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Increased Vulnerability to Soman Exposure in Aged Compared to Adult F344 Rats

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14. ABSTRACT
To determine the susceptibility of aged individuals to chemical warfare nerve agents (CWNAs), we evaluated the 24-hour median lethal dose (LD_{50}) of soman (GD) in adult (2 months of age) and aged (18 months of age) male F344 rats. Aged rats proved more sensitive to GD-induced lethality with an LD_{50} of 50.0 µg/kg (95% CI; 37.8-66.1), compared to adult rats with an LD_{50} of 70.5 µg/kg (95% CI; 63.8-78.00). To determine the etiology of these differences, we measured whole blood and brain acetylcholinesterase (AChE) as well as plasma carboxylesterase (CaE) activity in naïve aged and adult rats. Whole blood AChE revealed no differences; however, an 18% decrease in baseline plasma CaE activity was observed in aged rats compared to adult rats. Brain AChE was modestly reduced in aged compared to adult rats in the frontal cortex (82%), hippocampus (89%), striatum (92%), thalamus (91%), and pons (83%). Furthermore, aged rats showed a significantly greater sensitivity to the toxic effects of GD, displaying seizures and higher mortality rates at lower doses compared to adult rats. This increased sensitivity of aged rats to the toxic and lethal effects of GD has implications for dosing and efficacy of medical countermeasures in aged populations.

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Abstract

Aged individuals are one of the fastest growing segments of the population and represent a vulnerable group in the event of a terrorist attack using chemical warfare nerve agents (CWNAs). To determine the susceptibility of aged individuals to CWNAs, we evaluated the 24-hour median lethal dose (LD_{50}) of soman (GD) in adult (2 months of age) and aged (18 months of age) male F344 rats. Aged rats proved more sensitive to GD-induced lethality with an LD_{50} of 50.0 µg/kg (95% CI; 37.8-66.1), compared to adult rats with an LD_{50} of 70.5 µg/kg (95% CI; 63.8-78.00). To determine the etiology of these differences, we measured whole blood and brain acetylcholinesterase (AChE) as well as plasma carboxylesterase (CaE) activity in naïve aged and adult rats. Whole blood AChE revealed no differences; however, an 18% decrease in baseline plasma CaE activity was observed in aged rats compared to adult rats. Brain AChE was modestly reduced in aged compared to adult rats in the frontal cortex (82%), hippocampus (89%), striatum (92%), thalamus (91%), and pons (83%). Furthermore, aged rats showed a significantly greater sensitivity to the toxic effects of GD, displaying seizures and higher mortality rates at lower doses compared to adult rats. This increased sensitivity of aged rats to the toxic and lethal effects of GD has implications for dosing and efficacy of medical countermeasures in aged populations.

Introduction

According to the 2010 Census, the population of older Americans (≥ 65 years of age) is 40.3 million, 13.0% of the total population (Werner, 2011). Aged individuals are one of the fastest growing segments of the American population and represent a particularly vulnerable group in the event of a terrorist attack using chemical warfare nerve agents (CWNAs). However, the vast majority of research on medical countermeasures to CWNAs has been conducted on young adult male animal models of CWNA exposure, focusing on the potential for drug therapies to increase survival and terminate seizure activity to predict drug effects in the young adult male soldier. Little consideration has been given to the unique challenges associated with CWNA medical countermeasures in the elderly population.

Exposure to CWNA results in the irreversible inhibition of acetylcholinesterase (AChE) and subsequent excess of peripheral and central acetylcholine (ACh) at synapses and neuromuscular junctions. Symptoms of CWNA intoxication include miosis, hypersecretion, fasciculation, respiratory distress, cardiac dysfunction, and seizures progressing to *status epilepticus* (*SE*; reviewed in [Cannard, 2006]). CWNA-induced seizures result in neuronal death, affecting several brain regions, including the hippocampus, amygdala, thalamus, and piriform and perirhinal cortices (Carpentier, Delamanche, Le Bert, Blanchet, & Bouchaud, 1990; Petras, 1994; Shih, Duniho, & McDonough, 2003). A greater susceptibility to seizures is observed in both the aged human population and aged laboratory animals. Liang *et al.* (2007) reported a lower kainic acid-induced seizure threshold and increased oxidative stress in hippocampal CA1 neurons of aged rats compared to adult rats. Furthermore, following acute kainic acid-induced seizure activity, aged rats displayed a greater loss of pyramidal neurons in the CA1 layer of the hippocampus when compared to young adult rats. Aged rats are also more sensitive than young adult rats to the lethal effects of pilocarpine, a muscarinic acetylcholine receptor (mAChR) agonist used to induce seizures in animal models of epilepsy (Blair, Deshpande, Holbert, Churn, & DeLorenzo, 2009). In addition, a higher degree of acute toxicity is observed in aged rats exposed to the organophosphate insecticide parathion, despite similar levels of AChE inhibition and lower levels of ACh accumulation in the striatum of aged rats (Karanth *et al.,* 2007). Aged albino outbred rats are more susceptible to lethal effects of intramuscularly administered soman (GD) (Shih, Penetar, McDonough, Romano, & King, 1990).

Diverse alterations in cholinergic function may underlie the sensitivity of aged animals to CWNA intoxication. Older rats are more responsive to cholinergic-mediated behaviors *(e.g.*, salivation, lacrimation, and tremor) induced by cholinesterase inhibitors compared to young adult rats, in a manner that is independent of pharmacokinetic differences (Goh, Aw, Lee, Chen, & Browne, 2011; Karanth, Liu, Ray, & Pope, 2007). Kosaka *et al*. (1999) demonstrated a greater effect of the cholinesterase inhibitors donepezil and tacrine in the inhibition of AChE in the brains of aged rats compared to young adult rats. Furthermore, aged rats display a decrease in the density of brain mAChR (Yufu, Egashira *et al*., 1994), suggesting that a comparatively smaller increase in ACh in the aged population would result in a higher percentage of mAChR binding, thus leading to an increased susceptibility to CWNA toxicity in this population. Susceptibility to the toxic effects of organophosphorus agents may also be dependent upon the activity of choline acetyltransferase (ChAT), an enzyme crucial for the synthesis of ACh (Araujo, Lapchak, Meaney, Collier, & Quirion, 1990; Overstreet, 2000; Yufu, Egashira, & Yamanaka, 1994). The purpose of the current study was to investigate the differences to GD toxicity between young adult and aged F344 rats exposed subcutaneously using stagewise sequential design (Feder *et al*., 1991), as well as the biochemical variations relevant to the cholinergic system between these age groups.

Methods

Subjects: Male F344 adult (2 months old) and aged (18 months old) rats from the National Institute on Aging aged-rodent colony at Taconic or Charles River Laboratories were maintained on a normal (lights on from 9:00-21:00; Experiment 1) or reverse (lights on from 21:00-09:00; Experiment 2) light-dark schedule, with laboratory rat chow and water available *ad libitum.* 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.

Experiment 1: Median lethal dose (LD_{50}), cholinesterase and CaE activity in GD-exposed adult and aged rats.

LD_{50} Determination: Adult and aged rats were exposed subcutaneously to GD (70-200 µg/ml, 0.4-0.63 ml/kg) in a stage-wise manner using the methods described by Feder (P. Feder, Olson, Hobson, & Matthews, 1991; P. I. Feder, Hobson, Olson, Joiner, & Matthews, 1991; P. I. Feder, Olson, Hobson, Matthews, & Joiner, 1991). The initial stage included GD dosages expected to span the predicted range of lethality from 0-100% for both age groups. Each stage included 1-3 animals randomly assigned to each of the GD dose levels, and the results from each stage were used to guide dose selection for subsequent stages. This stage-wise approach continued until the ratio of the half width of the 95% confidence interval (CI) defined as (Upper bound – Lower bound)/(2 x LD_{50}), for the LD_{50} was less than 0.40. A probit regression using the maximum likelihood procedure was applied to the combined data from all stages to calculate the LD_{50}. The 95% CIs for the LD_{50} were calculated using the delta method.

Blood Sampling: Blood (100-300 µl) from rats was collected in EDTA-coated tubes via tail vein incision for the LD_{50} (baseline only) for comparison of blood ChE and CaE in adult and aged rats.

Brain Sampling: In a naïve set of rats, the brain was dissected into the frontal cortex, remaining cortex, hippocampus, thalamus, striatum, pons, and cerebellum and assayed for cholinesterase.

Cholinesterase and Carboxylesterase Assays: Rat plasma was prepared by centrifugation of whole blood at 3k x g for 10 minutes at 4°C, and then frozen on dry ice and stored at -80°C. Plasma carboxylesterase (CaE) activities were determined as described (Hashinotsume, Higashino, Hada, & Yamamura, 1978). Briefly, samples (10 µL) were pre-incubated with 10 µM eserine and 10 mM EDTA for 30 min in a reaction buffer of 50 mM HEPES pH 7.4 in a total volume of 0.28 mL. Treated samples were placed into wells of a 96-microtiter plate, and the reaction was initiated by the addition of 20 µL p-nitrophenyl acetate to 2.5 mM. The plate was immediately placed into a Molecular Dynamics model SpectraMax Plus plate reader under the control of SoftMax Ver. 5.4 software (Molecular Dynamics, Inc.), and the assay was conducted at 25°C. Plates were shaken for 60 sec before the first read at 1 min and then for 3 sec before each subsequent read at 1 min intervals for 10 min. Absorbance was measured at 400 nm. 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}. One unit is defined as 1 µmol product formed per minute. Samples were assayed in duplicate.

Acetylcholinesterase activity in whole blood and dissected brain regions (using acetylthiocholine as the substrate) was determined by an automated method (Feaster & Doctor, 2000; Feaster, Gordon, & Doctor, 2004) based on the manual method of Ellman *et al*. (1961) and modified for the microplate spectrophotometer. For the determination of brain AChE activity, naïve rats were deeply anesthetized with a sodium pentobarbital solution (Fatal-Plus®, Vortech

Pharmaceuticals, Ltd., Dearborn, MI) and perfused with ice cold heparinized saline in 0.1 M phosphate buffer.

Experiment 2: Seizure, blood cholinesterase and performance measures in GD-exposed adult and aged rats.

Surgery: Rats were anesthetized with isoflurane (4% induction, 1.5-3% maintenance) and surgically implanted with a telemetry transmitter (F40-EET, Data Sciences International, Inc.; New Brighton, MN) for the continuous monitoring and collection of electroencephalographic (EEG) activity and body temperature. Specifically, the rats were placed in a Kopf (Tujunga, CA) stereotaxic frame, and four stainless-steel screws were implanted into the skull (A/P, + 1.6; L, ± 2.0 and A/P, -4.0; L, ± 2.0). Stainless steel wires from the F40-EET transmitters were implanted subcutaneously, wrapped around the electrodes, and secured in place using dental acrylic. The body of the transmitter was positioned in a subcutaneous pocket in the left flank of the animal. Immediately following surgery, the rats were administered the analgesic buprenorphine (0.1 mg/kg, sc) and given a wet mash made of standard laboratory chow and sugar or grain based Nutra Gel Diet cubes (BioServ; Flemington, NJ). Animals were administered daily subcutaneous injections of saline (3 ml) for one week following surgery and allowed to recover from surgery for three weeks before exposure to GD.

Telemetry Equipment: The home cage was placed on a DSI Physiotel Receiver Model RPC-1 in the colony room for EEG acquisition (Dataquest ART™ 4.1; DSI). Data was collected continuously from 2 to 3 days prior to GD exposure and extended throughout the duration of the experiment. Body temperature was also recorded via the system described above.

GD Exposure: Following recovery from surgery, rats were administered GD (22-88 µg/kg; 0.5 ml/kg, sc) or saline (0.5 ml/kg, sc). Animals were continuously observed for toxic signs (*i.e.*, mastication, head myoclonus, forelimb myoclonus, rearing, falling and/or tonic-clonic seizures; Racine, 1972) for 2 hours following exposure and every 30 minutes afterward. Following exposure, rats were administered daily subcutaneous injections of saline (3 ml) and given a wet mash made of standard laboratory chow and sugar or grain-based Nutra Gel Diet cubes as needed for up to 2 weeks following exposure.

Blood Sampling and Assays: Blood (100-300 µl) from rats was collected in EDTA-coated tubes via tail vein incision at baseline, 30 minutes, 2 hours, 6 hours, 24 hours, 48 hours, 7 days, 14 days, and 21 days post-exposure in the rats exposed to 22 µg/kg GD. Additional blood was obtained via cardiac puncture in naïve animals deeply anesthetized with a pentobarbital solution prior to saline perfusion. Cholinesterase and carboxylesterase assays were conducted as described in Experiment 1.

Morris Water Maze: The rats were tested in a 150 cm radius circular pool filled with water (26 ± 1 °C) with a hidden platform submerged just below the surface of the water. Water was made opaque using black Tempera paint. Starting on the second week following exposure, spatial memory acquisition was assessed by the latency to find the platform over three days (2 sessions per day; 4 trials per session with 30 min rest period). Prior to the initial session the rats were placed on the platform for 10 s. The session began with the rats placed facing the wall of the pool in one of the four possible quadrants. Starting quadrants for each session were in a pseudorandom order with one trial of each session occurring in each of the four quadrants. Escape latency, path length and swim speed were determined using an imaging program (HVS, Inc.). On day 4, a probe trial was conducted in which the submerged platform was removed and measures of learning (# crossings of platform, latency to platform location, and time in platform quadrant) were recorded.

Statistical Analysis: All statistical analyses were performed using either IBM SPSS Statistics v20 (IBM Corporation; Endicott, NY) or GraphPad Prism v5 (GraphPad Software, Inc.; La Jolla, CA) unless otherwise stated. GD LD_{50} was estimated by probit analysis and compared using SAS (SAS Institute Inc.; Cary, NC) nonlinear regression and the specialized programs of Feder *et al*. (P. Feder et al., 1991; P. I. Feder, D. W. Hobson, et al., 1991; P. I. Feder, C. T. Olson, et al., 1991). Probit slopes were statistically compared using a Student's *t*-test. Survival curves were compared between ages at each GD dose using the Gehan-Breslow-Wilcoxon method. Plasma CaE activity was analyzed using a Welch-corrected unpaired *t*-test to account for unequal variances. Baseline whole blood AChE was compared using a Student's unpaired two tail *t*-test. Brain AChE activities were analyzed using a Student's unpaired two tail t-test by region. GD-inhibited blood AChE activity was analyzed using repeated measures ANOVA. Comparisons of the number of animals displaying toxic signs and seizures at each dose were made using two-sided Fisher's exact test. Latencies to seizure and death were compared between ages using a two-sided Mann Whitney *U* -test. Repeated measures ANOVAs were used to compare the latency to hit the platform and swim speed in the Morris water maze with age, session, and GD dose as factors.

Results

Experiment 1: Median lethal dose (LD_{50}), cholinesterase and CaE activity in GD-exposed adult and aged rats.

Median Lethal Dose (LD_{50}): Aged rats were more sensitive to the lethal effects of GD, with an LD_{50} of 50.0 µg/kg (95% CI; 37.8-66.1), compared to adult rats with an LD_{50} of 70.5 µg/kg (95% CI; 63.8-78.0) (Table 1). However, there was no statistically significant difference between the slopes of the curves.

Table 1: LD_{50} estimates from the probit analysis for adult and aged rats.

	LD_{50} (µg/kg)	95% CI	Slope	SEM	95% CI	PR	95% CI
Adult	70.55	63.83, 78.00	13.65	6.34	-0.47, 27.78	1.41	1.05, 1.90
Aged	50.00	37.81, 66.12	7.35	3.23	-0.94, 15.64		

Data presented as the estimate and the lower and upper 95% confidence intervals.

Blood AChE and CaE: Baseline plasma CaE activity (Figure 1) was significantly lower ($p < 0.05$) in aged rats than in young adult rats, while there was no difference in baseline whole blood AChE activity between age groups.

Brain AChE: Brain AChE activity in naïve controls was modestly, but significantly, lower in aged compared to young adult rats in the frontal cortex (82% of adult activity, t = 3.167, $p = 0.01$), hippocampus (89%, t = 3.723, $p < 0.01$), striatum (92%, t = 2.437, $p < 0.05$), thalamus (91%, t = 4.383, $p < 0.01$), and the pons (83%, t = 12.14, $p < 0.001$). However, AChE activity was not significantly reduced in the cerebellum (95%, $p = 0.12$) or the remaining cortex (92%, $p = 0.12$) (Figure 2).

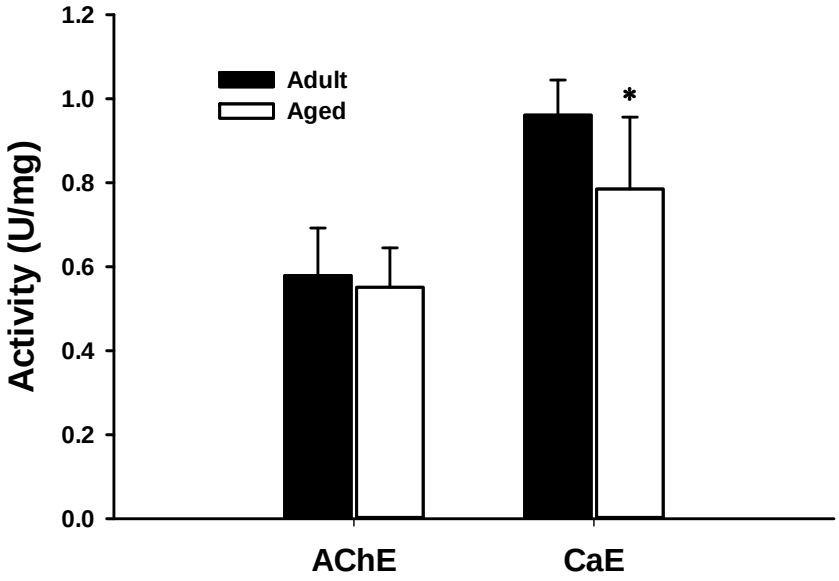

Figure 1: There were no age-dependent differences in whole blood AChE in naïve animals; however, aged rats had lower plasma CaE activity than did adults. The data are presented as the mean activity (U/mg) ± standard deviation with n=16-17/group. * $p < 0.05$

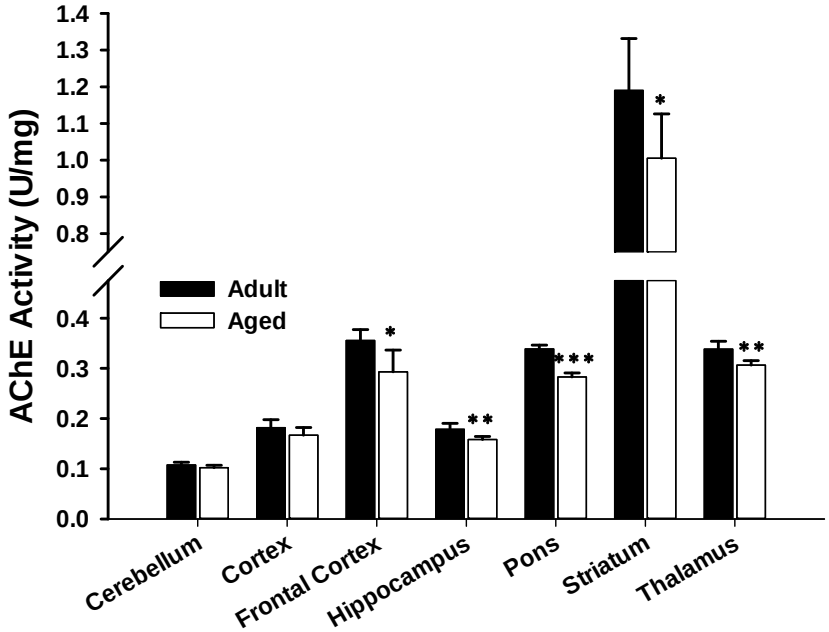

Figure 2: Brain AChE activity levels are reduced in the frontal cortex, hippocampus, striatum, thalamus, and the pons of naïve aged compared to adult rats. The data are presented as the mean activity (U/mg) ± standard deviation (n = 7 for each group). *p<0.05, **p<0.01, ***p<0.001.

Experiment 2: Cholinesterase activity, seizure and performance measures in GD-exposed adult and aged rats.

Blood AChE and CaE: When challenged with a sub-convulsive and sub-lethal dose of GD (22 µg/kg), both age groups showed a significant decrease in whole blood AChE activity following GD exposure (Time $F(9,18) = 21.864$, $p < 0.001$), with a trend (Age $F(1,2) = 13.122$, $p = 0.068$) for a greater decrease in AChE activity in aged compared to young adult rats (Figure 3).

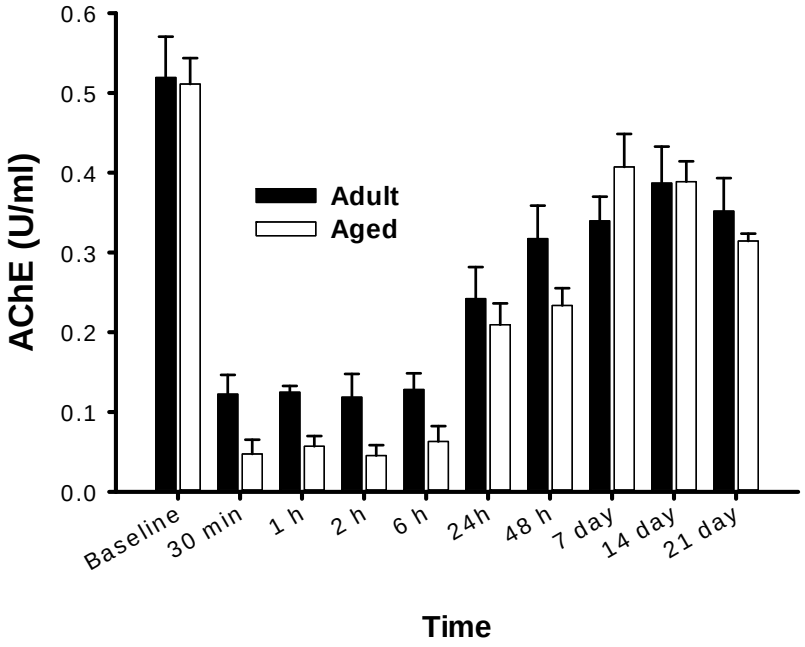

Figure 3: Exposure to GD (22 µg/kg) induced a significant decrease in whole blood AChE activity as compared to baseline.

Toxic Signs and EEG: The increased sensitivity observed in the older rats is also evident in survival curves (Figure 4) following exposure to GD (22-88 µg/kg). No age-dependent differences in survival were seen in rats exposed to saline or the lowest dose of GD (22 µg/kg) (Log-rank [Mantel-Cox] Test, $p = 0.37$ and $p = 1.00$, respectively). Age significantly impacted survival at the 44 µg/kg ($p < 0.05$; median survival: adult undefined, aged 72 h), 66 µg/kg ($p < 0.001$; median survival: adult undefined, aged 56.7 min), and 88 µg/kg ($p = 0.001$; median survival: adult 31 h, aged 24 min) doses of GD. Table 2 shows the incidence of signs of GD toxicity and seizure following exposure to saline or GD in adult and aged rats. Latencies were calculated only for animals displaying that outcome (*i.e.*, only rats that displayed seizures were included in the calculation for latency to seizure onset). One of the aged rats exposed to saline was moribund and euthanized eight days after exposure. Upon necropsy pituitary gland adenoma was suspected as a contributing factor to death. Two aged animals exposed to GD (44 µg/kg) were excluded from seizure data because excessive noise on the EEGs prevented the determination of seizure onset or termination. The time data are represented as the median (minimum, maximum).

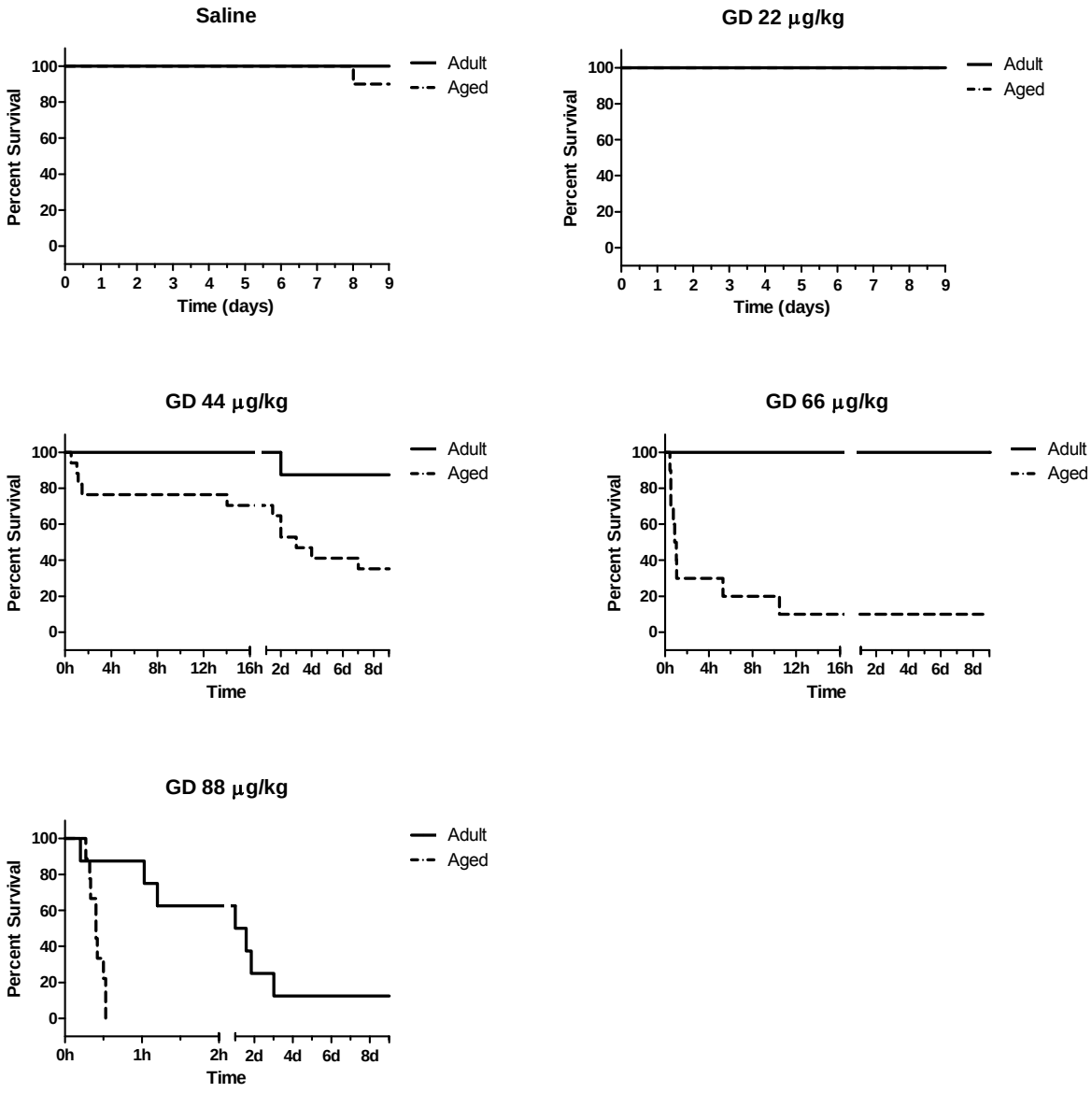

Figure 4: Survival curves following exposure to saline or GD (22-88 µg/kg) in adult and aged rats. Age-dependent differences in survival were seen following exposure to 44-88 µg/kg GD.

Table 2: Incidence of signs of GD toxicity and seizure following exposure to saline or GD in adult and aged rats.

Group	Age (months)	Toxic Signs (#/total)	Toxic Sign Latency (h:min)	Seizure (#/total)	Seizure Latency (h:min)	Seizure Duration (h:min)	Mortality Rate	Latency to Death (h:min)
Saline	2	0/8	--	0/8	--	--	0% (0/8)	--
	18	0/10	--	0/10	--	--	10% (1/10)	--
GD 22 µg/kg	2	2/7	0:17 (0:15, 0:19)	0/7	---	---	0% (0/7)	--
	18	4/9	0:08 (0:04, 0:40)	0/9	---	---	0% (0/9)	--
GD 44 µg/kg	2	3/8	0:15 (0:05, 1:10)	1/8	1:07	23:28	12.5% (1/8)	48:00
	18	17/17	0:28 (0:10, 0:37)	9/15	0:45 (0:24, 3:19)	--	64.7% (11/17)	35:21 (0:31, 168:00)
GD 66 µg/kg	2	7/8	0:09 (0:04, 0:16)	0/8	--	--	0% (0/8)	--
	18	10/10	0:13 (0:05, 1:36)	9/10	0:23 (0:11, 2:02)	--	90% (9/10)	0:52 (0:26, 10:28)
GD 88 µg/kg	2	8/8	0:07 (0:04, 0:10)	8/8	0:10 (0:07, 0:15)	12:49 (1:07, 18:17)	87.5% (7/8)	24:00 (0 :12, 72:10)
	18	9/9	0:10 (0:03, 0:18)	8/9	0:14 (0:03, 0:27)	--	100% (9/9)	0:24 (0:16, 0:32)

Time data is represented as median (minimum, maximum).

Mild signs of cholinergic toxicity were seen in 29% (2/7) of adult and 44% (4/9) of aged rats exposed to 22 µg/kg of GD (Table 2). Excessive chewing was the most common initial sign of toxicity at the two lower doses. All aged rats (17/17) displayed toxic signs following 44 µg/kg GD, whereas toxic signs were seen in significantly fewer (38%; 3/8) adult rats ($p = 0.001$). Seizures were first observed at 44 µg/kg GD with the majority of aged rats (9/15) and only one adult displaying seizure activity ($p = 0.074$). All rats that suffered seizure in both age groups died within 48 hours of exposure. Surprisingly, no young adult rats had seizures or died following 66 µg/kg GD despite 88% (7/8) displaying toxic signs. Median latency to toxic signs, seizure and lethality is shown in Table 2. Seizures were present in 9 of 10 aged rats (compared to young adult rats, $p < 0.001$) that subsequently died, with a median latency to death of 0:52 h:min. Severe toxicity was observed at the highest dose tested (88 µg/kg) with all rats in both age groups exhibiting toxic signs, and all young adult rats displaying seizures with a median latency of 0:10 minutes. The majority of aged rats (8/9) displayed seizures with a median latency of 0:14. Median latency to death was significantly shorter in aged (24 minutes) compared to young adult rats (24 hours; $p < 0.05$) with mortality rates of 100% for aged rats and 87.5% for young adults.

Morris Water Maze (MWM): Adult and aged animals that survived to be tested in the MWM did not display seizures following exposure, with the exception of one adult rat exposed to GD that had seizures and survived. Since GD-induced brain pathology following prolonged seizure activity is related to impairment in the MWM (Schultz *et al*., 2014), it is not surprising that in the present study GD exposure had no effect on performance. However, as expected, aged rats did not perform as well as adult rats in this test. Multi-variate analysis of the latency to hit the platform showed significant within-subjects effects of session [$F(2, 94) = 57.42$, $p<0.001$], and interaction between session and age [$F(2,94)=7.52$, $p = 0.001$]. Age [$F(1, 47) = 27.77$, $p <$

0.001] also significantly affected latency to hit. There was a significant effect of session in adult ($F(2,52) = 74.39$, $p<0.001$]) and in aged rats ($F(2,42) = 8.365$, $p <0.01$), as both adult and aged rats took less time to find the platform with repeated sessions (Figure 5). There was no effect of drug dose in either adult or aged rats, as expected since only one rat seized. All other effects and interactions were not significant. Aged rats had a significantly slower swim speed [$F (1, 47) = 76.90$, $p < 0.001$; Figure 6] regardless of GD exposure or session. There were no other significant effects or interactions on swim speed. On the test of visual acuity, aged rats performed less well (took longer to locate the visible platform; Figure 7). However, whether this is related to visual acuity or to reduced swim speed in aged rats is unclear.

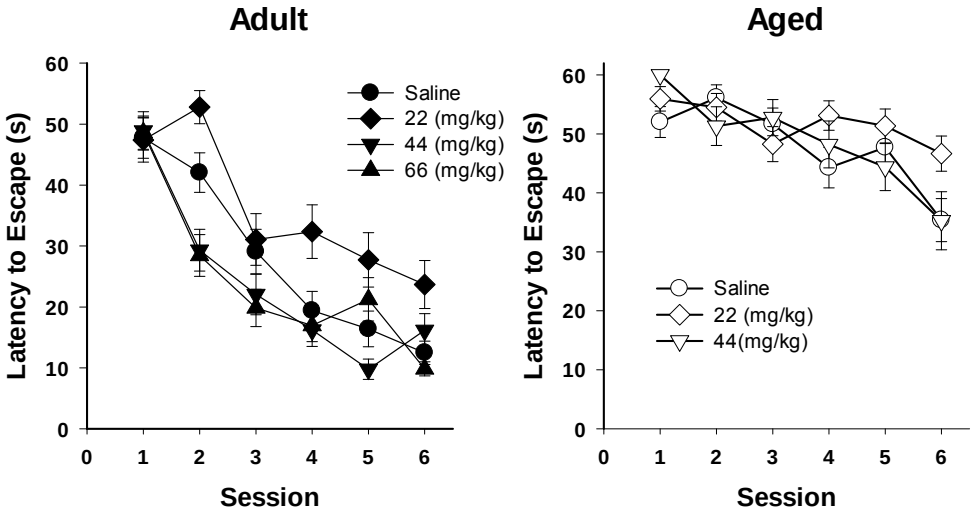

Figure 5: In the Morris water maze, rats had reduced latency to escape with repeated trials, with adult rats having better spatial memory acquisition compared to aged rats. There were no significant effects of GD exposure as rats that seized did not survive, and typically performance in this test is affected in rats with prolonged seizure and brain pathology. Data from 88 µg/kg in adult rats and 66 µg/kg in aged rats are not shown since only one survived in each of these groups.

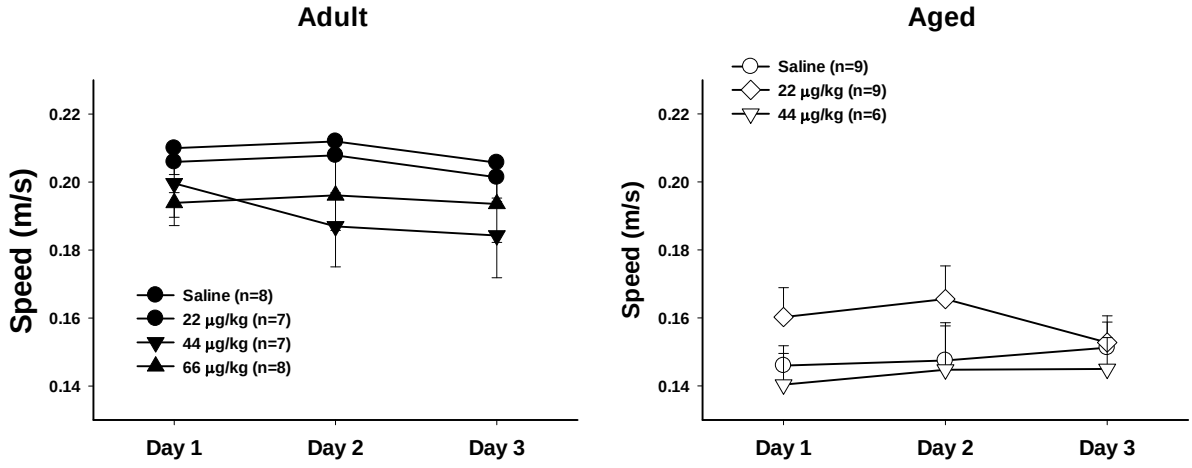

Figure 6: In the Morris water maze, adult rats (left) had greater swim speed compared with aged rats (right) ($p<0.001$). There was no effect of GD or session trial on swim speed.

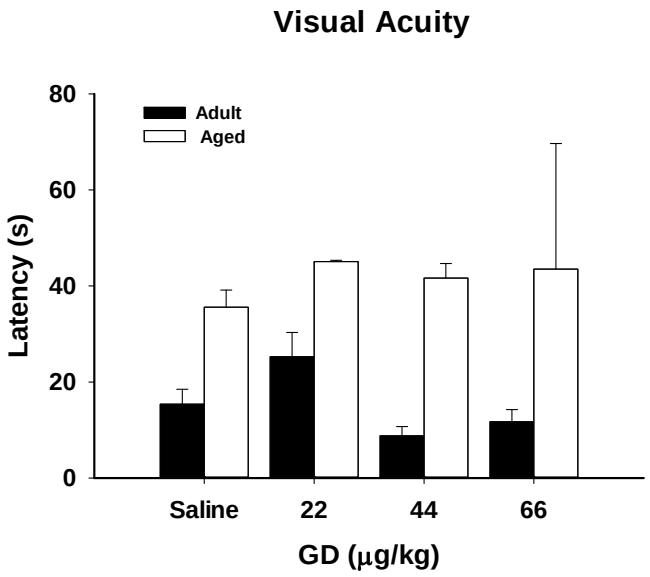

Figure 7: In the Morris water maze, aged rats (white bars) had poorer visual acuity compared to adult rats (black bars; $p<0.001$). There was no effect of GD on visual acuity.

Body Weight: Aged rats weighed more than young adult rats at the start of the study and throughout the study, as expected (Figure 8; $p<0.001$). Aged rats lost weight the first day after exposure but recovered within 3 days of exposure ($p<0.05$). Young adult rats gained weight over the weeks of the study, weighing more in week 3 compared to week 1, as is expected with growth and development ($p<0.05$).

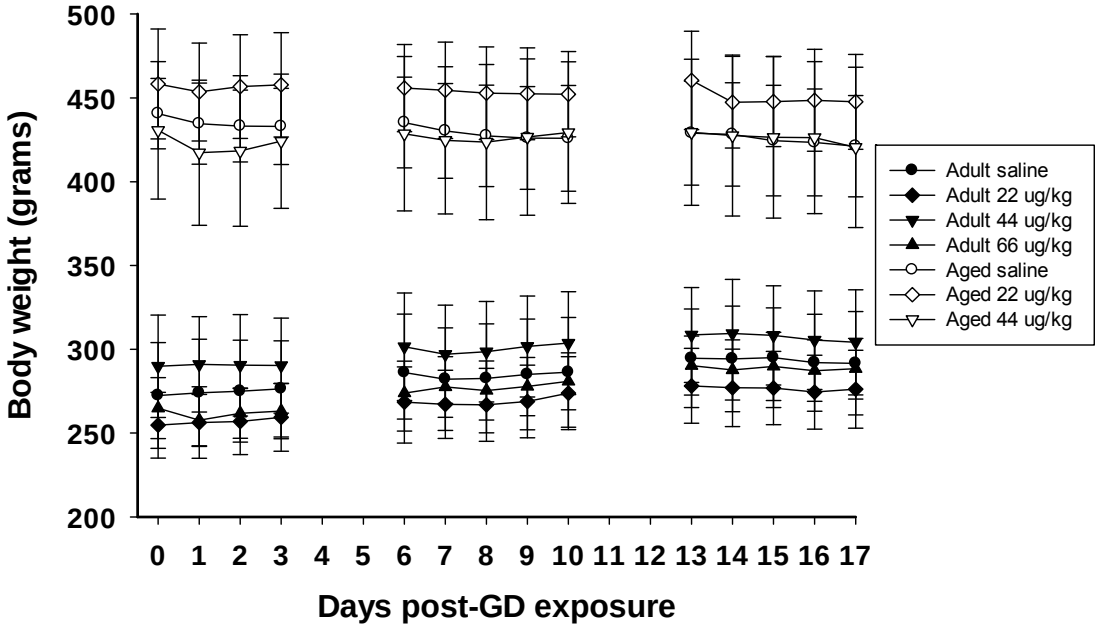

Figure 8: Body weights in aged rats (open shapes) and adult rats (solid shapes) following saline or GD exposure. Aged rats that received the higher dose of GD (44 μg/kg) tended to lose weight in the days following exposure.

Discussion

The present data show a lower LD_{50} for GD subcutaneous exposure in aged F344 rats compared to young adult rats. The lethality data obtained from inbred young adult and aged rats of the F344 strain exposed subcutaneously to GD (LD_{50} adult: 70.5 μg/kg; LD_{50} aged 50 μg/kg) are similar to lethality data in outbred male albino rats exposed intramuscularly to GD (LD_{50} adult 87 μg/kg; LD_{50} aged 59 μg/kg (Shih *et al*., 1990) in that aged rats were more susceptible to the lethal effects. Altogether, the data suggest that an aged human population may also be more sensitive to GD exposure toxicity.

The decline of brain AChE activity in aged rats observed in the current study is fairly consistent with the age-dependent decreases across rat strains reported in the literature (Das, Dikshit, & Nath, 2001; Ikarashi, Fujimori, Ohtake, Shiobara, & Maruyama, 1994; Michalek, Fortuna, & Pintor, 1989; Sastry, Janson, Jaiswal, & Tayeb, 1983; Scali *et al*., 1997; Scali, Vannucchi, Pepeu, & Casamenti, 1995; Shih *et al*., 1990; Yufu *et al*., 1994). We observed less AChE in the frontal cortex, hippocampus, pons, striatum and thalamus of aged compared to adult rats, but not less in the cerebellum or cortex, whereas in Shih *et al*. (1990) an age-dependent lowering of AChE was observed in all brain regions evaluated in albino rats, including the cerebellum and cortex. Differences observed between studies for AChE in the cortex could relate to removal of the frontal cortex in the current study and less AChE being observed in the frontal cortex of aged compared to adult rats. The reduction in brain AChE could underlie the ability of lower doses of cholinesterase inhibitors to reduce AChE activity below critical levels.

Although there was no age-dependent difference in baseline blood AChE, similar to previous findings (Shih *et al*., 1990), a moderate but statistically significant reduction in CaE was observed, which is consistent with the literature (Karanth & Pope, 2000). Plasma CaE can act as an agent sink, binding GD and thereby reducing the amount of free GD able to inhibit AChE. Studies by Maxwell *et al*. (1987) suggest that CaE activity in animals, especially in the plasma and lungs, is a key factor in accounting for interspecies differences in GD toxicity. Although only plasma CaE was measured in this study, Karanth and Pope (2000) reported lower activity in the plasma but not lung or liver CaE.

The general health of the aged rats can be a contributing factor to the increase in GD toxicity compared to younger rats. Inbred rat strains such as the F344 strain offer advantages over outbred strains such as uniform genetic backgrounds but also disadvantages including strain-specific lesions. There are common pathologies in F344 rats that worsen as the animal ages (Coleman, Barthold, Osbaldiston, Foster, & Jonas, 1977; Lipman, Dallal, & Bronson, 1999). For instance, pituitary gland adenoma is rarely observed in F344 rats younger than 18 months of age, but over a 25% incidence occurs with rats aged 18-24 months (Coleman *et al*., 1977). Two more common ailments afflicting aged F344 rats include cardiomyopathy (characterized by fibrosis and degeneration in the myocardium) in 35% of rats 12-18 months and 75% at 18-24 months of age, as well as chronic nephropathy, which was evident in all F344 rats over 6 months of age in the study by Coleman *et al*. (1977). Evaluating pathology in all rats was beyond the scope of this study; however, necropsies were performed on select rats. One aged rat exposed to saline gradually lost weight and was euthanized 8 days after exposure. Histologic evidence suggests pituitary gland adenoma and glomerulonephropathy as likely contributing factors. It may be that strain-associated health ailments contributed to the overall increase in mortality in our GD-exposed aged group, though this contribution likely does not account for all the differences observed.

In the current study, we did not observe an effect of GD exposure on performance in the Morris water maze in adult or aged rats. However, most of the rats that survived to perform in

the Morris water maze did not develop seizures. Rats exposed to seizure-inducing doses of CWNA are impaired in the Morris water maze (Schultz *et al*., 2014), while rats that do not seize after CWNA exposure are not impaired (Lumley, unpublished data). As expected, we did observe that aged rats were impaired compared to young adult rats in that they had longer latency to find the platform, had slower swim speed, and were impaired on the test of visual acuity. In general, the aged rats performed poorly on the paradigm that we used and may require additional training trials or other adjustments to the test design used to adequately learn the test, even in saline control rats.

The data suggest that in the event of a terrorist attack using organophosphorus agents, the elderly will represent an especially vulnerable population. This aged human susceptibility would likely be a result of a mixture of underlying pathologies (*e.g.,* cardiac and nephrotic lesions) and reduced brain AChE activity, similar to what we observed in our aged rat model. Unlike rats and mice, humans do not have plasma CaE (Bahar, Ohura, Ogihara, & Imai, 2012; Li *et al*., 2005), and there is not an effect of aging on plasma AChE or BChE activities in humans (Abou-Hatab, O'Mahony, Patel, & Woodhouse, 2001). However, the observed differences in brain AChE likely contribute to the increased sensitivity of aged rats to GD toxicity. It is known that declines in brain AChE function occur in at least a sub-population of elderly (*e.g.*, Alzheimer's patients), but AChE function does not appear to decrease in an age-dependent manner in a "healthy" population (Kuhl *et al*., 1999; Namba *et al*., 1999). Ultimately, the susceptibility of the elderly human population to CWNA exposure may be more strongly correlated with underlying age-related pathologies than strictly with reduced AChE levels.

Conclusion

Lethality data may not be easily interpreted because of differences in species cholinesterase and CaE levels; *i.e.*, rats possess CaE, while humans do not. Additional studies will need to take steps to "level the playing field" when drawing comparisons between aged animal models and human. For instance, the use of CaE knockout mice would eliminate one of the primary confounding factors of translational CWNA research. Future studies are required to investigate these concerns and to provide additional, crucial data on how CWNA exposure effects the elderly human population.

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