VX Toxicity in the Göttingen Minipig

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22 ABSTRACT

23 The present experiments determined the intramuscular LD₅₀ of VX in male Göttingen
24 minipigs at two stages of development. In pubertal animals (115 days old), the LD₅₀ of VX was
25 indeterminate, but approximated 33.3 µg/kg. However, in sexually mature animals (152 days
26 old), the LD₅₀ was estimated to be only 17.4 µg/kg. Signs of nerve agent toxicity in the
27 Göttingen minipig were similar to those described for other species, with some notable
28 exceptions (such as urticaria and ejaculation). Latencies to the onset of sustained convulsions
29 were inversely related to the administered dose of VX in both ages of minipigs. Additionally,
30 actigraphy was used to quantify the presence of tremor and convulsions and, in some cases,
31 was useful for precisely estimating time of death. The main finding indicates that in minipigs, as
32 in other species, even relatively small differences in age can substantially alter the toxicity of
33 nerve agents. Additionally, actigraphy can serve as a non-invasive method of characterizing the
34 tremors and convulsions that often accompany nerve agent intoxication.

35 Keywords: nerve agent; swine; Göttingen minipig; median lethal dose; age; actigraphy

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 1960's 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).

Appropriately, the use of the Göttingen minipig 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), however, the development of this model is far from complete. Hulet and colleagues (2007, 2014) have evaluated the toxic effects of a few key nerve agents (GB, GF, and VX) in the Göttingen minipig through inhalation, intravenous (IV), and subcutaneous (SC) routes of exposure. Some additional, but limited, progress in the development of this animal model has been made by Saxena and colleagues (2015; 2008; 2011a). These studies evaluated the protective efficacy of

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4 65 injected human serum butyrylcholinesterase against sarin (GB) vapor in the Göttingen minipig.
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6 66 In one in vitro experiment, Worek and colleagues (2008) have modeled the enzyme inhibition
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8 67 kinetics of several nerve agents and reactivation kinetics of oximes using minipig blood
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12 69 The establishment of predictive non-rodent animal models to evaluate the efficacy of
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14 70 medical countermeasures against nerve agents requires characterizing nerve agent toxicity in
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16 71 each model species to include the time course and progression of toxic manifestations and
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18 72 quantification of exposure for each route of exposure (FDA, 2015). Historically, non-human
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20 73 primates have been the preferred non-rodent species used to evaluate the efficacy of nerve
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22 74 agent medical countermeasures (Castro *et al.*, 1992; Despain *et al.*, 2007; Haigh *et al.*, 2010;
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24 75 Harvilchuck *et al.*, 2013; Lenz *et al.*, 2005; von Bredow *et al.*, 1991). The preferred route of
25
26 76 nerve agent exposure in these non-human primate studies has been intramuscular (IM)
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28 77 administration. Because the Göttingen minipig is intended to serve as a suitable large animal
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30 78 alternative to non-human primate species, it is imperative to establish and compare the
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32 79 *intramuscular* toxicity of VX across these species. Intramuscular administration provides a
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34 80 sound and reproducible exposure route that maximizes experimental control and minimizes
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36 81 inter-subject variability. The technical implementation is simple (the muscle is large) and
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38 82 individual variations in variables such as fat content at the injection site are rendered largely
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40 83 inconsequential. Thus, the current study was intended to estimate the median lethal dose
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42 84 (LD₅₀) of VX in the Göttingen minipig via intramuscular administration. The present study
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44 85 evaluated VX because it is among the most toxic nerve agents, and because of its physical
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46 86 properties (low volatility and oily liquid state under a broad range of environmental conditions),
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48 87 it poses a primarily dermal hazard.

48 88 METHODS

50 89 Subjects

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53 90 Nineteen male Göttingen minipigs were obtained from Marshall BioResources (North
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55 91 Rose, NY, USA). Minipigs were between 89 and 110 days old and weighed between 5.0 and 8.2
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57 92 kg at the time of shipping. The animals were acclimated to our facility and observed for
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59 93 evidence of disease for 5 days prior to initiating the study. Throughout the study, the minipigs

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4 94 were socially housed (except during feeding and experimental assessments) in cages with *ad*
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6 95 *libitum* access to water under a 12 h light/dark cycle (lights on at 0600) with temperature ($21 \pm$
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8 96 2°C) and relative humidity ($50 \pm 10\%$) controlled. The animals were fed twice daily in
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10 97 accordance with vendor/breeder recommendations and given a small amount of fruit or
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12 98 vegetable enrichment (e.g., string beans, kale) each afternoon. The experimental protocol was
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14 99 approved by the Animal Care and Use Committee at the United States Army Medical Research
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16 100 Institute of Chemical Defense (USAMRICD) and all procedures were conducted in accordance
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18 101 with the principles stated in the Guide for the Care and Use of Laboratory Animals and the
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20 102 Animal Welfare Act of 1966 (P.L. 89-544), as amended. The USAMRICD is a research facility fully
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22 103 accredited by the Association for Assessment and Accreditation of Laboratory Animal Care
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24 104 International.

25 26 105 Procedure

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28 106 The 24 h intramuscular LD_{50} of VX was determined using the up-and-down dosing
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30 107 method (Dixon and Massey, 1983). The initial dose was determined by calculating the ratios
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32 108 between published IV, SC, and IM LD_{50} values for VX from various species (e.g., rat, mouse, and
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34 109 guinea pig) to determine estimates for differences in lethality based on route of administration.
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36 110 The slope of the dose-response curve was estimated to be 10; thus the interval between doses
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38 111 was set to $0.1 \log_{10}$. VX (O-ethyl S-(2-(diisopropylamino)ethyl)methylphosphonothioate; $\geq 98\%$
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40 112 purity) was obtained from the Edgewood Chemical Biological Center (Aberdeen Proving
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42 113 Ground, MD, USA), diluted in ice-cold sterile saline, and stored at -80°C until the day of use. VX
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44 114 was administered IM in the lateral thigh at a volume $< 1 \text{ mL}$ while the animal was resting in a
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46 115 sling. Following injection, the animal was promptly returned to a holding cage and observed for
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48 116 toxic signs continuously during the first hour post-exposure and at hourly intervals thereafter.
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50 117 Animals surviving to 24 h were humanely euthanized by overdose of pentobarbital.

51 118 Animals were fitted with actigraphy devices (MotionWatch8; CamNtech Inc., Boerne, TX,
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53 119 USA) that provided information regarding gross activity. This small, wireless, wristwatch-sized
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55 120 device contained a tri-axial accelerometer (and a lux meter) and wirelessly recorded the sum of
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57 121 all motion counts each second. The actigraphy devices were secured to the animal's torso
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59 122 and/or to the animal's right rear leg using cohesive flexible bandage. The actigraphy devices

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4 123 were fitted to the animal prior to exposure to obtain baseline levels of activity for 24 hours. On
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6 124 the day of exposure, actigraphy devices were typically fitted to the animal following the onset
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8 125 of sustained prostration and remained on until death or just prior to euthanasia.
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10 126 Data Analysis

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12 127 The LD₅₀ estimate was calculated according to the method of Dixon & Massey (1983) for
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14 128 small sample sizes. Convulsion onset latencies were analyzed via t-test. Actigraphy data were
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16 129 summed within hourly blocks by animal for each sensor position separately (back and leg,
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18 130 where available). These hourly sums were then analyzed by fitting separate linear mixed-effects
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20 131 models with phase (baseline and exposure) and hourly interval as fixed-effects and the subject
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22 132 as the random effect for each sensor position. Because data were limited for the pubertal
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24 133 animals, actigraphy data only from the sexually mature animals are presented.

25 134 RESULTS

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27 135 The LD₅₀ determination was initiated using 10 animals that averaged 115 (range: 110 –
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29 136 118) days of age and had an average weight of 8.8 (range: 7.6 – 9.5) kg. This group of animals
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31 137 will be hereafter referred to as “pubertal.” Figure 1, panel A shows the results of the up-and
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33 138 down assay for this group of animals. Table 1 shows age and body weight at exposure, VX dose,
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35 139 and latency to convulsions for this group of animals. These 10 minipigs were administered
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37 140 doses of VX ranging from 11.7 µg/kg to 40.8 µg/kg. There was a single lethal outcome at the VX
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39 141 dose of 20.5 µg/kg. Following survival of the animal administered 40.8 µg/kg VX, the
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41 142 determination was made that an insufficient number of animals would remain to complete the
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43 143 required number of reversals to accurately estimate the LD₅₀. Based on experience and the
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45 144 available literature, an unknown factor was determined to be contributing to the survival of
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47 145 animals that were administered, ostensibly, supra-lethal doses of VX. To rule out the
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49 146 miscalculation of VX doses due to possible chemical degradation, aliquots of VX were submitted
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51 147 to three laboratories for independent analyses. These analyses revealed neither significant
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53 148 agent degradation nor significant heterogeneity across aliquots. Furthermore, there were no
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55 149 significant differences in the rate of enzyme inhibition caused by these stock solutions
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57 150 compared to a reference sample of VX. The remaining differences between this study and the
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59 151 only published study using minipigs to estimate the lethal doses of VX (Hulet, *et al.*, 2007) were
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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 the remarkably high rate of survival. 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 LD₅₀ 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 LD₅₀ 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 the first 4 hours following exposure were significantly increased compared to baseline counts for both sensor locations. The leg appeared to be the more sensitive sensor location. Activity counts of the leg sensor were 2 to 3.5-fold higher than those of the back sensor; however, the pattern of statistical significance did not differ between the two sensor locations.

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 $\mu\text{g}/\text{kg}$ resulted in nine of ten animals surviving (one lethality at a dose of 20.5 $\mu\text{g}/\text{kg}$). In contrast, in sexually mature minipigs, administration of VX doses from 13 to 32 $\mu\text{g}/\text{kg}$ resulted in only three of nine animals surviving (one each at 12.9 $\mu\text{g}/\text{kg}$, 16.2 $\mu\text{g}/\text{kg}$, and 25.7 $\mu\text{g}/\text{kg}$). The LD_{50} of VX in sexually mature animals was estimated to be 17.4 $\mu\text{g}/\text{kg}$ (95% CI 14.1-21.6 $\mu\text{g}/\text{kg}$). Despite increased survival of pubertal animals, latency to the onset of convulsions was similar in both age groups, and toxic signs were qualitatively and quantitatively similar.

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

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4 210 skin, resembling hives or another allergic skin reaction. A similar observation of “irregular pink-
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6 211 purple blotches” was reported following the administration of the oxime MMB-4 to swine
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8 212 (Stemler *et al.*, 1991). In the present study, this sign was usually observed following the onset of
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10 213 active convulsions and was transient, lasting between a few minutes to a few hours in duration,
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12 214 sometimes disappearing then reappearing before completely resolving.
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14 215 To our knowledge, this is the first study to quantify the behavioral and toxicological
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16 216 effects of VX using actigraphy (Mann *et al.*, 2005). The actigraphy devices recorded both
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18 217 luminance data and movement counts. Although actigraphy does not provide as detailed or
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20 218 specific behavioral data as many other non-invasive methods of data collection, it did allow for
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22 219 more precise estimates of time of death (Russo *et al.*, 2005). For the quantification of toxic
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24 220 signs of nerve agent exposure, actigraphy appears best suited to quantify increased activity that
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26 221 accompanies tremors and/or convulsions and may be useful for evaluating sleep disturbances
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28 222 (Landry *et al.*, 2015).
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30 223 Previously presented data (Hulet, *et al.*, 2007) estimated the LD₅₀ of VX in sexually
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32 224 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)
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34 225 for the IV and SC routes, respectively. An analysis of the potential differences between our
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36 226 initial LD₅₀ assessment in pubertal pigs and that of Hulet, *et al.* (2007), besides the obvious
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38 227 difference in route of administration, indicated that animal age could have been a factor.
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40 228 Specifically, male Göttingen minipigs undergo sexual maturation (puberty) within the 90 to 120
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42 229 day time frame (Ellegaard, *et al.*, 2010) and the first 10 minipigs in our study were exposed
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44 230 between 110-118 days of age. This is a period of rapid developmental change. Moreover, age
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46 231 has been shown to alter agent toxicity in other species, such as rats (Benke and Murphy, 1975;
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48 232 Karanth and Pope, 2000; Shih *et al.*, 1990) and guinea pigs (Myers and Langston, 2012). Hulet
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50 233 (personal communication, 2014) reported that they used sexually mature (approximately 4-5
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52 234 month old) minipigs weighing between 11 – 12.5 kg at the time of SC VX administration.
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54 235 In rats and mice, age-related differences in the toxicity of G-series nerve agents (i.e., GA,
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56 236 GB, GD, GF) and organophosphate pesticides are strongly correlated with age-related
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58 237 differences in plasma carboxylesterase (CaE) activity (Atterberry *et al.*, 1997; Benke and
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60 238 Murphy, 1975; Karanth and Pope, 2000; Mortensen *et al.*, 1996; Moser *et al.*, 1998; Peet *et*
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4 239 *al.*, 1987; Shih, *et al.*, 1990; Wright *et al.*, 2016). Perinatal and adult rats and mice are more
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6 240 sensitive to the lethal effects of G-series agents than are pubertal animals (Peet, *et al.*, 1987;
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8 241 Wright, *et al.*, 2016). In rats and mice, plasma CaE activity is low in perinatal animals (~7 d) and
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10 242 increases throughout development, reaching adult levels by ~30 d (Duysen *et al.*, 2001; Sterri
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12 243 *et al.*, 1985). In contrast to the G-series agents, there appears to be no age-related differences
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14 244 in V-series agent toxicities (i.e., VM, VR, VX) in rats (Wright, *et al.*, 2016). Additionally, when rats
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16 245 are administered the CaE inhibitor 2-(*O*-Cresyl)-4H-1,3,2benzodioxaphosphorin-2-oxide (CBDP)
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18 246 there are significant alterations in the toxicities of G-series agents, but no change in the toxicity
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20 247 of VX (Maxwell, 1992). This difference is likely due to the observation that CaE has low
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22 248 reactivity with polar compounds, such as VX, and does not protect acetylcholinesterase (AChE)
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24 249 from irreversible inhibition by VX (Maxwell and Brecht, 2001).

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26 250 In contrast to rats and mice, swine, like humans, non-human primates, and guinea pigs,
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28 251 have very little or no plasma CaE (Bahar and Imai, 2013; Bahar *et al.*, 2012; Li *et al.*, 2005;
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30 252 Mendoza *et al.*, 1976). Swine, like most mammals, also have other plasma esterases that could
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32 253 confer some protection against organophosphates, notably arylesterase (a historic term for
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34 254 paraoxonase-1 [PON-1]) and butyrylcholinesterase (BuChE) (Augustinsson and Olsson, 1959a;
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36 255 Augustinsson and Olsson, 1959b; Bahar and Imai, 2013; Bahar, *et al.*, 2012; Li, *et al.*, 2005;
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38 256 Peng *et al.*, 2016; Richter *et al.*, 2008). It is widely known that BuChE hydrolyzes certain drugs,
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40 257 such as cocaine and heroin (Li, *et al.*, 2005), and functions as a scavenger of toxic
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42 258 organophosphorus compounds (Lenz, *et al.*, 2005; Lenz *et al.*, 2007). Several investigators have
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44 259 demonstrated that exogenously administered BuChE can protect against the toxic effects of
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46 260 multiple LD₅₀ doses of nerve agents, including VX, when administered either prophylactically or
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48 261 as post-exposure therapy (Lenz, *et al.*, 2005; Lenz, *et al.*, 2007; Mumford *et al.*, 2013;
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50 262 Mumford *et al.*, 2011; Mumford *et al.*, 2010; Mumford and Troyer, 2011; Saxena, *et al.*, 2015;
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52 263 Saxena, *et al.*, 2008; Saxena *et al.*, 2011b). However, to be effective, BuChE must be
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54 264 administered in relatively large doses due to its stoichiometric binding with nerve agents
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56 265 (Ashani *et al.*, 2016; Lenz, *et al.*, 2007; Masson *et al.*, 2008).

57 266 In rats, plasma BuChE activity is influenced by sexual development and sex hormones. In
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59 267 male rats, serum BuChE activity decreased by approximately 50% concurrently with the onset
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of sexual maturity (Leeuwin, 1965; Vetter-O'Hagen and Spear, 2012). In contrast, the serum BuChE activity of female rats increased throughout sexual maturation and, in adulthood, was higher than that of males (Alves-Amaral *et al.*, 2010; Gordon and Mack, 2003; Leeuwin, 1965; Schmidt and Schmidt, 1978; Smith *et al.*, 2015; Tsuchiya *et al.*, 1995; Vetter-O'Hagen and Spear, 2012; Wang *et al.*, 2000; Wang *et al.*, 2001; Wang and Weinstock, 2001). Gonadectomy reversed the effects of sexual maturity on BuChE activity (Alves-Amaral, *et al.*, 2010; Illsley and Lamartiniere, 1981; Wang, *et al.*, 2001; Wang and Weinstock, 2001) and administration of sex hormones restored the sexually dimorphic BuChE activity (Illsley and Lamartiniere, 1981). Furthermore, testosterone administration to gonadectomized rats decreased BuChE activity (Leeuwin and Groenewoud, 1970; Wang, *et al.*, 2001; Wang and Weinstock, 2001). Testosterone and some of its metabolites are reversible BuChE inhibitors (Al-Aboudi *et al.*, 2009). The BuChE-inhibiting property of testosterone may partially account for the relationship between puberty and BuChE activity in male rats. Gonadectomy and/or sex hormone administration have been shown to alter the effects of parathion, carbamates, and the protection afforded by medical countermeasures against soman toxicity in rats (Agarwal *et al.*, 1982; Lundy *et al.*, 1989; Wang, *et al.*, 2000; Wang, *et al.*, 2001; Wang and Weinstock, 2001).

The present experiments were conducted with male swine in the age ranges reported for puberty and post-puberty (Ellegaard, *et al.*, 2010), depending on the definition of puberty used (Howroyd *et al.*, 2016). Plasma testosterone levels in male Göttingen minipigs increase by 2.4-fold from 11 to 25 weeks of age (Christoffersen *et al.*, 2013). In large swine, there was a 25-fold increase in plasma testosterone concentration from pre-puberty to puberty (At-Taras *et al.*, 2006). Interestingly, male swine also have high plasma levels of estrogen that increase concurrently with testosterone levels during sexual maturation (At-Taras, *et al.*, 2006; Christoffersen, *et al.*, 2013; Christoffersen *et al.*, 2007). Notably, swine are reported to have 13-21-fold lower BuChE activity than humans (Peng, *et al.*, 2016; Worek, *et al.*, 2008). Further, polyacrylamide gel electrophoresis (PAGE) stained for esterase activity by β -naphthylacetate revealed undefined bands corresponding to unidentified esterases in swine plasma (Bahar, *et al.*, 2012). It has also been reported that swine plasma cholinesterases lack substrate specificity (Myers, 1953), are resistant to inhibition by diisopropylfluorophosphate, paraoxon, and

dichlorvos (Ecobichon and Comeau, 1973), and consist of both AChE and BuChE (Arpagaus *et al.*, 1991). Dorandeu *et al.* (2008) reported a rapid and unexplained rebound in plasma BuChE activity in domestic swine following IV challenge with approximately 0.5 – 1.0 LD₅₀ VX. A similar observation was made by Tenberken *et al.* (2010) in swine following the IV administration of tabun (GA). These authors suggested that the rebound in BChE activity was likely due to an increase in its release from producing organs. Thus, it is possible that our pubertal animals had lower plasma testosterone levels than the mature animals and those differences in testosterone level could have influenced BuChE activity and hence VX toxicity. However, according to a recent investigation on the effects of exogenously administered BuChE in minipigs, a 248-fold increase in BuChE activity was required to protect against the lethal effects of ~3 LCT₅₀ of sarin (Saxena, *et al.*, 2015). Furthermore, in previous studies 93-219-fold increases in BuChE activity failed to protect minipigs from the lethal effects of 1 LCT₅₀ of sarin (Saxena, *et al.*, 2008; Saxena, *et al.*, 2011a). This suggests that extremely large changes in swine BuChE activity related to sexual development would have to occur to appreciably alter nerve agent toxicity.

A current focus in the development of catalytic bioscavengers to protect against nerve agent toxicity is focused on developing a mutated form of PON-1 or other phosphotriesterases that will hydrolyze both G- and V-series agents (Ashani, *et al.*, 2016; Lenz, *et al.*, 2007; Masson and Lushchekina, 2016; Masson, *et al.*, 2008). In a proof of concept experiment, Broomfield (1993) demonstrated that there was a protective effect of organophosphorus acid anhydrolase (OPAH) in mice challenged with soman. In pigs, plasma arylesterase hydrolysis of p-nitrophenylacetate (PNPA) is 10-fold higher than that in humans (Bahar, *et al.*, 2012). Augustinsson (1961) reported that in swine, puberty and exogenous testosterone administration decreased arylesterase activity whereas castration increased arylesterase activity. The maximum change in arylesterase activity in that study was approximately 5-fold. The generality of this finding was confirmed in dogs (Augustinsson and Henricson, 1965). Carr *et al.* (2015) reported that pig serum PON-1 had a moderate level of activity toward chlorpyrifos-oxon and low levels of activity against paraoxon and methyl paraoxon. Recent studies examining nerve agent hydrolysis by native human PON-1 have revealed limitations in the both

the rate and substrate specificity as well as the protection afforded against nerve agents (diTargiani *et al.*, 2010; Hodgins *et al.*, 2013; Otto *et al.*, 2009). Specifically, the catalytic efficiency (k_{cat}/K_M) of native human PON-1 hydrolysis of VX was reported to be $\sim 10^5$ -fold lower (Otto, *et al.*, 2009; Peterson *et al.*, 2011) than that estimated to be required to afford complete protection against nerve agent toxicity (Ashani, *et al.*, 2016). To our knowledge, no studies of swine PON-1 hydrolysis of nerve agents have been published; however, given the low hydrolytic activity of swine PON-1 against paraoxon, it is unlikely that a 5-fold difference in activity due to sexual maturation, or the lack thereof, would have been sufficient to account for the differences in survival observed in the present study. Ashani, *et al.* (2016) estimated that a catalytic efficiency of $2.5 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ would likely confer protection against lethality.

The acute lethal effects of VX were dependent on the developmental stage of male Göttingen minipigs. Pubertal animals were less sensitive to the lethal effects of VX than sexually mature animals. The mechanism underlying the observed difference in sensitivity to VX is unclear, but may be related to hormone levels associated with sexual maturation. Additional research will be required to identify the mechanism underlying the interaction between sexual maturity and VX toxicity in the Göttingen minipig. Clearly, more research is required to fully characterize the esterase profile of swine plasma and delineate species and strain differences where they exist. Although additional research is required to identify the contributing mechanism, the results from the present study emphasize the importance of age on the toxicity of organophosphorus compounds.

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Figure 1. LD₅₀ determinations in Götting minipigs as a function of 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 LD₅₀ estimate and the dotted lines indicate the 95% confidence intervals.

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631 Table 1: Record of LD₅₀ determination in pubertal minipigs.

		Exposure	VX Dose	Convulsion
Pig	Age (days)	BW (Kg)	(µg/Kg)	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

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634 Table 2: Record of LD₅₀ determination in sexually mature minipigs.

		Exposure		VX Dose	Convulsion
Pig	Age (days)	BW (Kg)	(µg/Kg)	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	

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637 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%)

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Figure 2. Total activity counts ($\pm$ SEM) as a function of hours post-exposure, actigraphy device placement, and experimental phase in sexually mature animals. The symbols “*” denote a statistically significant increase from baseline values. Note the scaling of the ordinate is different for the two sensor locations.

Figure1

Figure2,