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Hormone-dependence of sarin lethality in rats: Sex differences and stage of the estrous cycle

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ABSTRACT

Chemical warfare nerve agents (CWNAs) are highly toxic compounds that cause a cascade of symptoms and death, if exposed casualties are left untreated. Numerous rodent models have investigated the toxicity and mechanisms of toxicity of CWNAs, but most are limited to male subjects. Given the profound physiological effects of circulating gonadal hormones in female rodents, it is possible that the daily cyclical fluctuations of these hormones affect females' sensitivity to the lethal effects of CWNAs, and previous reports that included female subjects did not control for the stage of the hormonal cycle. The aim of the current study was to determine the 24-hour median lethal dose (LD_{50}) of the CWNA sarin in male, ovariectomized (OVEX) female, and female rats during different stages of the estrous cycle (diestrus, proestrus, and estrus). Additionally, baseline activity levels of plasma acetylcholinesterase, butyrylcholinesterase, and carboxylesterase were measured to determine differences among the groups. Results indicated that females in proestrus had a significantly higher LD_{50} of sarin compared to OVEX and estrous females. Although some sex differences were observed in the activity levels of plasma esterases, they were not consistent and likely not large enough to significantly affect the LD_{50}s. These results suggest that hormonal cyclicity can influence the outcome of CWNA-related studies using female rodents, and that this variability can be minimized by controlling for the stage of the cycle. Additional research is necessary to determine the precise mechanism of the observed differences because it is unlikely to be solely explained by plasma esterase activity.

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Introduction

Chemical warfare nerve agents (CWNAs), such as sarin, irreversibly bind to acetylcholinesterase and induce a “cholinergic crisis” that causes numerous physiological events including miosis, salivation, respiratory failure, tremors, seizures, and death. Treatment regimens that typically include an anticholinergic (atropine), oxime (2-PAM chloride), and anticonvulsant (diazepam) reduce symptoms and lethality when administered before or quickly after an exposure (Lee, 2003). Limited efficacy and a short therapeutic window of these treatments, however, require the development of new, more effective medical countermeasures. Numerous animal models are currently used to identify novel countermeasures (for review, see Pereira et al., 2014). However, these are largely limited to male animals, and relatively little is known regarding the toxic effects of CWNAs and the therapeutic efficacy of countermeasures in females. Furthermore, developing medical countermeasures exclusively in males may result in a product that is not as safe for use in females. There is precedence for this safety concern because past FDA-approved pharmaceuticals have been withdrawn because of unintended adverse effects in women (Heinrich et al., 2001). These facts underscore the need to include female subjects in scientific research, and to incentivize this inclusion, the National Institutes of Health plans to mandate the use of female subjects for preclinical trials (Clayton and Collins, 2014). Thus, understanding how biological differences between males and females can affect outcomes is necessary for developing safe and effective medical countermeasures for CWNA exposure.

The relative sensitivity to the lethal effects of CWNAs between male and female rats is consistent in some reports but not others, and depends on factors including route of exposure, agent, and species. For example, the median lethal concentration (LC_{50}) for whole-body sarin (Mioduszewski et al., 2002) and cyclosarin (Anthony et al., 2004) exposures is lower in female rats compared to males. In at least one report, female rats are also more sensitive than males to subcutaneous injections of soman (Sket, 1993). In contrast, no significant sex differences emerge from whole-body exposures of VX (Benton et al., 2006) or subcutaneous injections of tabun (Lundy et al., 1989). The latter study also reported only a small difference in the subcutaneous median lethal dose (LD_{50}) of soman between the sexes (Lundy et al., 1989), but the significance of this difference is difficult to determine because statistical analyses were not performed. Species is also a factor; in guinea pigs, the LD_{50} of sarin and VX is lower in males compared to females, whereas no sex

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difference is observed following exposure to soman (Fawcett et al., 2009).

Why some studies report sex differences after CWNA intoxication and others do not is unknown. One contributing factor may involve differences in sex-specific gonadal hormones. Males secrete androgens at a relatively constant rate that slowly declines with age (Mooradian et al., 1987). Females, in contrast, secrete estradiol and progesterone on a cyclical schedule that can significantly change from day to day. For example, the four-day estrous cycle in female rats is defined by two days of diestrus, one day of proestrus, and one day of estrus (Goldman et al., 2007). Estradiol and progesterone secretions remain relatively quiescent during the first two days of diestrus before peaking in proestrus and then declining by the morning of estrus. Given the widespread distribution of estrogen and progesterone receptors throughout the body, and their effects on physiological functions (Jensen and DeSombre, 1972), endogenous fluctuations of these hormones could possibly affect females' sensitivity to intoxication with CWNAs from day to day. One commonality among studies using females in CWNA research is that none have controlled for the stage of the estrous cycle (Lundy et al., 1989; Sket, 1993; Mioduszewski et al., 2002; Anthony et al., 2004; Benton et al., 2006; Fawcett et al., 2009).

The aim of the current study was to determine the toxicity of sarin throughout these hormonal stages in rats by comparing the 24-hour LD_{50} of sarin in male, ovariectomized (OVEX) female, and cycling female rats at different stages of the estrous cycle (diestrus, proestrus, and estrus). In addition, baseline plasma acetylcholinesterase (AChE), butyrylcholinesterase (BuChE), and carboxylesterase (CE) activity levels were measured. These esterases bind to CWNAs, and numerous studies report sex differences in baseline levels (Leeuwin, 1965; Schmidt and Schmidt, 1978; Illsley and Lamartiniere, 1981; Sterri and Fonnum, 1989; Chanda et al., 1997), which may affect sensitivity to sarin. If lethality significantly fluctuates throughout the estrous cycle in female rats, consideration should be taken in future studies to reduce variability by controlling for hormonal state.

Material and methods

Subjects. Age-matched adult (200–300 g) male ($n = 23$), cycling female ($n = 76$), and ovariectomized female (OVEX; $n = 19$) Sprague Dawley rats were purchased from Charles River Laboratories (Kingston, NY) and housed under normal 12:12 h light cycle with food and water available *ad libitum*. OVEX females had their ovaries surgically removed by Charles River Laboratories by an incision through the rear dorsum. To control for the surgical procedures, males and cycling females received a sham procedure, whereby an incision was made on the rear dorsum and immediately stapled. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the USAMRICD, 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.

Determination of the stage of estrus. The estrous cycle in females was monitored daily by vaginal smears. To perform the smear, a cotton swab moistened in 0.9% saline was briefly inserted into the vaginal opening of female rats. The cotton swab was removed, and the sample applied to a slide. Slides were dipped in a solution of 0.025% methylene blue and examined under a light microscope at 10×. The ratio of cornified, epithelial, and/or leukocytic cells was used to determine the stage of estrus as described in Goldman et al. (2007). Briefly, diestrus was determined by the presence of leukocytes with or without cornified, nucleated epithelial, or non-nucleated epithelial cells; proestrus by the presence of nucleated epithelial cells without leukocytes; and estrus by the presence of cornified cells without leukocytes.

Blood draws and agent exposures. Approximately 4 weeks before exposure to sarin, OVEX female and cycling female rats received vaginal smears for 8 days to determine their estrous cycle. OVEX females were smeared to confirm the lack of cyclicity. To control for the perturbation of the smearing procedure, male rats were removed from their cages and immediately replaced. At the end of the monitoring period, all rats were habituated to the blood collection procedure by gentle restraint in a chux. Four to five days later between 0830 and 0930 h, a subset of rats received blood draws (250 μL per day) from a tail incision. To minimize the number of animals required for blood draws, cycling females ($n = 14$) received repeated daily blood draws throughout their 4-day estrous cycle (diestrus I, diestrus II, proestrus, and estrus), while OVEX ($n = 10$) and male rats ($n = 10$) received a single blood draw. Only cycling females with regular 4-day cycles were included in blood draws. One cycling female was removed from the entire study because evidence of hormonal cyclicity was not observed. OVEX and male rats received "sham" blood draws for the remaining 3 days, where a tail incision was made but no blood collected. All other rats that did not receive blood draws but were included in the LD_{50} determination were given tail incisions for 4 days. All animals then recovered for 2 weeks before exposure to sarin.

Five days before exposure to sarin, all females received vaginal smears to confirm hormonal stage. On the day of exposures, cycling females were designated to a group by the stage of their estrous cycle into diestrus, proestrus, or estrus. Given the similar hormonal milieu during the morning on the two days of diestrus (Butcher et al., 1974; Schank and McClintock, 1992), rats in diestrus I and diestrus II were combined to reduce the overall number of required animals. Between 0930 and 1200 h, all rats were subcutaneously injected with sarin (65.8–239.1 μg/kg; 0.5 mL/kg). Each rat was then continuously monitored by an observer for 2 h and then every 30 min thereafter until 1700 h. Rats surviving the 24-hour time period were euthanized by injection of a pentobarbital-based euthanasia solution followed by perforation of the diaphragm.

Enzyme assays. Cholinesterase assays: Blood AChE and BuChE were measured using a modification of the WRAIR whole blood cholinesterase assay (Gordon et al., 2005). Briefly, fresh rat whole blood was diluted 1:10 in water and 10 μL were added into 96-well plates. The reactions were initiated by the addition of 290 μL of 50 mM sodium phosphate buffer (pH 8.0) containing one of the following substrates at 1 mM: acetylthiocholine, propionylthiocholine, or butyrylthiocholine. The reactions were conducted at 25 °C and monitored at 324 nm in a Molecular Dynamics model SpectraMax Plus plate reader (Sunnyvale, CA) under the control of SoftMax Ver. 5.4 software (Molecular Dynamics, Inc.) by tracking hydrolysis of 4,4′-dithiodiopyridine (DTP), added at 0.2 mM. AChE and BuChE activities (in μmol/min/mL) were calculated for each sample. The product concentration was determined using the molar extinction coefficient $M\varepsilon_{324} = 12{,}517\ cm^{-1}\ M^{-1}$.

Carboxylesterase (CE) assay: Rat plasma was prepared by centrifugation of whole blood at 3000 × *g* for 10 min at 4 °C, and then flash frozen on dry ice and stored at −80 °C. Plasma CE activities were determined as described by Hashinotsume et al. (1978). Samples (10 μL) were preincubated with 10 μM eserine and 10 mM EDTA for 30 min in reaction buffer containing 50 mM HEPES pH 7.4 in a total volume of 280 μL. Treated samples were loaded into a 96-microtiter plate well, and the reaction was initiated by the addition of 20 μL p-nitrophenyl acetate (2.5 mM final concentration). Samples were analyzed at 25 °C by monitoring absorbance at 400 nm using a Molecular Dynamics model SpectraMax Plus plate reader with SoftMax Ver. 5.4 software. The product concentration was determined with the molar extinction coefficient at pH 7.4 for p-nitrophenol ($M\varepsilon_{400} = 7860\ cm^{-1}\ M^{-1}$).

Data analyses. A stagewise, adaptive dose design was used to determine the 24 h LD_{50} of sarin (Feder et al., 1991a, 1991b, 1991c). In the first stage, agent doses were selected to span the predicted range of lethality from 0% to 100%. Animals were allocated randomly to agent doses per

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