fifteen age group comparisons per exposure time. SigmaPlot 12.3 (Systat Software, San Jose, CA) was used to conduct these t-tests, as well as plot the concentration–response curves.

Results

LC50 estimates

Table 2 shows the LC50 values for male and female rats exposed via their whole bodies to sarin for 10 or 60 min on PND 7, 14, 21, 28, 42 or 70. PND 14 rats were the most susceptible (lowest LC50 value) to the lethal effects of sarin, whereas PND 28 rats were the least susceptible (highest LC50 value). Male rats were less susceptible than females, but significant sex differences were only observed for PND 42 at 10 min and PND 21 and 70 at 60 min.

Concentration–response curves

Figure 1 shows the concentration–response curves for male and female rats exposed via their whole bodies to sarin for 10 or 60 min on PND 7, 14, 21, 28, 42 or 70. For both exposure times, the concentration–response curves for PND 28 and 42 rats were shifted to the right compared to PND 7, 14, 21 and 70 rats. For rats exposed to sarin for 10 min, no sex differences were observed in the slopes of these concentration–response curves (Table 3). However, the slope of the concentration–response curve for male rats exposed to sarin for 60 min was significantly different from their female counterparts for PND 7. No other sex differences were
Table 2. 24 h LC50 and 95% CI for male and female rats exposed via their whole bodies to sarin for 10 or 60 min at various times in development.

| Group     | Male 10 min | Female 10 min | Male 60 min | Female 60 min |
|-----------|-------------|---------------|-------------|---------------|
| LC50 (mg/m^3) | 17.5^{a,c,d} | 16.5^{a,c,d} | 4.2^{a,c,d,e} | 3.7^{a,c,d,e} |
| 95% CI     | 15.0–20.3   | 13.5–20.2     | 3.3–5.3     | 3.5–3.9       |
| N          | 19          | 16            | 18          | 22            |
| LC50 (mg/m^3) | 12.6^{b,c,d,e} | 12.0^{b,c,d,e} | 2.5^{b,c,d,e} | 2.3^{b,c,d,e} |
| 95% CI     | 10.4–15.2   | 10.7–13.4     | 2.2–2.7     | 2.1–2.5       |
| N          | 25          | 26            | 24          | 24            |
| LC50 (mg/m^3) | 17.4^{c,d}  | 14.9^{d,e}    | 4.8^{c,d,e} | 3.7^{c,d,e}   |
| 95% CI     | 15.1–20.2   | 13.9–16.0     | 3.9–6.0     | 3.3–4.2       |
| N          | 17          | 17            | 20          | 20            |
| LC50 (mg/m^3) | 36.0^{e}    | 34.1^{d,e}    | 9.6         | 8.6^{e}       |
| 95% CI     | 34.4–37.8   | 32.0–36.2     | 8.4–10.9    | 8.0–9.3       |
| N          | 17          | 17            | 20          | 20            |
| LC50 (mg/m^3) | 33.1^{e}    | 27.7^{n}      | 9.1         | 8.2^{e}       |
| 95% CI     | 30.2–36.3   | 24.3–31.6     | 8.2–10.0    | 7.6–8.9       |
| N          | 23          | 23            | 27          | 27            |
| LC50 (mg/m^3) | 20.8        | 18.6          | 8.6         | 7.2           |
| 95% CI     | 17.3–25.0   | 17.3–20.2     | 8.1–9.2     | 6.9–7.5       |
| N          | 18          | 18            | 25          | 20            |
| LC50 (mg/m^3) | 33.1^{e}    | 27.7^{n}      | 9.1         | 8.2^{e}       |
| 95% CI     | 30.2–36.3   | 24.3–31.6     | 8.2–10.0    | 7.6–8.9       |
| N          | 23          | 23            | 27          | 27            |
| LC50 (mg/m^3) | 20.8        | 18.6          | 8.6         | 7.2           |
| 95% CI     | 17.3–25.0   | 17.3–20.2     | 8.1–9.2     | 6.9–7.5       |
| N          | 18          | 18            | 25          | 20            |

For each exposure time and age group, comparisons were made between genders and significant (p < 0.05) differences are highlighted in bold. For each exposure time and gender, comparisons were made between age groups and the differences are notated as follows.

- ^{a}Significantly different from PND 7, p < 0.05.
- ^{b}Significantly different from PND 14, p < 0.05.
- ^{c}Significantly different from PND 21, p < 0.05.
- ^{d}Significantly different from PND 28, p < 0.05.
- ^{e}Significantly different from PND 42, p < 0.05.
- ^{f}Significantly different from PND 70, p < 0.05.

Figure 1. Concentration–response curves for male and female rats exposed via their whole bodies to sarin at various times in development. (A) 10 min – Males; (B) 10 min – Females; (C) 60 min – Males and (D) 60 min – Females.
dependencies of the LC50 values for sarin were not inversely proportional to age, with PND 14 rats being the most susceptible (and PND 28 rats being the least). The age-related differences were significant and were observed across all exposure times. For each exposure time and age group, comparisons were made between male and female rats at six different times during development (PND 7, 14, 21, 28, 42, and 70) that were selected to span the brain growth-spurt period into adolescence and early adulthood. We found PND 14 rats were the most susceptible (lowest LC50 values) to the lethal effects of sarin, PND 28 rats were the least susceptible (highest LC50 values) and sex differences were minimal. In addition, the LC50 values for PND 70 rats were similar to those previously published by Mioduszewski et al. (2002) for 8–10 week-old rats.

In general, susceptibility to the lethal effects of whole-body exposure to sarin decreased with age as we hypothesized. Using the equation listed in Alexander et al. (2008), delivered doses of 350, 163, 341, 569, 457 and 369 μg/kg were calculated for each age group (PND 7–70, respectively) from the LC50 values for male rats exposed via their whole bodies to sarin for 60 min. Given that PND 14 rats were the most susceptible (and PND 28 rats were the least), the age-dependencies of the LC50 values for sarin were not inversely related to body weight or body surface area. Instead, the age-dependencies of the LC50 values for sarin may correlate with maturational expression of detoxification enzymes as previously observed with soman (Sterri et al., 1985) and organophosphorus pesticides (Atterberry et al., 1997; Benke & Murphy, 1975; Furlong, 2007; Karanth & Pope, 2000; Moser et al., 1998). Although we did not determine basal activity levels for these enzymes in this study, Karanth & Pope (2000) reported that the levels of carboxylesterase and paraoxonase in the plasma and tissues of PND 7 and 21 rats were significantly lower than in adult (three month old) rats. Carboxylesterase stoichiometrically eliminates while paraoxonase catalytically inactivates anticholinesterase compounds, and lower activity levels of these enzymes in postnatal rats result in lesser detoxification and higher lethality compared to adults (Vidair, 2004).

The end of the second postnatal week is a critical period for respiratory development in rats due to a drastic imbalance in the expression of excitatory and inhibitory neurotransmitters, a switch in the expression of GABA<sub>A</sub> receptor subunits and a sudden drop in cytochrome oxidase activity in several brainstem respiratory nuclei at PND 12 (Wong-Riley & Liu, 2008). These neurochemical changes are closely followed by significant increases in ventilation (Liu et al., 2006) and metabolic rate (Liu et al., 2009) at PND 13, which require too much energy to maintain under hypoxic conditions. This weakened hypoxic ventilatory response may explain why PND 14 rats were the most susceptible to the lethal effects associated with whole-body exposure to sarin.

It is less clear why PND 28 were the least susceptible to sarin, but it is tempting to speculate that the surge of hormones during adolescence may have played a role. Uddin & Titchener (1968) showed that hepatic carboxylesterase activity is under the control of sex hormones, which become detectable in female rats at PND 28 and surge in male rats from PND 40 to 44 (Vetter-O’Hagen & Spear, 2012). Future studies, however, are needed to determine whether carboxylesterase activity levels change during the adolescent period of a rat.

Sex differences in the lethality associated with whole-body exposure to sarin were minimal with the exception of a few age groups, PND 42 at 10 min and PND 21 and 70 at 60 min. In each of these instances, females had lower LC<sub>50</sub> values than males. Adult, female rats have previously been shown to be more susceptible than males to the lethal effects of whole-body exposure to sarin (Mioduszewski et al., 2002) and cyclosarin (Anthony et al., 2004). Sex differences in the slopes of the concentration–response curves were also minimal suggesting that the rate of toxicity associated with whole-body exposure to sarin was similar between male and female rats. Although the lethal potency of sarin changes throughout the estrous cycle (Smith et al., 2015), this would only have been a confounding variable for our oldest age group of rats as female do not regularly start cycling until PND 48 (Vetter-O’Hagen & Spear, 2012).

In conclusion, this study provides the first evidence in an exposure model relevant to battlefield or terrorist situations that younger rats (PND 14) are more susceptible to the lethal effects of sarin than adults (PND 70). This study is also the first to report that adolescent rats (PND 28) are less susceptible. Although future studies are needed to determine the mechanism of these age-related differences in susceptibility, these results underscore the importance of controlling for age (and to a lesser extent sex) in research on the toxic effects associated with CWNA exposure.

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Declarations of interest

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