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VX Toxicity in the Göttingen Minipig

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ABSTRACT

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INTRODUCTION

Swine have anatomical, physiological, and functional characteristics that are similar to humans, making them an acceptable non-rodent species for use in pharmacology and toxicology research (Dorandeu *et al.*, 2007; Svendsen, 2006; Swindle *et al.*, 2012; USDA, 2000). In particular, porcine cardiovascular, digestive, urinary, and dermal anatomy and physiology are well-suited for drug development systems (USDA, 2000). Because of the similarity in the structural and physical properties of porcine skin, swine are a good model for predicting human skin response to dermally applied pharmaceuticals and toxicants (Qvist *et al.*, 2000; Svendsen, 2006).

Swine commonly used as research subjects are *Sus scrofa domesticus*, whether they are domestic farm breeds or specialized miniature breeds (USDA, 2000). Typical farm breeds include Landrace, Yorkshire, Duroc, Hampshire, and Chester White strains and up to four-way cross-breeds. These farm breeds often reach adult weights in excess of 300 kg, making them less wieldy and economical research subjects. Miniature breeds, such as the Yucatan, Sinclair, and Göttingen, are attractive alternative swine models. The Göttingen minipig is a composite breed, whose development was begun in the 1960s with the principal aim of advancing it as a model experimental animal for medical and pharmacological research (Simianer and Köhn, 2010). The Göttingen minipig reaches sexual maturity within 3 to 5 months and has an average adult body weight of 35 kg at approximately 24 months (Köhn, 2012). The Göttingen is used primarily in regulatory toxicology studies; however, they are also used for cardiovascular, diabetic, orthopedic, dental, surgical, and other studies (Köhn, 2012).

The use of Göttingen minipigs for the evaluation of the toxicity of organophosphorus nerve agents has increased in the last decade (Dorandeu*et al.*, 2007; Hulet *et al.*, 2002; Hulet *et al.*, 2006; Hulet *et al.*, 2007; Hulet *et al.*, 2014). Hulet and colleagues (2007, 2014) have evaluated the toxic effects of several nerve agents (GB, GF, and VX) in the Göttingen minipig through inhalation, intravenous (IV), and subcutaneous (SC) routes of exposure. Saxena and colleagues (2008; 2011) evaluated the protective efficacy of injected human serum butyrylcholinesterase against sarin (GB) vapor in the Göttingen minipig. Worek and colleagues (2008) have modeled the enzyme inhibition kinetics of several nerve agents and reactivation kinetics of oximes using minipig blood samples. The establishment of predictive non-rodent animal models to evaluate the efficacy of medical countermeasures against nerve agents requires determining estimates of the nerve agent toxicity. In this vein, the current study was intended to estimate the median lethal dose (LD_{50}) of VX in the Göttingen minipig via intramuscular (IM) administration. The present study evaluated VX because it is among the most toxic nerve agents, and because of its physical properties (low volatility and oily liquid state under a broad range of environmental conditions), it poses a primarily dermal hazard. We chose the IM route because it is the controlled injection exposure route preferred for many previous medical chemical defense studies (Despain *et al.*, 2007) using non-human primates (a historically important large animal model).

METHODS

Subjects

Nineteen male Göttingen minipigs were obtained from Marshall BioResources (North Rose, NY, USA). Minipigs were between 89 and 110 days old and weighed between 5.0 and 8.2 kg at the time of shipping. The animals were acclimated to our facility and observed for evidence of disease for 5 days prior to initiating the study. Throughout the study, the minipigs were socially-housed (except during feeding and experimental assessments) in cages with ad libitum access to water in fully AAALAC-accredited facilities under a 12 h light/dark cycle (lights on at 0600) with temperature (21 ± 2 °C) and relative humidity (50 ± 10%) controlled. The animals were fed twice daily in accordance with vendor/breeder recommendations and given a small amount of fruit or vegetable enrichment (e.g., string beans, kale) each afternoon.

Procedure

The 24 h intramuscular LD_{50} of VX was determined using the up-and-down dosing method (Dixon and Massey, 1983). The initial dose was determined by calculating the ratios between published IV, SC, and IM LD_{50} values for VX from various species (e.g., rat, mouse, and guinea pig) to determine estimates for differences in lethality based on route of administration. The slope of the dose-response curve was estimated to be 10; thus the interval between doses was set to 0.1 $\log_{10}$. VX (0-ethyl S-(2-(diisopropylamino)ethyl)methylphosphonothioate, ≥ 98% purity) was obtained from the Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD, USA), diluted in ice-cold sterile saline, and stored at -80 °C until the day of use. VX was administered IM in the lateral thigh at a volume < 1 mL while the animal was resting in a sling. Following injection, the animal was promptly returned to a holding cage and observed for toxic signs continuously during the first hour post-exposure and at hourly intervals thereafter. Animals surviving to 24 h were humanely euthanized by overdose of pentobarbital.

Animals were fitted with actigraphy devices (MotionWatch8; CamNtech Inc., Boerne, TX, USA) that provided information regarding gross activity. This small, wireless, wristwatch-sized device contained a tri-axial accelerometer (and a lux meter) and wirelessly recorded the sum of all motion counts each second. The actigraphy devices were secured to the animal's torso and/or to the animal's right rear leg using cohesive flexible bandage. The actigraphy devices were fitted to the animal prior to exposure to obtain baseline levels of activity for 24 hours. On the day of exposure, actigraphy devices were typically fitted to the animal following the onset of sustained prostration and remained on until death or just prior to euthanasia.

Data Analysis

The LD_{50} estimate was calculated according to the method of Dixon and Massey (1983) for small sample sizes. Convulsion onset latencies were analyzed via t-test. Actigraphy data were summed within hourly blocks by animal for each sensor position separately (back and leg, where available). These hourly sums were then analyzed by fitting separate linear mixed-effects models with phase (baseline and exposure) and hourly interval as fixed-effects and the subject as the random effect for each sensor position. Because data were limited for the pubertal animals, actigraphy data only from the sexually mature animals are presented.

RESULTS

The LD_{50} determination was initiated using 10 animals that averaged 115 (range: 110 – 118) days of age and had an average weight of 8.8 (range: 7.6 – 9.5) kg. This group of animals will be hereafter referred to as "pubertal." Figure 1, panel A shows the results of the up-and down assay for this group of animals. Table 1 shows age and body weight at exposure, VX dose, and latency to convulsions for this group of animals. These 10 minipigs were administered doses of VX ranging from 11.7 µg/kg to 40.8 µg/kg. There was a single lethal outcome at the VX dose of 20.5 µg/kg. Following survival of the animal administered 40.8 µg/kg VX, the determination was made that an insufficient number of animals would remain to complete the required number of reversals to accurately estimate the LD_{50}. Based on experience and the available literature, an unknown factor was determined to be contributing to the survival of animals that were administered, ostensibly, supra-lethal doses of VX. To rule out the miscalculation of VX doses due to possible chemical degradation, aliquots of VX were submitted to three laboratories for independent analyses. These analyses revealed no significant agent degradation nor significant heterogeneity across aliquots. Furthermore, there were no significant differences in the rate of enzyme inhibition caused by these stock solutions compared to a reference sample of VX. The remaining differences between this study and the only published study using minipigs to estimate the lethal doses of VX (Hulet *et al.*, 2007) were animal age at time of dosing and route of administration. Data from other species and other nerve agents suggested that the route of administration (SC vs IM) would not account for the direction or magnitude of the difference in toxicity. Therefore, it was concluded, that age at the time of VX administration must be the factor responsible for decreased toxicity. According to the available literature, male Göttingen minipigs undergo sexual maturation between 3 – 4 months of age (Ellegaard *et al.*, 2010), and these animals were likely undergoing puberty at the time of exposure.

Subsequently, a second LD_{50} determination was undertaken using 9 minipigs that were 152 (range: 152 – 153) days of age and weighed an average of 12.9 (range: 10.9 – 15.8) kg. Figure 1, panel B shows the results of the up-and down assay for this group of animals. In these sexually mature animals, the IM LD_{50} of VX was estimated to be 17.4 µg/kg (95% CI = 14.1 – 21.6 µg/kg). Table 2 shows age and body weight at exposure, VX dose, and latency to convulsions for this group of mature animals.

Toxic Signs

The progression of toxic signs following VX administration in the minipig can be generally described as follows: localized fasciculations (at the injection site), mastication, ataxia, hyper-salivation, tremor, vocalizations, hyperactivity, generalized fasciculations, prostration, and convulsions. Table 3 shows the prevalence of toxic signs in both age groups of minipigs. As seen in Tables 1 – 3, all animals experienced convulsions. There was also a high prevalence of fasciculations, mastication, excessive salivation, vocalizations, ataxia, tremor, hyperactivity, and prostration. The vocalizations consisted mostly of loud barking or grunting that was typically coincident with the onset of convulsions. The hyperactivity could be characterized as a cluster of behaviors consisting of circling, ballistic movements within the cage (with concomitant

ataxia), and/or rearing with support of the cage walls with associated forelimb movements (i.e., wall climbing).

The average latencies to the onset of convulsions for the pubertal animals and sexually mature animals were 13.29 min (7.85 min SD) and 10.19 min (3.48 min SD), respectively. The difference between these convulsion onset latencies for the two age groups was not significantly different ($p > 0.29$).

Actigraphy

Figure 2 shows group averages of total activity counts within one-hour blocks as a function of phase and sensor location from sexually mature minipigs. As seen in these figures, activity counts increased dramatically for both sensor locations for approximately 5 hours following exposure before returning to roughly baseline levels. The activity counts during 0900 to 1200 following exposure were significantly increased compared to baseline counts for both sensor locations.

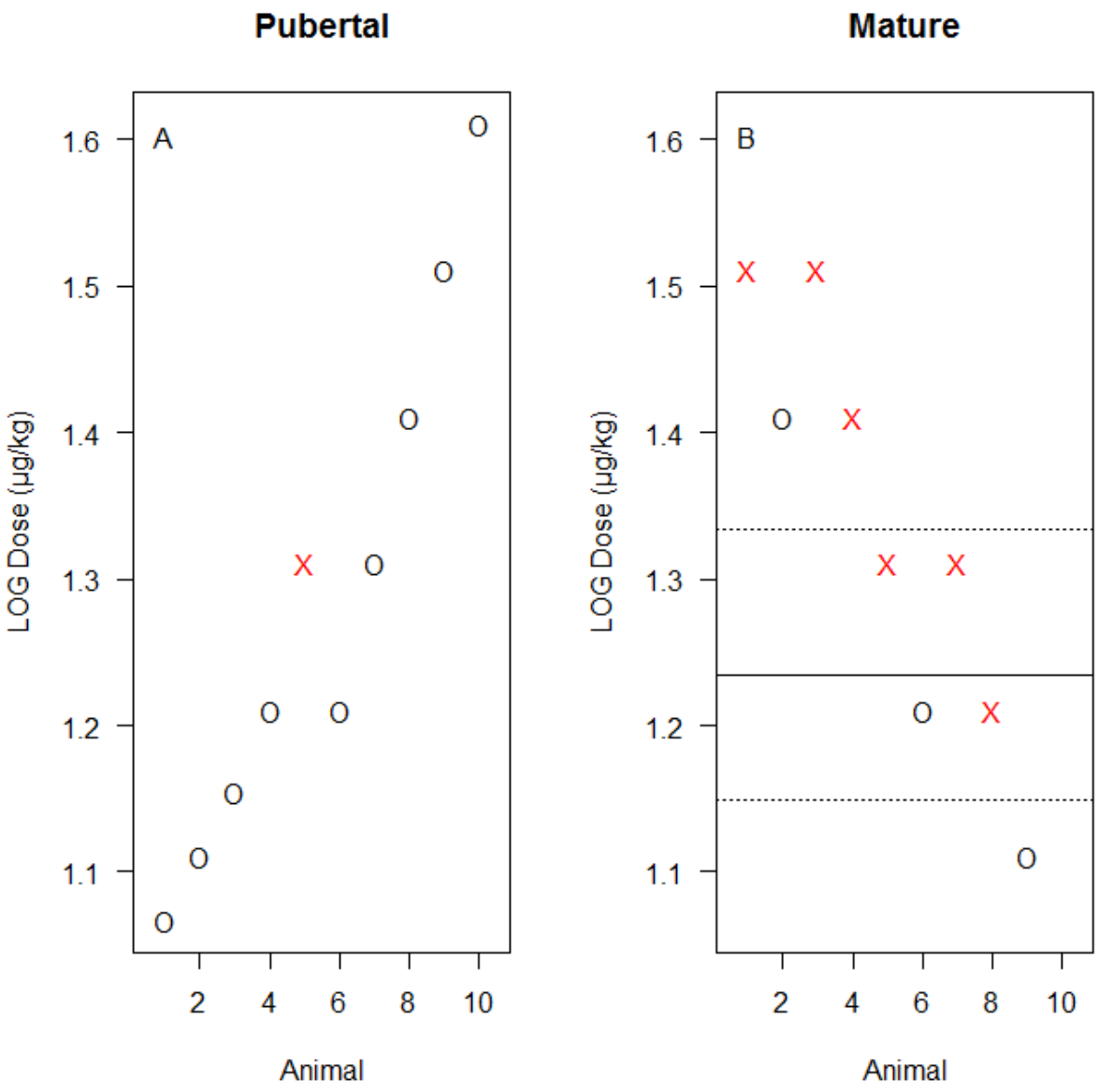

Figure 1. LD_{50} determinations in Göttingen minipigs as a function age. Panel A shows the sequence of VX administration and outcome for pubertal animals. The symbols "O" and "X" indicate 24 h survival and lethality, respectively. Panel B shows the sequence of VX administration in sexually mature animals. The solid horizontal line in Panel B indicates the LD_{50} estimate and the dotted lines indicate the 95% confidence intervals.

Table 1: Record of LD_{50} determination in pubertal minipigs.

Pig	Age (days)	Exposure BW (Kg)	VX Dose (µg/Kg)	Convulsion Onset (min)
1	116	8.9	11.7	23.2
2	117	7.6	12.9	20.8
3	117	8.6	14.3	22.5
4	117	9.5	16.2	22.4
5	118	8.9	20.5	9.9
6	118	8.4	16.2	9.8
7	110	9.3	20.5	6.7
8	111	8.7	25.7	7.4
9	111	9.3	32.4	5.2
10	112	8.7	40.7	5.1

Table 2: Record of LD_{50} determination in sexually mature minipigs.

Pig	Age (days)	Exposure BW (Kg)	VX Dose (µg/Kg)	Convulsion Onset (min)
1	152	12.4	32.4	6.7
2	153	11.5	25.7	9.5
3	152	12.4	32.4	9.1
4	152	13.6	25.7	8.1
5	152	13.2	20.5	7.7
6	153	15.8	16.2	10.8
7	153	15.2	20.5	8.0
8	152	10.9	16.2	15.9
9	152	11.0	12.9	16.0

Table 3: Prevalence of toxic signs in Göttingen minipigs

Toxic Sign	Pubertal Minipigs	Mature Minipigs
Mastication	9/10 (90%)	7/9 (78%)
Salivation	8/10 (80%)	8/9 (89%)
Fasciculation	10/10 (100%)	9/9 (100%)
Tremor	8/10 (80%)	7/9 (78%)
Ataxia	9/10 (90%)	8/9 (89%)
Convulsions	10/10 (100%)	9/9 (100%)
Vocalizations	9/10 (90%)	8/9 (89%)
Prostration	10/10 (100%)	9/9 (100%)
Hyperactivity	8/10 (80%)	7/9 (78%)
Urticaria	4/10 (40%)	6/9 (67%)
Penile Erection	2/10 (20%)	4/9 (44%)
Ejaculation	3/10 (30%)	5/9 (56%)
Defecation	9/10 (90%)	3/9 (33%)
Urination	1/10 (10%)	3/9 (33%)
Wet dog shake	1/10 (10%)	2/9 (22%)
Straub tail	3/10 (30%)	1/9 (11%)
Nystagmus	2/10 (20%)	1/9 (11%)

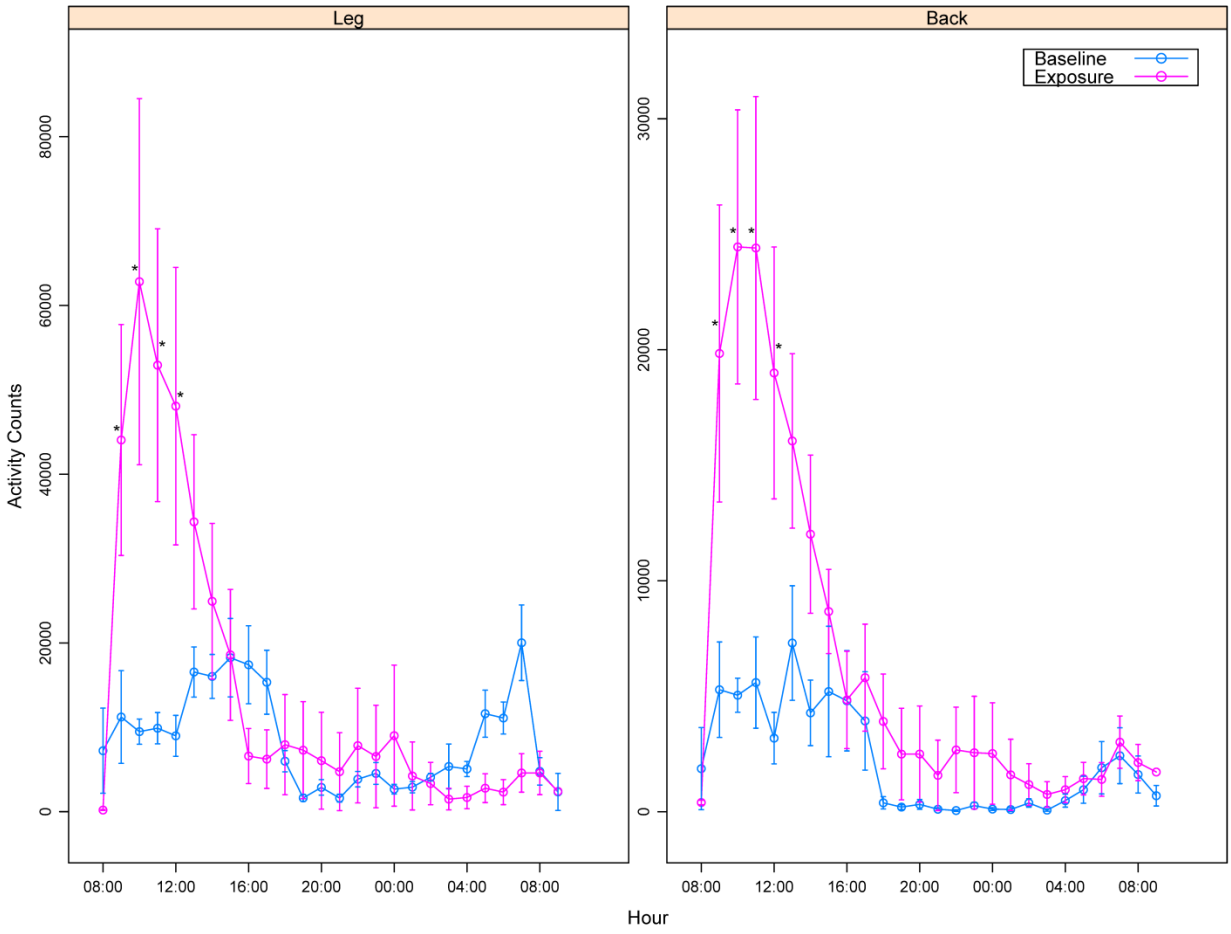

Figure 2. Total activity counts (± SEM) per hour as a function of actigraphy device placement and experimental phase for sexually mature animals. The symbols "*" denote a statistically significant increase from baseline values.

DISCUSSION

The data from the present study indicate that the toxicity of VX in the male Göttingen minipig is dependent upon the age and/or sexual maturity of the animal at the time of poisoning. In pubertal minipigs, administration of VX doses from 11 to 41 µg/kg resulted in only one death (at a dose of 20.5 µg/kg). In contrast, in sexually mature minipigs, administration of VX doses from 13 to 32 µg/kg resulted in only three of nine animals surviving (one each at 12.9 µg/kg, 16.2 µg/kg, and 25.7 µg/kg). The LD_{50} of VX in sexually mature animals was estimated to be 17.4 µg/kg (95% CI 14.1-21.6 µg/kg). Despite reduced lethality in pubertal animals, latency to the onset of convulsions was similar in both age groups, and toxic signs were qualitatively and quantitatively similar.

Previously presented data (Hulet *et al.*, 2007) estimated the LD_{50} of VX in sexually mature Göttingen minipigs to be 11.8 µg/kg (9.7-14.5 µg/kg) and 16.1 µg/kg (12.9-19.9 µg/kg) for the IV and SC routes, respectively. An analysis of the potential differences between our initial LD_{50} assessment and that of Hulet *et al.* (2007), besides the obvious difference in route of administration, indicated that animal age could have been a factor. Specifically, male Göttingen minipigs undergo sexual maturation (puberty) within the 90 to 120 day time frame (Ellegaard *et al.*, 2010). The first 10 minipigs were exposed between 110-118 days of age. This is a period of rapid developmental change. Age has been shown to alter agent toxicity in other species, such as rats (Benke and Murphy, 1975; Karanth and Pope, 2000; Shih *et al.*, 1990) and guinea pigs (Myers and Langston, 2012). Hulet (personal communication, 2014) reported that they used sexually mature minipigs that were in the 11 – 12.5 kg weight range at the time of SC VX administration, and the animals were approximately 4-5 months old.

Compared to rodent species, the Göttingen minipig appears to be less sensitive to the lethal effects of VX. In rats, the IM LD_{50} of VX has been reported to be approximately 8 µg/kg (Misik *et al.*, 2015), and the SC LD_{50} is between 8 µg/kg and 16.25 µg/kg (Maxwell, 1992; Shih and McDonough, 1999). In the guinea pig (Dunkin-Hartley) the IM LD_{50} of VX has been reported to be 7.07 µg/kg (Bajgar *et al.*, 2007) and the SC LD50 of VX has been reported to be approximately 8.5 µg/kg (Lenz *et al.*, 2005; Shih and McDonough, 1999). Dorandeu *et al.* (2008) reported a rapid and unexplained rebound in plasma butyrylcholinesterase (BChE) activity in domestic swine following IV challenge with approximately 0.5 – 1.0 LD_{50} VX. The authors suggest that the rebound in BChE activity was likely due to an increase in its release from producing organs. Exogenously administered BChE is known to be highly efficacious against VX poisoning in multiple species (Lenz *et al.*, 2007). Given the observations of Dorandeu *et al.* (2008), the decreased toxicity of VX in swine compared to rodent species could possibly be due to a spontaneous increase in BChE release in response to poisoning.

Clawn miniature swine (a crossbreed between Göttingen and Ohmini minipigs; Köhn, 2012) have been reported to have no plasma carboxylesterase (Bahar *et al.*, 2012); however, this breed of minipig has high levels of plasma BChE in addition to several unidentified esterases. Bahar *et al.* (2012) also reported that total plasma esterase activity of Clawn minipigs, as quantified by the hydrolysis of ρ-nitrophenyl acetate (PNPA), was approximately 10-fold higher than that of human plasma. Worek *et al.* (2008) reported that AChE activity in Göttingen minipig whole blood was approximately half that in human whole blood. In contrast, BChE

activity in the plasma of human blood was approximately 20-fold greater than that in minipig plasma. There are no known published studies that have examined the inhibition, reactivation, and aging kinetics of nerve agents in Göttingen minipig plasma. Taken together, these observations suggest that the decreased sensitivity of the Göttingen minipig to VX toxicity, compared to rodents, may be due to BChE activity levels or its affinity for VX; however, at this time the mechanism responsible for this difference remains unknown.

Signs of VX toxicity in the minipig were qualitatively similar to those observed in other species. Typical signs of OP intoxication (hyper-salivation, mastication, tremor, fasciculations, convulsions, ataxia, and prostration) were evident. However, a few toxic signs resulting from VX administration appeared to be unique to this species. The appearance of hyperactivity/hyper-locomotion was a toxic sign that has not frequently been observed during similar experiments with rodents. This particular toxic sign may be a species-specific phenomenon that is largely due to disorientation, since similar behavior has been observed in this species during recovery from anesthesia. A more puzzling toxic manifestation that was frequently observed was what might be termed cholinergic urticaria. This toxic sign was characterized by the sudden appearance of discontinuous (and apparently unraised) areas of dark pink discoloration of the skin, resembling hives or another allergic skin reaction. This sign was usually observed following the onset of active convulsions and was transient, lasting between a few minutes to a few hours in duration, sometimes disappearing then reappearing before completely resolving.

To our knowledge, this is the first study to quantify the behavioral and toxicological effects of VX in swine using actigraphy (c.f. Mann *et al.*, 2005). The actigraphy devices recorded both luminance data and movement counts. Although actigraphy does not provide as detailed or specific behavioral data as many other non-invasive methods of data collection, it did allow for more precise estimates of time of death (Russo *et al.*, 2005). For the quantification of toxic signs of nerve agent exposure, actigraphy appears best suited to quantify increased activity that accompanies tremors and/or convulsions.

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