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

Carl D. Smith, **Linnzi K.M. Wright**, **Gregory E. Garcia**, **Robyn B. Lee**, and **Lucille A. Lumley**

US Army Medical Research Institute of Chemical Defense (USAMRICD), Aberdeen Proving Ground, Maryland 21010

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.

Keywords

sarin; nerve agents; sex differences; estrous cycle; LD50

Corresponding author: Carl D. Smith, Ph.D., US Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010-5400, Tel: 1 410 417 3760, Fax: 1 410 436 8377, carl.d.smith179.mil@mail.mil.

Disclaimers

The views expressed in this article are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government. The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense 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.

1. 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 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.

2. Material and methods

2.1 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 hr 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.

2.2 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 10X. 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.

2.3 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 0830–0930 hrs, 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 0930–1200 hrs, 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 hrs and then every 30 min thereafter until 1700 hrs. Rats surviving the 24-hour time period were euthanized by injection of a pentobarbital-based euthanasia solution followed by perforation of the diaphragm.

2.4 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 BChE activities (in μmol/min/mL) were calculated for each sample. The product concentration was determined using the molar extinction coefficient $M\varepsilon_{324}$=12517 cm^{-1} M^{-1}.

Carboxylesterase (CE) assay: Rat plasma was prepared by centrifugation of whole blood at 3,000 × 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 pre-incubated 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}).

2.5 Data analyses

A stagewise, adaptive dose design was used to determine the 24 hr LD_{50} of sarin (Feder *et al.*, 1991a; Feder *et al.*, 1991b; Feder *et al.*, 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 stage (where the first stage used 2 animals each per 5 agent doses). The lethality results from the first stage were used to select agent doses for the second stage. In the subsequent stages, agent doses were selected to further focus on doses with a 50% lethality response and/or to better estimate the dose response curve. After each stage, probit dose response models using maximum likelihood methods were fitted to the combined data for all stages (Finney, 1971; Feder *et al.*, 1991b). The stage process continued until the half width of the 95% confidence interval (CI) defined as (Upper Bound – Lower Bound)/(2 × LD_{50}) for the 24 hr LD_{50} was less than 0.4 or a maximum of 32 animals were used. Output from the stagewise adaptive dose program for each group was used to calculate a ratio of the LD_{50} values for pairs of groups and the respective 95% CI. This ratio and the respective 95% CI was used to compare LD_{50} values between groups. If the 95% CI did not encompass the value of 1.0, then the LD_{50} values of the groups were determined to be significantly different.

Statistical comparisons of the activity levels of AChE, BuChE, and CE were tested with one-way ANOVAs. Four separate analyses were conducted with male and OVEX females and one group of cycling females from each stage of the estrous cycle (diestrus I, diestrus II, proestrus, and estrus) because cycling females provided data for each stage of the estrous cycle. Since the male and OVEX female groups were used in all four analyses, statistical significance of the main effect of group was determined using a Bonferroni adjusted p-value ($p \leq 0.0125$). Significant main effects of esterase activities were followed by a Tukey's multiple comparison test to compare all group pairs with a Bonferroni adjusted p-value ($p \leq 0.0167$).

3. Results

3.1 24 hr Sarin LD_{50}

Female rats in proestrus had a significantly higher 24 hr sarin LD_{50} compared to females in estrus or females with their ovaries removed (Table 1; $p < 0.05$). No differences were observed among any of the female groups compared to the male group.

3.2 Plasma AChE, BuChE, and CE

Baseline activity levels of plasma AChE, BuChE, and CE varied throughout the estrous cycle when compared to male and OVEX rats (Figure 1). Significant main effects of esterase activities after Bonferroni adjustment ($p \leq 0.0125$; Table 2) were observed for most of the esterases in all four comparisons (male, OVEX, and one stage of the estrous cycle) except

for the levels of AChE in proestrous and CE in diestrous II females when compared to males and OVEX females. Tukey's multiple comparison test revealed that females in diestrus I ($p = 0.004$), diestrus II ($p < 0.001$), and estrus ($p = 0.001$) had significantly lower plasma AChE activity than males. Activities of AChE were not different in OVEX females compared to males or cycling females. Females at each stage of the estrous cycle had significantly more BuChE activity than males ($p < 0.001$), while levels of BuChE ($p = 0.001$) were lower in OVEX females compared to females in diestrus II. Activities of CE did not differ in cycling females compared to males, but OVEX females had lower CE (on average, $p = 0.012$) than did males.

4. Discussion

The purpose of this study was to determine if the 24 hr sarin LD_{50} changes throughout different hormonal states in female rats and in male rats. In addition, we measured the baseline activity levels of AChE, BuChE, and CE during all hormonal states (male, OVEX, diestrus I, diestrus II, proestrus, and estrus) to determine if activity of these esterases fluctuated among the groups. Our results indicated that the sarin LD_{50} changed throughout the estrous cycle. Females in proestrus were significantly less sensitive to the lethal effects of sarin compared to OVEX or estrous females, although no overall sex difference was observed. In addition, levels of AChE were generally lower, while BuChE levels were generally higher, in all females compared to males, and OVEX females had lower levels of CE than did males.

Although no sex differences were observed in the LD_{50} values, the variability in the cycling females could affect outcomes in CWNA-related toxicity or treatment studies. If one conducted a toxicity study in females, and all subjects were in the same day of the estrous cycle, results from that study could skew future experiments. For example, if all of our female subjects were in estrus during the sarin LD_{50} determination, we may have concluded that the female LD_{50} of sarin is ~110 μg/kg. If, however, all of the female subjects were in proestrus, we may have concluded that the female LD_{50} of sarin is ~124 μg/kg. Although a relatively small difference, using an LD_{50} of sarin of 110 μg/kg in proestrus female rats may be too low to fully or reliably induce toxicity. If one tested potential therapeutics in this scenario, one may incorrectly conclude that the therapeutics possess a higher level of efficacy than actually exists. In light of this possibility, the probability of females cycling together is determined by more than simply chance. Some strains of rats (Schank and McClintock, 1992) but not others (Schank, 2001) exhibit partial estrous cycle synchrony by the third cycle (~12 days), which is likely mediated by pheromones. Environmental stimuli, such as initiating vaginal smears or introducing females to a novel environment, also influence synchrony (Shirley, 1978), suggesting that it is not uncommon for female rats to exhibit similar cycles.

As indicated by LD_{50} values, protection from sarin in proestrous females compared to OVEX and estrous females may be imparted by the presence of high levels of circulating gonadal hormones, particularly estradiol. Indeed, plasma levels of estradiol peak in the morning of proestrus and decline rapidly by the morning of estrus (Gorski *et al.*, 1975; Schank and McClintock, 1992). It is difficult to determine if progesterone exhibits a

protective effect; progesterone levels rapidly peak and decline during the evening of proestrus and early morning of estrus (Gorski *et al.*, 1975), and none of the cycling females received sarin during this time period. Incidentally, the LD_{50} of sarin in diestrous rats was in between those of proestrus and estrus females, which could have resulted from the gradual increase of estradiol secretion throughout the two days of diestrus. The LD_{50} of males resembled that of diestrous rats, which suggests that androgens may also impart some protection against sarin.

The exact mechanism underlying the observed differences in LD_{50} values remains to be determined, but it is unclear if the toxic effects of sarin are affected by variation in levels of plasma AChE, BuChE, and/or CE. These esterases varied little throughout the estrous cycle and, as deduced from Sweeney *et al.* (2006), endogenous levels of AChE and BuChE in the plasma are likely not high enough to significantly influence the LD_{50} of sarin. In addition, the levels of esterase activity do not appear to be associated with LD_{50} values. Males had higher AChE levels, and lower BuChE levels, than females, even though no difference between sexes existed in LD_{50} values. Furthermore, OVEX females only differed in BuChE activity compared to diestrous II females but the LD_{50} of these two hormonal states did not differ, and none of the esterase levels differed between OVEX and proestrous females. Nevertheless, these results do not necessarily preclude endogenous esterases from affecting the observed LD_{50} values. Additional studies examining esterase activity throughout the estrous cycle in tissues could address this issue.

Alternatively, the presumably high levels of estradiol in the proestrous rats may have imparted other protective effects that are independent of esterase activity. It is well established that estradiol has potent protective properties against a variety of physiological insults by reducing inflammation, inhibiting apoptotic messengers, and improving mitochondrial function (for review, see Spence and Voskuhl (2012)). Respiratory failure, a major contributing factor to CWNA-induced lethality (Rickett *et al.*, 1986), may be delayed or reduced in severity by estradiol. Indeed, estradiol increases bronchodilation by relaxing smooth muscle in the airway (Townsend *et al.*, 2010) possibly by potentiating the effects of norepinephrine on acetylcholine-induced contractions (Townsend *et al.*, 2012). Testosterone also relaxes the airway (Montano *et al.*, 2014), which may account for the higher LD_{50} of sarin in males compared to OVEX females.

Additional research is required to elucidate the precise underlying mechanisms affecting females' sensitivity to the lethal effects of sarin throughout the estrous cycle. Future studies could examine the potential role of estradiol and progesterone, as well as other hormones that fluctuate with the estrous cycle, such as follicle stimulating hormone and luteinizing hormone. Gonadal hormones are also known to affect seizures (Scharfman and MacLusky, 2006), and the stage of the estrous cycle may affect how the epileptogenic properties of CWNAs manifest in females. Finally, the efficacy of currently fielded (atropine, 2-PAM, and diazepam) and potentially novel therapeutics could be examined to determine if their therapeutic properties fluctuate throughout the cycle. Nevertheless, the current study supports the hypothesis that the lethality of sarin in female rats is affected by the stage of the estrous cycle. If including female subjects in CWNA research, these results suggest that

controlling for the stage of the estrous cycle can reduce potential variability most likely due to cycling gonadal hormones.

5. Conclusion

The 24 hr LD_{50} of sarin changes throughout the estrous cycle in female rats. Females in proestrus exhibit a significantly higher LD_{50} compared to females in estrus or those females with their ovaries removed. Furthermore, this finding does not appear to be dependent on differences in plasma activity levels of acetylcholinesterase, butyrylcholinesterase, or carboxylesterase. The mechanism underlying the higher LD_{50} in proestrus females is unclear but may be related to the presence of estradiol during that stage of the cycle. Although future research is required to elucidate these mechanisms, results from this study underscore the importance of monitoring the estrous cycle when using female rats in CWNA-related research.

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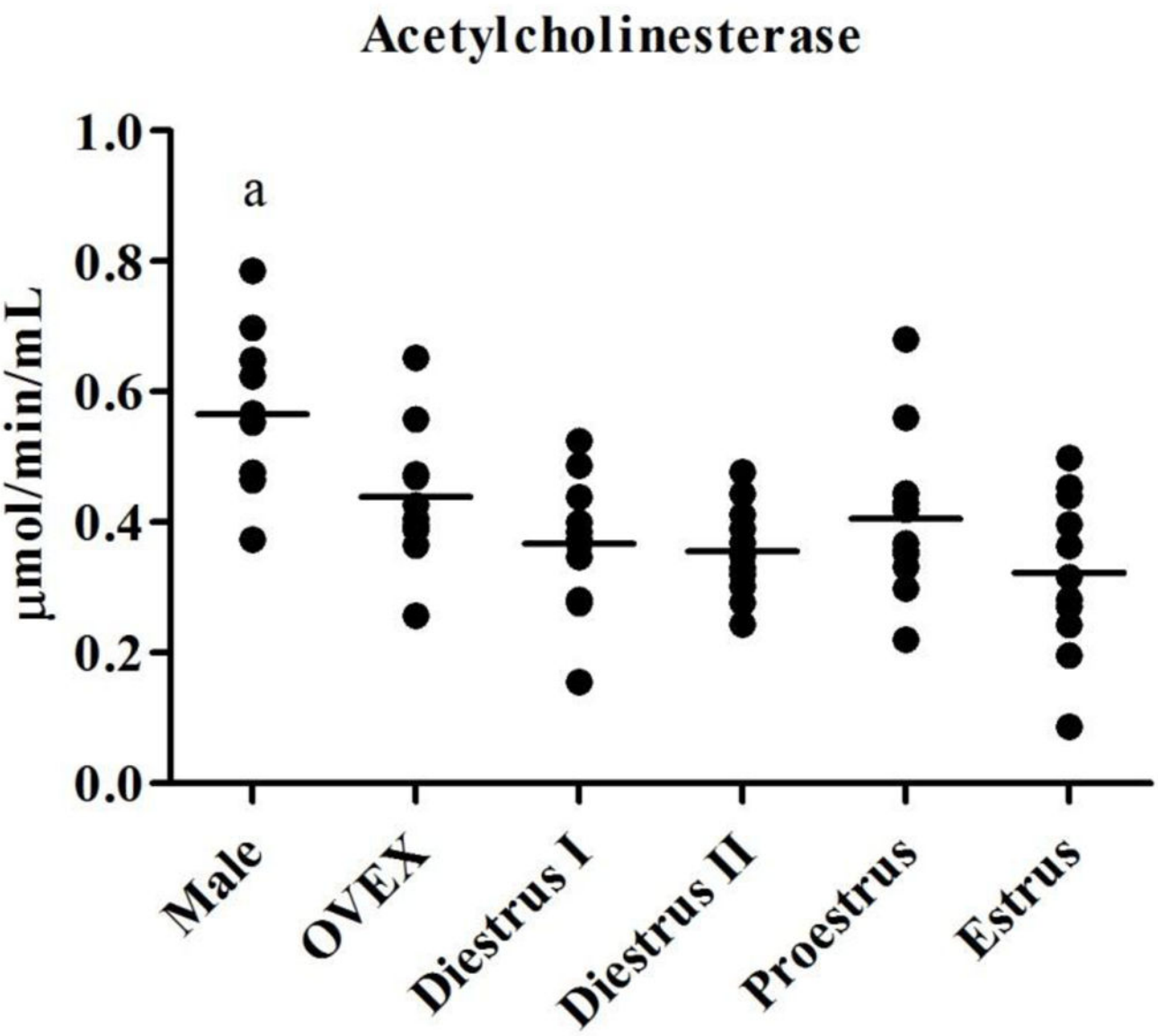
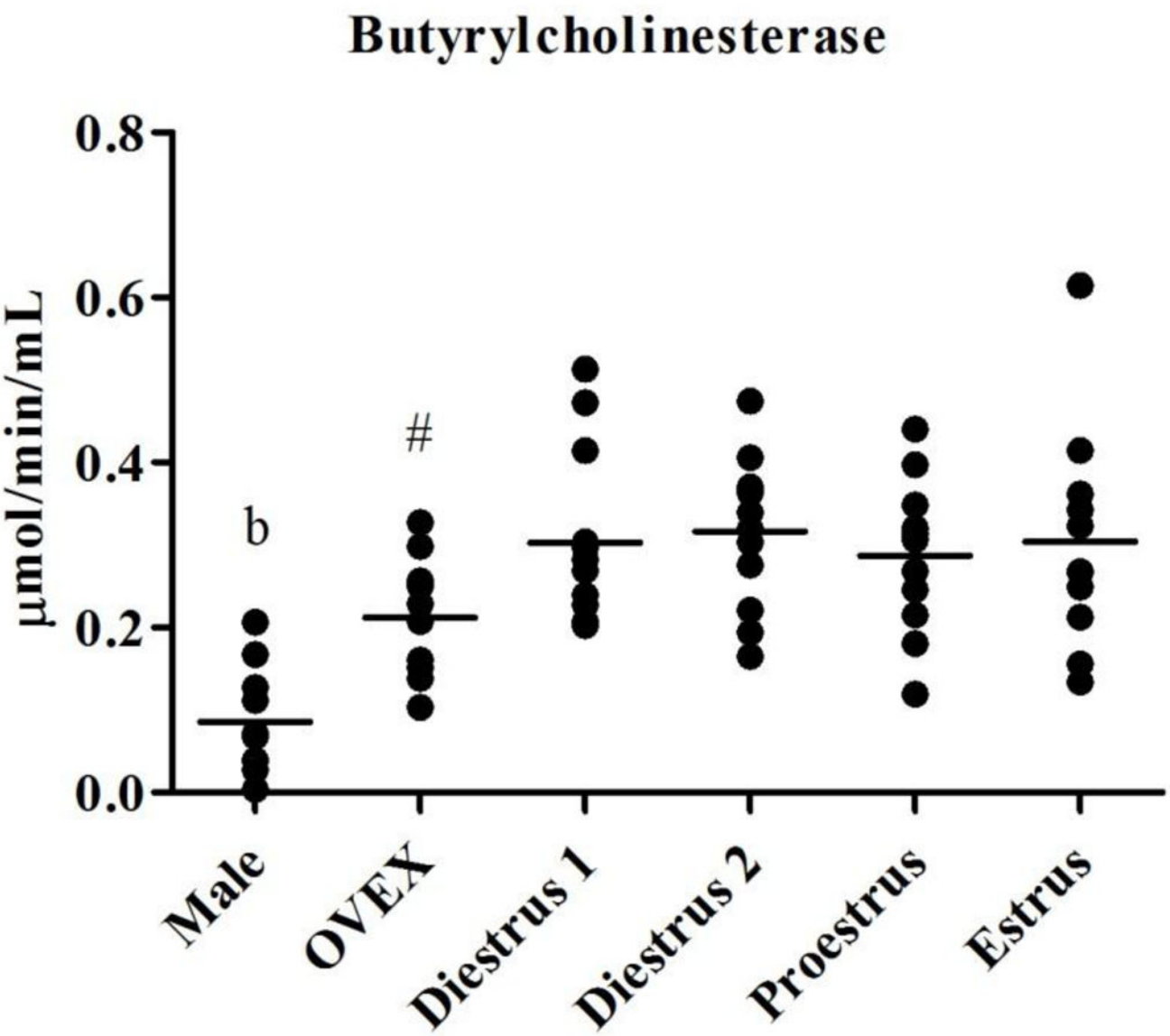
Highlights

- The LD_{50} of sarin was determined in female rats throughout the stages of the estrous cycle.
- Females in proestrus had a significantly higher LD_{50} compared to estrous or ovariectomized females.
- No sex differences were observed between male and female rats.
- It is unlikely that plasma esterase activity underlies the observed differences in LD_{50}s





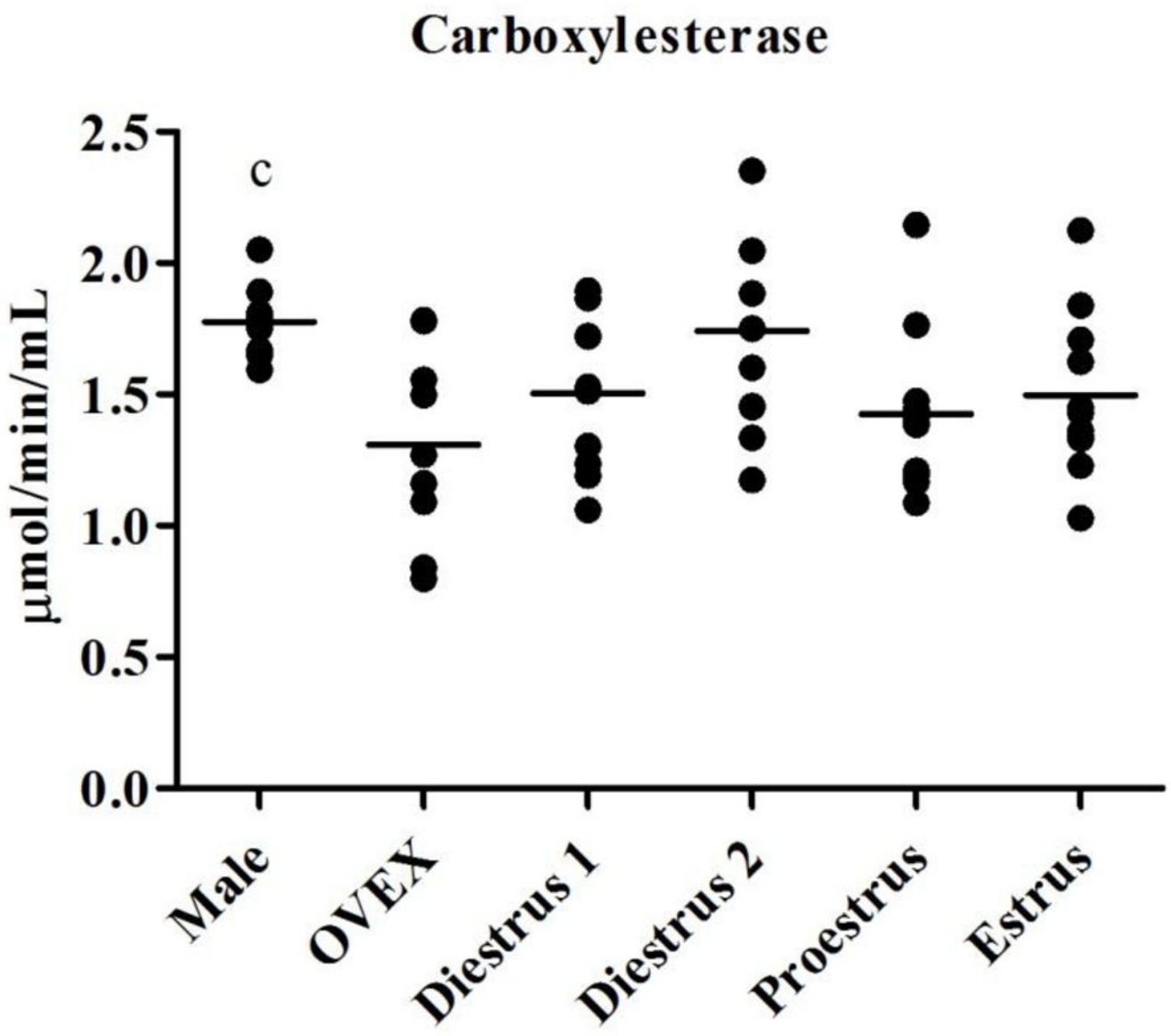


Figure 1.

Activity levels of (1.1) AChE, (1.2) BuChE, and (1.3) CE in male, OVEX, diestrous I, diestrous II, proestrous, and estrous rats. Significant post-hoc comparisons are shown in letters and symbols.

a: AChE in males differed from females in diestrus I, diestrus II, and estrus

b: BuChE in males differed from all females

c: CE in males differed from OVEX females

#: BuChE in OVEX females differed from females in diestrus II

Table 1

The 24 hr 1.0 × LD_{50} of sarin in male, OVEX, diestrous, proestrous, and estrous rats. Protective ratios and confidence bounds as determined by the maximum likelihood model are shown on the right.

Group	*n*	LD_{50} (μg/kg) (95% CI)	Protective Ratio (Confidence Bounds)			
			OVEX	Diestrus	Proestrus	Estrus
Male	23	116 (105–127)	0.92 (0.82–1.03)	1.00 (0.85–1.17)	1.08 (0.98–1.18)	1.05 (0.91–1.20)
OVEX	19	106 (99–113)		0.92 (0.79–1.06)	1.18* (1.07–1.26)	0.96 (0.84–1.08)
Diestrus	23	116 (102–132)	-	-	1.08 (0.94–1.23)	0.95 (0.81–1.13)
Proestrus	32	125 (122–127)	-	-	-	1.13* (1.02–1.25)
Estrus	21	111 (100–122)	-	-	-	-

* indicates a significant difference ($p < 0.05$).

Table 2

Means and standard deviations of AChE, BuChE, and CE plasma activity levels (μmol/min/mL) in male, OVEX, diestrous I, diestrous II, proestrous, and estrous rats. The two right columns show *F* and *p* values for esterase comparisons among each cycling female group with the male and OVEX rats.

Enzyme Activity (μmol/min/mL)						
Group	**AChE**	**BuChE**	**CE**			
Male	0.57 ± 0.13	0.086 ± 0.066	1.77 ± 0.15			
OVEX	0.44 ± 0.11	0.21 ± 0.073	1.31 ± 0.37		***F***	***p***
Diestrus I	0.37 ± 0.098	0.30 ± 0.11	1.50 ± 0.28	AChE	6.57	0.005
				BuChE	12.14	< 0.001
				CE	5.71	0.009
Diestrus II	0.36 ± 0.071	0.32 ± 0.090	1.74 ± 0.39	AChE	9.93	0.001
				BuChE	26.84	< 0.001
				CE	5.09	0.014
Proestrus	0.41 ± 0.13	0.29 ± 0.094	1.42 ± 0.30	AChE	4.00	0.031
				BuChE	12.61	< 0.001
				CE	5.75	0.009
Estrus	0.32 ± 0.12	0.30 ± 0.13	1.50 ± 0.31	AChE	9.03	0.001
				BuChE	9.77	0.001
				CE	5.33	0.012

Significant main effects were Bonferroni corrected at $p \leq 0.0167$.