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Full Length Article

Survey of drug therapies against acute oral tetramethylenedisulfotetramine poisoning in a rat voluntary consumption model

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ABSTRACT

Ingestion of the noncompetitive $GABA_A$ receptor antagonist tetramethylenedisulfotetramine (TETS) results in arrhythmias, respiratory depression, and life-threatening convulsive status epilepticus. We have previously developed a realistic model of voluntary TETS consumption, in which rats promptly consumed a piece of cereal containing a dose of TETS that led to rapid progression of toxic signs (including convulsions) and profound and enduring behavioral suppression. Recently, this model was used to survey nine different drugs from distinct drug classes over a large range of doses to identify possible therapeutics. The drugs included three benzodiazepines (diazepam, midazolam, and lorazepam), two barbiturates (phenobarbital and pentobarbital), the $GABA_A$ allosteric modulator allopregnanolone, and three non-traditional therapeutics (dexmedetomidine, ketamine, and ethanol). Treatment was administered intraperitoneally 10 min after consumption of the cereal morsel containing TETS (600 μg/kg). This exposure model resulted in a survival rate of 30% in vehicle-treated rats. Diazepam (12.5 mg/kg) and midazolam (25 mg/kg), compared to vehicle, significantly increased survival (75 and 100% respectively) but at only one of the three doses tested. Lorazepam increased survival across a wide range of doses (1.56–25 mg/kg) with survival rates between 80–100%. Phenobarbital (100 mg/kg) was the only other drug and non-benzodiazepine to improve survival rates (80%). Although the four aforementioned therapeutics increased survival, TETS-induced weight loss, food wastage, and behavioral deficits remained in survivors.

1. Introduction

Tetramethylenedisulfotetramine (TETS) is a highly toxic and potent convulsant compound that in the past has been used as a rodenticide, mainly in Asia. Despite a worldwide ban on its use enacted in 1984, TETS remains available on the black market in some regions due to the ease of synthesis which requires only a few uncontrolled reagents (Fraga et al., 2011) and thus remains a serious threat. In the years since its ban, TETS has been responsible for thousands of poisonings and hundreds of deaths from accidental and intentional ingestion around the world (Li et al., 2012). TETS is a tasteless, odorless white powder which is stable in solution and environmentally persistent (Croddy, 2004). TETS binds non-competitively and reversibly to the α1 and γ2 subunits of the γ-aminobutyric $acid_A$ ($GABA_A$) ionophore complex (Bowery et al., 1975; Cao et al., 2012), occluding the chloride channel and acting as a potent antagonist (Zhao et al., 2014). Symptoms of TETS poisoning include nausea, arrhythmias, tremor, convulsions, respiratory failure and in severe cases, life-threatening status epilepticus (SE) (Lu et al., 2008; Zhang et al., 2011; Li et al., 2012). Patients poisoned by TETS who develop SE require aggressive anti-epileptic drug therapy to control seizures (Barrueto et al., 2003; Li et al., 2012).

Numerous animal studies have been carried out to assess various drug treatments for TETS poisoning, including $GABA_A$ agonist drugs such as diazepam, allopregnanolone, and GABA itself (Sun et al., 2007; Shakarjian et al., 2012; Vito et al., 2014; Bruun et al., 2015; Shakarjian et al., 2015), NMDA receptor antagonists ketamine and dizocilpine (Shakarjian et al., 2012, 2015; Laukova et al., 2018), and sodium dimercaptopropane sulfonate (Chen and Lu, 2004). There has yet to be a study published that directly compares the efficacy of numerous drugs from numerous distinct classes at different doses against a standard TETS challenge. With this in mind, a survey of therapeutics was planned using nine separate drugs at three or more doses each to test against lethality when administered 10 min after a voluntary oral TETS exposure in rats (Rice et al., 2017). Factors that influenced drug selection included previous FDA approval, current availability in an emergency room (ER) or intensive care unit (ICU) setting, potential for

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practical use in a pre-hospital emergency medical services (EMS) setting, and relevance to ongoing and recent research on TETS poisoning and refractory SE. In some cases, lower or higher doses were further explored beyond the three initial doses to determine lowest effective doses or the ceiling effects of a particular drug. The highest doses of all drugs were chosen based on available toxicity data to allow for the highest dose possible without significant toxic effects. To this end, a safety group (no TETS, drug only; n = 3) was included for every drug at every dose to provide a preliminary safety assessment.

Benzodiazepines such as midazolam, diazepam, and lorazepam are the most common first-line drugs for treating seizures, including SE, both in the EMS and ER setting and therefore were included (reviewed in Rogalski and Rogalski, 2015). Two barbiturates, phenobarbital and pentobarbital, were chosen for inclusion in this drug survey due to their actions on the $GABA_A$ receptor via a binding site that is distinct from those of the benzodiazepines (Twyman et al., 1989; Sieghart, 1992). In addition to the benzodiazepines and barbiturates, allopregnanolone was included in the drug survey. Allopregnanolone is an endogenous neurosteroid that acts as a positive allosteric modulator of the $GABA_A$ receptor (Pinna et al., 2000; Puia et al., 2003), with well-documented anxiolytic and anticonvulsant effects similar to benzodiazepines and barbiturates (Brot et al., 1997; Czlonkowska et al., 2000; Reddy and Rogawski, 2001; Lonsdale et al., 2006; Lonsdale and Burnham, 2007).

Additionally, three drugs that have anticonvulsive activity outside of the action on the $GABA_A$ receptor were included. Ethanol was included due to the availability across the globe and documented anticonvulsant effects in a wide variety of models (Workman et al., 1958; Nutt and Lister, 1987; Kulkarni et al., 1990; Fischer and Kittner, 1998). Ethanol, while largely nonspecific in its pharmacology, is both a GABA agonist and an *N*-methyl-*D*-aspartate (NMDA) antagonist (Grant and Lovinger, 1995; Krystal et al., 2003), both of which are implicated in SE (Naylor et al., 2005; Chen and Wasterlain, 2006; Niquet et al., 2016). Ketamine, a clinically available noncompetitive NMDA antagonist, was likewise chosen for inclusion in this drug survey. Ketamine is an effective adjunct medication for treating refractory SE in both adult and pediatric human patients (Synowiec et al., 2013; Zeiler, 2015), including patients poisoned with TETS (Chau et al., 2005). Additionally, in animal models ketamine has shown some efficacy when combined with other treatments to treat SE induced by electrical stimulation of the hippocampus (Borris et al., 2000), lithium-pilocarpine (Martin and Kapur, 2008), organophosphates (Klemm, 1985; Dorandeu et al., 2005, 2007; Niquet et al., 2016) and TETS (Shakarjian et al., 2012). Dexmedetomidine is a highly selective α_2-adrenergic agonist with both analgesic and sedative properties (Giovannitti et al., 2015). However, a limited number of studies have shown that dexmedetomidine displays efficacy as an anticonvulsant in multiple models of SE (Halonen et al., 1995; Kan et al., 2013), and as an adjunct treatment for nerve agent-induced SE (McCarren et al., 2018).

The purpose of including such a wide variety of drug treatments in the survey, including those not typically thought of as anticonvulsants, was to obtain efficacy data as a foundation for determining the best therapeutic agent for the treatment of TETS poisoning. A single study with identical exposure and treatment methods offers a high degree of internal validity that cannot be achieved by comparing therapeutic outcomes across diverse separate studies. Moreover, including multiple doses of each drug at the upper end of the therapeutic range provides a fair assessment of each drug's potential to treat TETS poisoning. Additionally, overnight food consumption, weight changes, and behavioral acquisition measures were collected to inform not only the general safety of all surveyed therapeutics but to also provide a preliminary indication of therapeutic outcome following acute treatment.

2. Material and methods

Chemicals: TETS (anhydrous) was obtained from the Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD) at ~78% purity and stored at 4 °C. The primary impurity (approximately 16% of the sample) within the powder was hexamethylenetrisulfohexamine (HEXS), a common TETS contaminant (Hondrogiannis and Cullinan, 2011) believed to be approximately 50-fold less toxic than TETS and therefore thought to have little if any impact on the current study. TETS was dissolved into acetone at a concentration of 2 mg/mL. Acetone (≥99.5%) was purchased from Sigma Aldrich (St. Louis, MO).

USP grade drugs and solvents were preferentially used for treatments when available. A combination vehicle was used for diazepam, phenobarbital, and pentobarbital (by volume): 62% propylene glycol (Fisher Chemical; Pittsburgh, PA), 18% polyethylene glycol 400 (AppliChem Inc; Maryland Heights, MO), 10% ethanol (Sigma Aldrich), 8% water, and 2% benzyl alcohol (Sigma Aldrich). The two different vehicles used for lorazepam consisted of (by volume) 80% propylene glycol, 18% polyethylene glycol 400, 2% benzyl alcohol, or 45% (2-hydroxypropyl)-β-cyclodextrin (HPBCD, Sigma Aldrich) in sterile water. Allopregnanolone (4 mg/ml; Tocris Bioscience; Bristol, UK) was freshly prepared before use in 45% HPCD by heating to 50 °C and sonicating until in solution. Saline (0.9% sodium chloride) vehicle was used for midazolam, ketamine, dexmedetomidine, and ethanol.

Ketamine (Ketaset©, 100 mg/ml) and dexmedetomidine (Dexdomitor©, 0.5 mg/ml) were purchased from Zoetis (Parsippany, NJ). Lorazepam (4 mg/ml), midazolam (5 mg/ml) and diazepam (5 mg/ml) were purchased from Hospira (Lake Forest, IL). Phenobarbital sodium (130 mg/ml) was purchased from West-Ward Pharmaceutical (Eatontown, NJ). Nembutal© (sodium pentobarbital 50 mg/ml) was purchased from Akorn Pharmaceuticals (Lake Forest, IL). Fatal Plus© (sodium pentobarbital 390 mg/ml) was purchased from Vortech Pharmaceuticals (Dearborn, MI). Pure ethanol was purchased from Sigma Aldrich (St. Louis, MO) and diluted to 60% (v/v) with sterile water. The ethanol solution was stored in a refrigerator at 4 °C until the day of use.

Subjects: Male Sprague Dawley rats (SAS SD 400) were obtained from Charles River (Wilmington, MA) and housed individually on a 12 h light/dark cycle. All experiments were conducted during the light phase. Food regulation was employed to increase the likelihood of rapid consumption of a small piece of food (Froot Loops®; Kellogg Company, Battle Creek, MI) and to help ensure little to no stomach contents during oral TETS exposure. A measured portion of rat chow was provided daily in the afternoons to maintain the subjects at 90% of the free-feeding weights determined from established growth-curves. Any food remaining in the cage the following morning was weighed and recorded as food wastage. The rats weighed between 250–340 g at the time of exposure (M = 295 g, SD = 14 g). Water was available ad libitum in the home cages.

The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense (USAMRICD), and all procedures were conducted in accordance with the principles stated in the 2011 Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89–544), as amended. The USAMRICD is a research facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.1. Voluntary consumption training and exposure

One week prior to exposure the rats were trained daily (Mon.-Fri.) to rapidly consume a whole piece of Froot Loops® cereal as previously described (Rice et al., 2017). On the day of exposure, approximately 90 µL (adjusted for individual rat weights) of TETS in acetone (2 mg/ml) or acetone alone was applied in increments to the piece of cereal to achieve a 400 or 600 µg/kg (312 or 468 µg/kg adjusted for purity) dose of TETS. Approximately 40 min to 1 h was allotted to allow the acetone to evaporate prior to placing it within in the exposure cage; exposure was considered to have occurred once the entire piece of cereal was consumed. Toxic signs were observed continuously for the first hour

following exposure and once an hour through the end of the working day (up to 5 h post-exposure). Additionally, toxic signs were observed at 24 h after exposure, immediately before the shuttlebox avoidance session was initiated.

2.2. Treatment

Cohorts were composed of rats receiving TETS and test drug, a drug safety group receiving a cereal piece with acetone applied and test drug, a positive control group receiving a TETS cereal piece and drug vehicle, and a vehicle control group receiving an acetone cereal piece and the drug vehicle. Rats were randomly assigned to groups within the cohort prior to exposure using the random number function of Microsoft Excel. The test drugs (or vehicles) were administered intraperitoneally 10 min following exposure. The volumes of drugs and vehicle controls were not held constant due to the wide variation in solubility of the drugs tested. The range of volumes was from 1 ml/kg rat body weight to 15 ml/kg (60 mg/kg allopregnanolone), depending on the solubility of the drug. In the event that a dose volume was less than 1 ml/kg, the drug was diluted in saline to a volume that ensured a dose volume of at least 1 ml/kg.

2.3. Shuttlebox avoidance

Twenty-four hours after TETS exposure, animals were evaluated in a shuttlebox active avoidance task to measure learning and performance. The active avoidance session was conducted in GEMINI™ test chambers (San Diego Instruments Inc., San Diego, CA) which have two separate compartments and an array of photobeams for detecting the location of the rat. The avoidance session began with a 5-minute acclimation period during which all chamber illumination and sound were off. In each test session, 50 discrete trials were presented and were separated by an inter-trial interval (20 ± 5 s). On each trial, the location of the rat was determined, and a warning stimulus (WS) consisting of a light and a ~75 db SPL tone was presented in that compartment. Failure to ambulate to the dark compartment within 5 s resulted in a 1.2 mA scrambled shock to the grid floor while the WS continued. A cross into the dark compartment within 15 s terminated all stimuli (WS, shock) and was registered as an escape response. Failure to ambulate to the dark compartment within the 15 s maximal shock duration was recorded as a "failed escape." If 10 consecutive "failed escape" trials occurred, the animal was considered incapacitated and the session was terminated. Metal grids were cleaned immediately after each session using 70% isopropyl alcohol and a scrubbing pad. Following conclusion of the shuttlebox avoidance task, rats were euthanized with an overdose of a pentobarbital-based solution (Fatal Plus©) administered intraperitoneally.

2.4. Data analysis

TETS-exposed animals that were moribund (i.e., unresponsive, prostrate and gasping) at or before the time of treatment were excluded from the study. The survival data (percent surviving) were analyzed using a z test with the significance level (α) set at .05. A *post hoc* power analysis was also conducted on survival data using a one-tailed z test with an arcsin transformation (G*Power v. 3.1.92). A statistically significant z test ($p < .05$) and a power ≥ .80 were considered to denote greater survival compared to vehicle. Statistical analysis for food wastage, percent change in body weight, and percent avoidance were performed for each treatment using a one-way ANOVA with Dunnett's multiple comparisons test to compare dose groups to the control group. Experimental data suggested a potential vehicle effect for food wastage and percent change in body weight. Statistical analysis to explore the potential differences between vehicles was performed using a one-way ANOVA with Dunnett's multiple comparisons test to compare different vehicle groups to the saline vehicle control group.

3. Results

3.1. TETS exposure

In initial experiments, rats in one cohort were exposed to 400 µg/kg of TETS and the severity of toxic signs was less than expected based on past experiments; therefore, four additional rats were added as positive controls (TETS + Vehicle). The added rats also showed less severe signs of intoxication, including a lower than expected percentage of animals with convulsions. Therefore, the dose of TETS was increased to 600 µg/kg in all further experiments to achieve severe intoxication in most animals. The rats receiving the lower dose of TETS (TETS + drug, n = 11; TETS + vehicle, n = 5) were excluded from data analysis. The data from the rats in these cohorts that were assigned to the drug safety group (acetone + drug, n = 2) or negative control (acetone + vehicle, n = 4) were included in all analyses.

Only 5 of the 510 rats participating in the study were excluded for not rapidly or completely consuming the cereal. Seven rats were moribund or dead prior to treatment (< 10 min after cereal consumption) and subsequently excluded. Due to an error in scheduled feeding outside of the experimental protocol, 8 rats were given larger food rations the night prior to exposure. All of these rats that consumed the TETS-tainted cereal failed to show profound signs of intoxication and were therefore excluded, however two rats were drug safety controls (Acetone + Drug) and were included in the study. Three rats from various cohorts were excluded following partial injections of the therapeutic drug.

Onset of toxic signs occurred within 1–5 minutes of consumption, beginning with lethargy and myoclonic jerks, typically progressing to tonic-clonic convulsions. Approximately 71.3% of the rats experienced tonic-clonic convulsions prior to or at the time of treatment (M = 7 min, SD = 2 min) after exposure. Initial convulsions occurring after treatment were observed in 28 of 363 (7.7%) rats. Convulsions were not observed within the first hour of continuous observation after exposure or during any of the hourly checks in the remainder of animals (20.9%).

The large doses of therapeutics administered often led to large volumes of vehicles administered in control animals. Preliminary results suggested a possible vehicle effect following large injections of vehicles containing propylene glycol, which has been demonstrated to have some anticonvulsant activity (Gasior et al., 1997). The experiment was originally designed with the intent of combining all vehicle controls into one group. However, the possibility of a vehicle effect on survival required additional animals to be added to vehicle control groups. The animals that received saline (n = 19) fared the worst with a 24 h survival percentage of 14%, while survival under all other vehicles ranged between 30–40%. Survival in each of the vehicle groups (HPBCD, n = 14; combination, n = 35; and lorazepam vehicle, n = 13) significantly differed from saline ($p < .001$ for each) but did not significantly differ from each other. However, the observed power comparing the vehicles to saline was .51 or lower, and therefore the vehicles were combined (n = 53) for assessment of drug efficacy on 24 h survival.

3.2. Efficacy

A number of treatments increased 24 h survival following 600 µg/kg TETS exposure, but results from only a few drugs and doses were statistically significant and sufficiently powered to suggest substantial efficacy (Fig. 1). As a class, the benzodiazepines were most efficacious at promoting survival after TETS ingestion (Fig. 1A). Single doses of diazepam (12.5 mg/kg, $p < .01$, observed power = .92) and midazolam (25 mg/kg, $p < .001$, observed power = .99) provided ample protection with survival rates of 80% and 100% respectively. Lorazepam was efficacious at 12.5 mg/kg (100% survival; $p < .001$, observed power = .99) and 25 mg/kg (90% survival; $p < .01$, observed power = .89). Diazepam (25 mg/kg) significantly improved survival

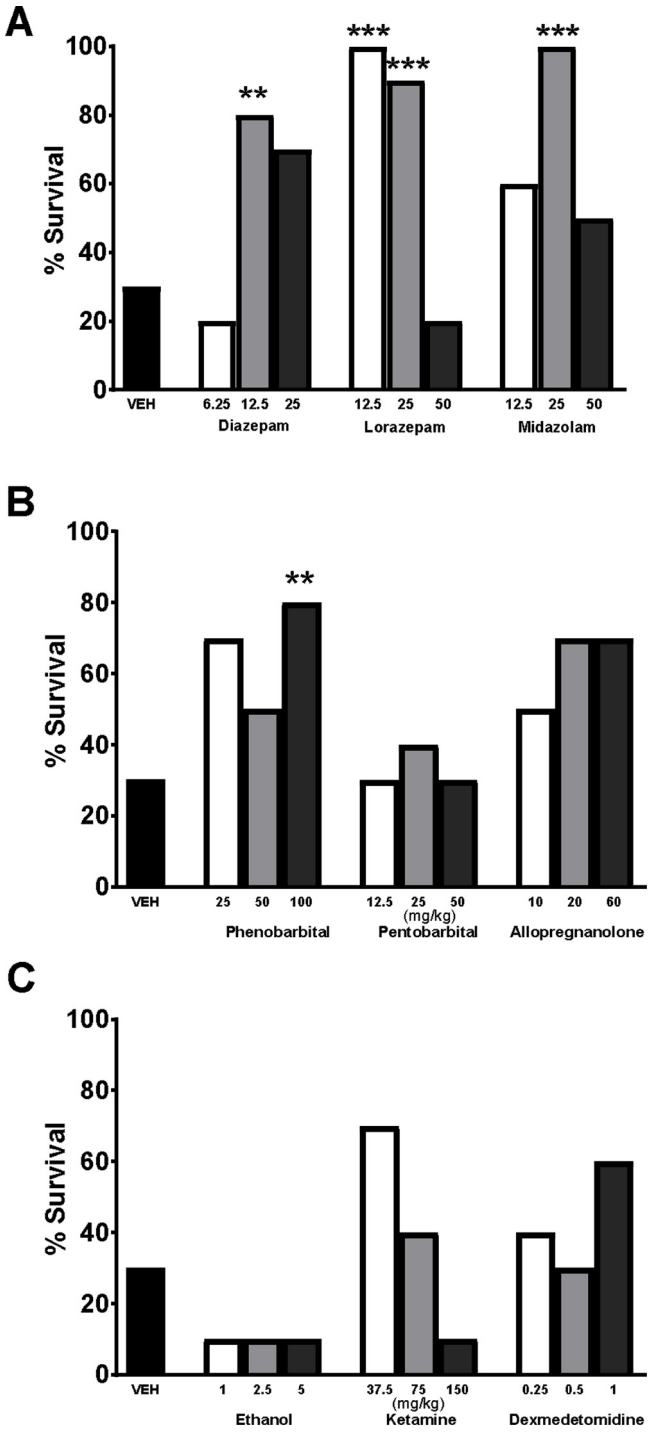

Fig. 1. Survival following ingested TETS (600 μg/kg) and treatment 10 min later with a benzodiazepine (A), barbiturate or allopregnanolone (B), or atypical therapeutic (C) compared to combined vehicle controls (n = 53). Ten animals were assigned to each dose. Asterisks denote statistically significant p values (** $p < .01$, *** $p \leq .001$) and an observed power ≥ .80.

but did not reach our criterion of an observed power ≥ .80 (observed power = .77). Phenobarbital was the most efficacious barbiturate tested (Fig. 1B). A survival rate of 80% was achieved with the highest dose tested (100 mg/kg, $p < .01$, observed power = .92), whereas the 25 mg/kg dose improved survival compared to vehicle (70% compared to 30%, $p < .05$) but had an observed power of only .77. Similarly, both the 20 and 60 mg/kg doses of allopregnanolone and the 37.5 mg/kg dose of ketamine (Fig. 1C) had significant survival rates of 70%

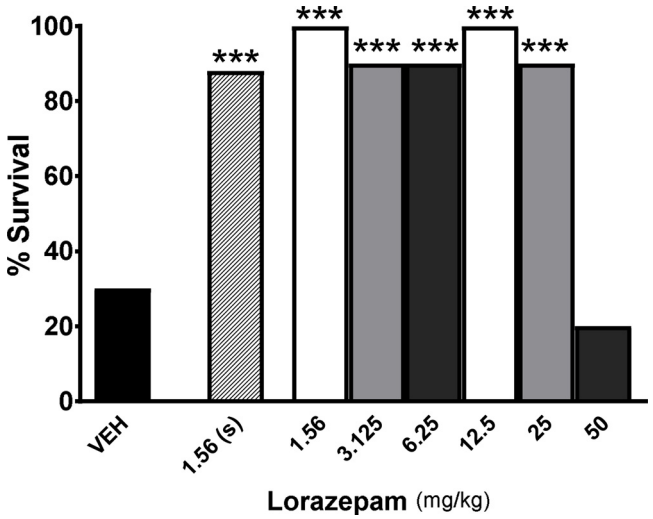

Fig. 2. Percent survival following treatment with lorazepam 10 min after exposure. Doses as low as 1.56 mg/kg were effective against TETS lethality compared to combined vehicle controls (n = 53) To ensure that the propylene glycol in the commercial formulation of lorazepam was not contributing to the observed efficacy an additional group at 1.56 mg/kg [1.56(s)] was included using 45% (2-hydroxypropyl)-β-cyclodextrin as the vehicle. Ten animals were assigned to each dose. Asterisks denote statistically significant p values (*** $p \leq .001$) and an observed power ≥.80.

compared to vehicle-treated rats ($p < .05$ for all), but fell short of our observed power criterion. Neither ethanol nor dexmedetomidine provided significant protection against TETS lethality at any dose tested.

Lorazepam was very efficacious at the 12.5 mg/kg dose with a 100% survival rate at 24 h and warranted further study at lower doses. The dose of lorazepam was halved in subsequent cohorts until the lowest dose tested (1.56 mg/kg) was reached. Remarkably, survival rates were high (> 88%) across all doses of lorazepam tested (Fig. 2). The vehicle of lorazepam contained 80% propylene glycol which could have possibly contributed to the efficacy of lorazepam. To rule out this possibility, lorazepam was administered at the lowest dose tested (1.56 mg/kg) with 45% HPCD solution and no propylene glycol. The 88% survival rate of lorazepam (1.56 mg/kg) in HPCD was not significantly different from the 100% survival of animals treated with the same dose of lorazepam in a solution including propylene glycol, suggesting that lorazepam and not the vehicle was responsible for the high survival rates observed.

The vast majority of surviving rats exposed to TETS regardless of treatment showed signs of physical and behavioral impairment including but not limited to: hunched posture, piloerection, and hypersensitivity or reduced sensitivity to stimuli. Food wastage remained high even in survivors administered the four most effective drugs (Fig. 3A). Only lorazepam 12.5 mg/kg (mean ± SD; 46.5 ± 37.4%, $p < .0001$) and phenobarbital 100 mg/kg (44.6 ± 38.6%, $p < .0001$) showed an improvement (reduced food wastage) compared to untreated controls. Other drugs or dosages that were outside of the top four therapeutics including ketamine 37.5 mg/kg (62.7 ± 23.7%, $p < .001$, data not shown), pentobarbital 12.5 mg/kg (73.9 ± 14.8%, $p < .05$, data not shown), and phenobarbital 25 mg/kg (57.4 ± 25.1%, $p < .01$, data not shown) showed reduced food wastage relative to controls (92.8 ± 11.6%). Significant adverse changes in body weights were observed in vehicle controls as well as in survivors administered the four most efficacious therapeutics following TETS exposure (Fig. 3B). Phenobarbital at 25 mg/kg (-6.3 ± 2.9%, $p < .05$; data not shown) and 100 mg/kg (-4.7 ± 5.1%, $p < .05$) was the only therapeutic that showed a smaller reduction in percentage body weight than the vehicle controls exposed to TETS (-10.8 ± 3.3%). Similar to what was seen with food wastage and body weight loss, the top four

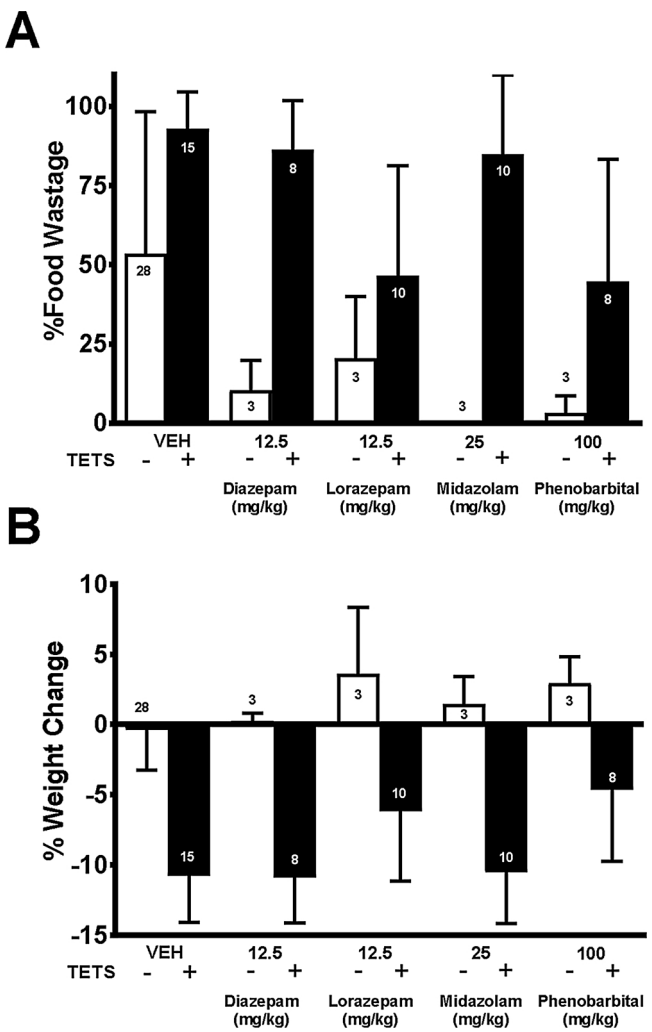

Fig. 3. Food wastage (A) and percent change in body weight (B) of the top four therapeutics alone (indicated by a minus sign) and in rats exposed to TETS (indicated by a plus sign). Vehicle-treated animals without TETS exposure had high rates of food wastage likely due to the toxic effects of large doses of propylene glycol. Despite the therapeutics being highly efficacious attenuating TETS lethality, food wastage and weight loss remained high. The data are presented as the mean + SD with the number of animals appearing in the bar.

therapeutics did not significantly improve performance in the shuttlebox test (Fig. 4). Severe impairments were observed in surviving animals regardless of treatment with 24.8% (38/153) animals meeting the escape failures criterion.

3.3. Safety

Nearly all of the drug and vehicle safety control animals completely recovered (defined by showing no toxic signs) by the 24 h mark following drug administration, with a few notable exceptions. The experiment was designed with a minimal number of animals assigned to the drug safety groups because all of the drugs tested are currently FDA approved or are currently undergoing clinical trials. The highest doses of both lorazepam and ethanol (50 mg/kg and 5.0 g/kg, respectively) were invariably fatal when administered in the absence of TETS. Administration of the highest volume (12.5 ml/kg, equivalent to 50 mg/kg lorazepam) of the commercial lorazepam vehicle (80% propylene glycol, 18% polyethylene glycol 400, 2% benzyl alcohol) led to one of three animals dying, with a second animal being rendered unresponsive, severely hypothermic, and nearly apneic at 24 h post-injection. The severe observed toxicity suggests that the commercial

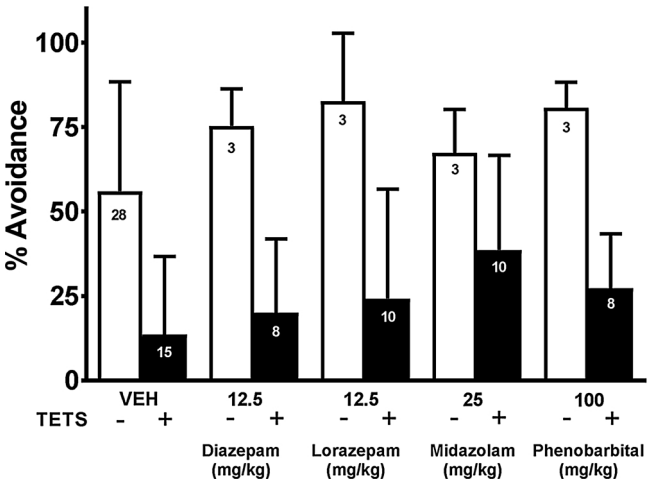

Fig. 4. Percent avoidance in the shuttlebox test for the top four therapeutics alone and in rats exposed to TETS. Despite the success of treatments in significantly reducing TETS lethality, there was no statistically significant difference in shuttlebox performance between treated and untreated animals that consumed TETS. Data are presented as the mean + SD with the number of animals appearing in the bar.

lorazepam vehicle, most likely the propylene glycol content of the formulation, contributed significantly to the toxicity of lorazepam.

Although there was no statistical difference in survival rates when vehicle-treated animals ingested TETS, there were clear effects of the vehicles on food wastage and change in body weight (Fig. 5). There was a marked difference in percentage food wastage ($F_{3,14} = 7.283$, $p < .01$) among vehicle-treated animals (Fig. 5A). Rats wasted significantly more food compared to those administered saline as a vehicle (0%) when administered propylene glycol-containing vehicles (combination vehicle, 67%; lorazepam vehicle, 98%; $p < .01$ for both) or HPBCD (79%, $p < .05$). Vehicle type significantly affected percent change in body weight (Fig. 5B; $F_{3,\ 23} = 3.908$, $p < .05$), although the lorazepam vehicle group was the only group to significantly differ from the saline group ($p < .05$). There were no significant differences between vehicles in percent avoidance in the shuttle box.

While there were some minor differences in effects between drugs and doses with regards to food wastage, and weight gain, none of the changes indicated a lasting or significant impairment caused by the drugs outside of those that caused obvious toxicity discussed above. None of the drugs had a significant effect on shuttlebox performance (data not shown). Food wastage differed from vehicle controls in groups administered pentobarbital ($F_{3,\ 31} = 3.931$, $p < .05$), and phenobarbital ($F_{3,\ 31} = 3.778$, $p < .05$). Multiple comparison tests did not find significant differences between individual doses of any of the drugs compared to vehicle controls (data not shown). Food wastage was nominally lower in all the above-mentioned groups compared to vehicle controls. Fewer differences were seen in percent change in body weight, with only rats receiving lorazepam differing from vehicle controls ($F_{3,\ 30} = 4.795$, $p < .01$). Rats administered the 25 mg/kg dose of lorazepam on average exhibited a 4.71% increase in body weight which was greater than the -0.377% change observed in vehicle controls ($p < .05$).

4. Discussion

This study was designed to survey a diverse group of drugs at high therapeutic doses to identify those that may be most efficacious in preventing TETS lethality. All test drugs were investigated across a minimum of three dose levels with the highest dose chosen from the literature and the upper limit of safety based on each drug's published LD_{50} values. Four of the nine drugs tested were efficacious when

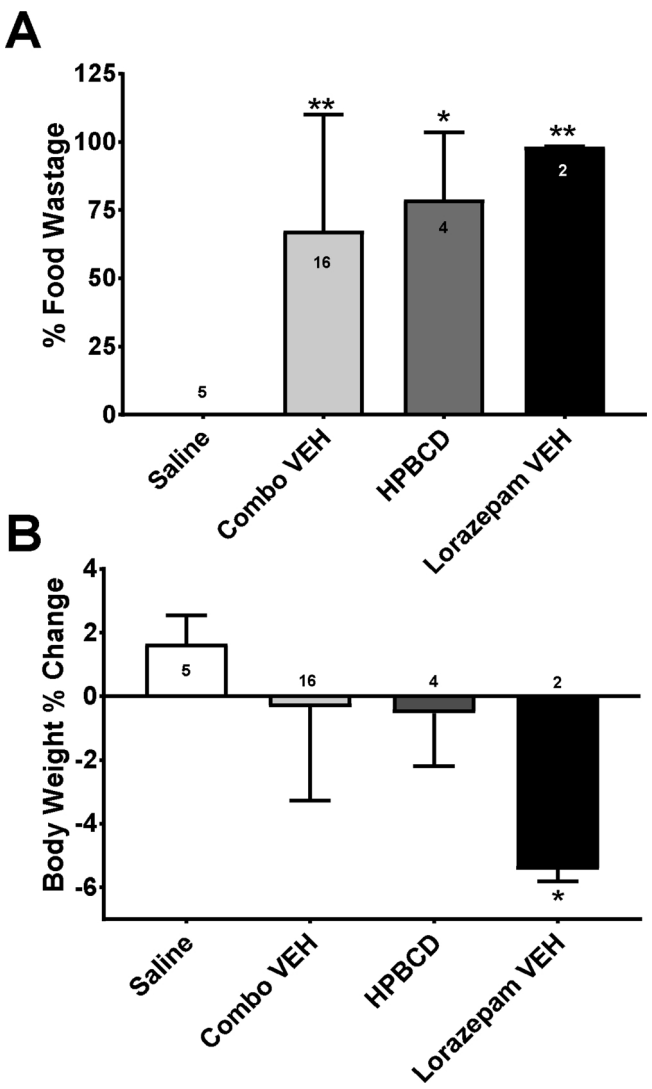

Fig. 5. Food wastage (A) and percent body weight change (B) comparison between vehicles in the safety studies (no TETS exposure). Rats receiving saline did not waste food and gained weight following acetone (TETS vehicle) exposure, whereas the vehicle animals administered larger volumes (10–15 ml) wasted the majority of food. Only the lorazepam vehicle group showed a significant decrease in body weight compared to the saline group. The data are presented as the mean + SD with the number of animals appearing in the bar. * $p < .05$, ** $p < .01$. VEH, vehicle; HPBCD, 45% (2-hydroxypropyl)-β-cyclodextrin.

administered 10 min after ingestion of TETS, and are all considered standard therapeutics for the initial treatment of SE (Chapman et al., 2001; Chen and Wasterlain, 2006; Arif and Hirsch, 2008). The benzodiazepines diazepam, midazolam, and lorazepam as well as the barbiturate phenobarbital all target the $GABA_A$ receptor, the site of action of TETS.

The efficacy of a single administration of any one of the four leading compounds is especially impressive when it is noted that three of the four have relatively short elimination half-lives in rats. Diazepam has an elimination half-life of 1.1 h (Klotz et al., 1976), whereas lorazepam and midazolam have elimination half-lives of 0.3 h and 0.5 h respectively (Kotegawa et al., 2002; Atack et al., 2007). Phenobarbital has the longest elimination half-life of the four, approximately 17 h in female rats (Brandt et al., 2004). Importantly, TETS has an extremely long plasma elimination half-life of 56.9 h following intravenous administration and 262.5 h after oral administration in rabbits (Zhang et al., 2005) and remains sustained at plasma levels corresponding to about 10% of the administered dose for greater than 4 h after TETS administration in mice (Vasylieva et al., 2017). Using mice, Radwan and Dodge (1970) showed that TETS is rapidly absorbed following oral administration with peak plasma levels occurring within 10 min and remaining unchanged for 48 h. Similar to the observations made in laboratory animals, TETS is persistent in humans, detectable in the vomitus and plasma of two patients one week after consuming TETS (Chau et al., 2005). Despite the persistence of TETS and the short half-lives of the efficacious therapeutics, high rates of survival were achieved with just one dose of the therapeutic. It is important to note that the majority of animals that survived to 24 h were behaviorally and physically impaired and most would likely have required aggressive supportive care to survive. Nevertheless, the present study offers promise that, in a mass casualty event involving TETS, a single administration of a therapeutic in the immediate aftermath could increase survival even if more extensive medical care is greatly delayed.

With the exceptions of lorazepam 50 mg/kg and ethanol, the therapeutics were well tolerated in the absence of TETS despite being administered at high doses. Most of the negative effects on body weight and food wastage were also observed in vehicle controls, suggesting that the vehicle at least contributes to, if not causes, these adverse effects. It is important to note that the most severe toxicity was observed with ethanol and the highest dose of lorazepam. Ethanol proved ineffective at attenuating TETS lethality, and lorazepam was efficacious at a dose (1.56 mg/kg) 32-fold lower than the toxic dose. The lethality of the 50 mg/kg dose of lorazepam came as a surprise, as the 24 -h intraperitoneal LD_{50} of lorazepam in rats is often reported in Safety Data Sheets as 805 mg/kg based on the work of Owen et al. (1971). In that study, the median lethal doses of two forms of lorazepam were determined. When lorazepam was administered in 0.4% carboxymethlcellulose suspension the LD_{50} was 805 mg/kg; however, the LD_{50} was lowered to 48 mg/kg when lorazepam was formulated in 5 mg/ml injectable preparation. The injectable preparation of lorazepam was administered at a volume of 10.6 ml/kg. The LD_{50} (i.p.) of the injectable vehicle alone was 11.6 ml/kg, suggesting that the vehicle contributed significantly to the lethality of the injectable formulation. Although the components of the vehicle were not listed, propylene glycol was likely a major component with an LD_{50} (i.p.) in the rat of 13.0 ml/kg (Bartsch et al., 1976).

The combination vehicle used for lorazepam, diazepam, phenobarbital, and pentobarbital contained (by volume) 62% propylene glycol, 18% polyethylene glycol 400, 10% ethanol, 8% water, and 2% benzyl alcohol. This vehicle was chosen to accommodate multiple drugs containing different proportions of shared components such as propylene glycol and to conserve animals, as no profound vehicle effect was expected. All of the compounds used in the combination vehicle are commonly used in pharmaceutical preparations. Once testing began it became clear that the large volumes of the combination vehicle produced severe and prolonged sedation. After all the rats receiving the high dose of lorazepam died, a group administered the vehicle used in commercial preparations was added to the study. The commercial vehicle contained even higher amounts of propylene glycol (80%) than the combination vehicle and resulted in a fatality occurring shortly after the 24 h period during behavioral testing when administered at 12.5 mL/kg, the volume used for rats administered 50 mg/kg lorazepam. Propylene glycol toxicity, often presenting as an osmolar gap (Barnes et al., 2006), is frequently observed in case reports of patients receiving large doses of commercially formulated lorazepam, especially in patients with impaired renal function (Cawley, 2001; Al-Khafaji et al., 2002; Wilson et al., 2005).

The reported ability of propylene glycol to shift convulsive thresholds of pentylenetetrazol and strychnine (Zaroslinski et al., 1971) as well as to delay the onset of hyperbaric oxygen-induced seizures (Beckman and Crittenden, 1981) deserves recognition. Exploring the effects of propylene glycol on TETS-induced seizures was well beyond the scope of this study; however, to ensure that propylene glycol was

not contributing to the anticonvulsant activities of lorazepam the 1.56 mg/kg dose of lorazepam was replicated using a 40% HPBCD solution. The near perfect replication (90% survival compared to 100% of the commercial preparation) strongly suggests that propylene glycol does not contribute to the efficacy of lorazepam and low doses of lorazepam can effectively treat TETS poisoning regardless of the vehicle used.

The therapeutics chosen for evaluation were selected for anticonvulsant action at the $GABA_A$ receptor or elsewhere. Other TETS investigators have evaluated the efficacy of a subset of the drugs reported in this study. Phenobarbital was first identified as an efficacious therapeutic for TETS intoxication by Voss et al. (1961) and later shown to increase the LD_{50} by 5.5 fold when administered as a pretreatment (Casida et al., 1976). Diazepam has been demonstrated to successfully attenuate TETS lethality in mice (Vito et al., 2014; Shakarjian et al., 2015). It is therefore not surprising that the most efficacious drugs in the current study included benzodiazepines and phenobarbital. Shakarjian et al. (2012, 2015) found that high doses of an NMDA antagonist (i.e., ketamine or dizocilpine) decreased tonic-clonic seizures following a lethal dose of TETS when administered after the first clonic seizure. In this study, ketamine (at the 37.5 mg/kg dose only) showed some promise, falling just short of our statistical survival criteria for further study. Allopregnanolone has worked in combination with diazepam against a lethal concentration of TETS in mice (Bruun et al., 2015) but at significantly lower doses of each, 0.03 mg/kg diazepam and 0.03 mg/kg allopregnanolone, compared to the present study. Allopregnanolone, ketamine, and dexmedetomidine all showed some promise in the present study but were not statistically efficacious enough to be considered as a sole therapeutic. Addition of an adjunct therapeutic with a different or complementary mechanism of action may improve survival; however, the current study demonstrates that a single dose of lorazepam, diazepam, midazolam, or phenobarbital is enough to significantly increase survival in the first 24 h after TETS exposure. Ongoing studies are evaluating the efficacy of these drugs when administered at delayed time points following TETS ingestion. It may become apparent as these studies progress that the top four therapeutics may not be as efficacious as desired when administered alone at the longer delays, warranting the study of allopregnanolone, dexmedetomidine, or ketamine as an adjunct therapeutic.

The present survey of therapeutics against the acute toxicity of TETS demonstrated that the three benzodiazepines (lorazepam, midazolam, and diazepam) and the barbiturate phenobarbital were the most efficacious in preventing TETS lethality. Lorazepam especially was efficacious over a wide range of doses with the lower limits yet to be found. In the case of all four top therapeutics, a single administration ten minutes after TETS ingestion was enough to ensure survival in the vast majority of rats. The data suggest that although TETS lethality was attenuated, the therapeutics did not prevent TETS-induced weight loss, food wastage, and behavioral deficits. Studies examining the longer-term functional and quality-of-life outcomes beyond simple survival following TETS ingestion and treatment are necessary before deciding the most beneficial therapeutic.

Transparency document

The Transparency document associated with this article can be found in the online version.

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