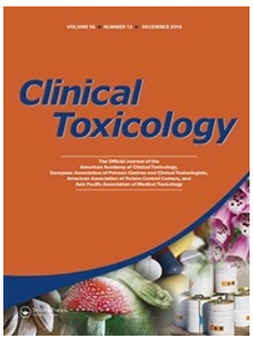

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BASIC RESEARCH

Neurotoxic effects of nephrotoxic compound diethylene glycol

Courtney N. Jamison, Robert D. Dayton, Brian Latimer, Mary P. McKinney, Hannah G. Mitchell and Kenneth E. McMartin

Department of Pharmacology, Toxicology and Neuroscience, LSU Health Sciences Center, Shreveport, LA, USA

ABSTRACT

Context: Diethylene glycol (DEG) is an organic compound found in household products but also as an adulterant in medicines by acting as a counterfeit solvent. DEG poisonings have been characterized predominately by acute kidney injury (AKI), but also by delayed neurological sequelae such as decreased reflexes or face and limb weakness.

Objectives: Characterizing the neurological symptoms of DEG poisoning in a subacute animal model would create a clearer picture of overall toxicity and possibly make mechanistic connections between kidney injury and neuropathy.

Methods: Male Wistar-Han rats were orally administered doses of 4 – 6 g/kg DEG every 12 or 24 h and monitored for 7 days. Urine was collected every 12 h and endpoint blood and cerebrospinal fluid (CSF) were collected for a renal plasma panel and total protein estimation, respectively. Motor function tests were conducted before and after treatment. Kidney and brain tissue was harvested for metabolic analysis.

Results: Of the 43 animals treated with DEG, 11 developed AKI as confirmed by increased BUN and creatinine levels. Renal and brain DGA accumulation was markedly increased in animals that developed AKI compared to animals without AKI. The total protein content in CSF in animals with kidney injury was markedly elevated compared to control and to treated animals without AKI. Significant decreases in forelimb grip strength and decreases in locomotor and rearing activity were observed in animals with AKI compared to control and to animals without AKI.

Discussion: Repeated dosing with DEG in an animal model produced nephrotoxic effects like those in studies with acute DEG administration. The decrease in motor function and increase in CSF protein were only present in animals that developed AKI.

Conclusions: These studies show development of neurotoxicity in this DEG animal model and suggest that neurological symptoms are observed only when DGA accumulation and kidney injury also occur.

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Diethylene glycol; diglycolic acid; nephrotoxicity; neurotoxicity; cerebrospinal fluid; motor function; metabolite toxicity

Introduction

Diethylene glycol (DEG) is an organic compound that can be found in household products, including chafing fuels and brake fluid, but has also been a contaminant in some medicines as a counterfeit solvent [1,2]. Unfortunately, this adulteration has led to cases of accidental ingestion around the world, including China, Africa, and Panama, resulting in long term kidney damage, neurologic sequelae and death [3]. While DEG poisonings are not prevalent in the United States in comparison to more recent DEG outbreaks, an adulterated DEG poisoning was instrumental in the creation of the 1938 Federal Food, Drug, and Cosmetic Act due to the Massengill sulfanilamide disaster of 1937 [3–5] Since then, there have been numerous mass poisoning incidences involving DEG, with Brazil in late 2019 [6] being the most recent and Bangladesh from 1990 to 1992 being the deadliest [7].

Common symptoms of DEG poisoning include oliguria or anuria, inebriation, gastrointestinal distress, fever, fatigue, breathing issues, and limb weakness, with the hallmark clinical sign being acute kidney injury, particularly in the proximal tubular cells [3]. These signs and symptoms are applicable to many other conditions and there is no biomarker or common test that easily detects DEG. Blood work shows increased BUN and creatinine levels and osmolal gap along with a metabolic acidosis, but such results could allude to a spectrum of illnesses [4]. Additionally, there are delayed neurological sequelae such as decreased reflexes, facial muscle and limb weakness, or ataxia, demonstrated in patients 2–7 days after DEG ingestion. The neurotoxic characteristics of DEG toxicity have been well described from patients in the Panama epidemic [3]. 40 out of the 46 patients included in the study exhibited neurological symptoms at some point during their DEG-induced illness, with over 60% experiencing limb weakness and decreased or absent reflexes. Nerve conduction studies performed on 21 patients produced results that suggest severe sensorimotor axonopathy, including "unexcitable motor and sensory responses", and "decreased motor and sensory amplitudes with relatively normal distal latencies and conduction velocities, with variable prolongation or absence of F waves" [3,8]. This phenomenon has also been previously reported in a 2002 case study of a man who intentionally ingested a can of

CONTACT Kenneth E. McMartin kmcmar@lsuhsc.edu Department of Pharmacology, Toxicology and Neuroscience, LSU Health Sciences Center – Shreveport, 1501 Kings Highway, Shreveport, LA71130-3932, USA

chafing fuel containing DEG [9,10]. Another nerve conduction study performed on a patient who intentionally ingested DEG similarly showed no motor or sensation response through the 29th hospital day, with slow recovery of the distal limbs through discharge of the patient after a 7-month recovery [11]. In the Panama case study, an increase in protein concentration in the cerebrospinal fluid (CSF) without pleocytosis or change in CSF glucose was measured in 14 patients, 13 of which later developed neurological signs. Elevated CSF protein levels have also been noted in case studies in South Africa [12] and Colorado [10]. Cranial nerves 3, 5–7, and 9–12 [9] have been documented as being involved in this neuropathy, as well as in a case that resulted in basal ganglia hemorrhage, and lobe damage confirmed *via* MRI [1,8,13].

Similar to ethylene glycol, DEG is initially metabolized by alcohol dehydrogenase and treatment with the alcohol dehydrogenase inhibitor, fomepizole, can diminish DEG toxicity, but this action must be taken rapidly after DEG intake [14,15]. This is often not possible when doctors or patients are unaware of the contaminant consumed or medical facilities are slow to diagnose. DEG is metabolized by alcohol dehydrogenase to 2-hydroxyethoxyacetaldehyde [16] and then to 2-hydroxyethoxyacetic acid (2-HEAA), with a minor metabolite, diglycolic acid (DGA) being produced as well. HEAA is found at a higher concentration in the blood and is responsible for the metabolic acidosis during DEG poisoning [17] However, at toxic doses of DEG, DGA was shown to accumulate in the kidney and cause dose-dependent cell necrosis, unlike the parent compound or other metabolites [18,19]. Recent studies have confirmed that DGA accumulation is responsible for the nephrotoxicity and hepatotoxicity from DEG [19,20]

The mechanism for the neurotoxicity of DEG is not understood at present. There is some evidence suggesting that neurological dysfunction from DEG ingestion only presents after renal failure, which could lead to a link between these pathologies [21]. Further investigation surrounding the neuropathy of DEG ingestion could uncover toxicity pathways related to both the neurologic and kidney injuries. An *in vivo* animal model of neurological dysfunction during DEG poisoning is necessary to fully understand the breadth of this toxicity. As such, these studies were designed to evaluate an animal model by comprehensively studying the neurological and nephrotoxic aspects. This study used the case study on the Panama DEG mass poisoning as a guide for parameters to assess [3] and employed subacute dosing schedule of DEG over the course of 7 days in order to better mimic a clinical presentation of DEG ingestion and possible neurotoxicity. Our hypothesis is that during DEG poisonings, DGA is distributed throughout the body, including the kidney, liver, and brain, and in the later stages contributes to neurological sequelae.

Materials and methods

Materials

DEG (≥99% purity by gas chromatography analysis) for gavage was obtained by Sigma-Aldrich Corporation (St. Louis, MO) and prepared in deionized water.

Table 1. Total number of animals and AKI development by DEG dose schedule.

Treatment	Number of animals	Developed AKI*	No AKI
Water control	9	0	9
DEG 4 g/kg	6	0	6
DEG 4 g/kg every 12 h	7	1	6
DEG 5 g/kg	7	1	6
DEG 5 g/kg every 12 h	8	2	6
DEG 6 g/kg	5	1	4
DEG 6 g/kg every 12 h	10	6	4
Total	52	11	41

*Animals included in the group that developed acute kidney injury had elevated BUN and creatinine endpoint levels.

Animal protocol

Fifty-two adult male Wistar-Han rats (Envigo, Indianapolis, IN) had baseline motor function established as described below and were randomly placed into a treatment group (Table 1) and administered their dose *via* oral gavage for up to 7 days. Rats were single-housed in metabolic chambers for urine collection starting at −12 h before dose administration and collected every 12 h through 168 h. Standard conditions of humidity, temperature (25 °C ± 2 °C) and light (12:12 h light:-dark) were maintained in the animal facility and rats were allowed free access to food (normal rat chow, Envigo Teklad Diet) and water. The overall study was conducted as a sum of nine different experiments in which rats between ~6 and 12 weeks of age were used. Weight ranges for the nine experimental sets were 205–420 g, although the mean weight among the various groups remained similar (see Figure 1). DEG doses were based on previous DEG acute oral administration animal studies [15,22], which showed that single doses of 5 g/kg were not toxic while 10 g/kg induced nephrotoxicity within 48 h. The animal protocols were approved by the Institutional Animal Care and Use Committee (Louisiana State University Health Sciences Center, Shreveport (LSUHSC-S)) and were in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Animal observations

Animals were regularly monitored for signs of morbidity and behavioral changes such as decreased food and water intake, decreased urine output (oliguria), as well as diminished response to stimuli during each dosing. Animals that showed a decrease in urine volume by at least 50% or a change in behavior were not continued on their dose schedule and were evaluated for motor function prior to euthanasia.

Urine collection and analysis

Urine was collected in chilled tubes every 12 h from −12 to 168 h. Metabolic urine chambers were rinsed between each collection. Following collection, urines were centrifuged at 1000 rpm at 4 °C for 10 min and transferred to clean 50 mL conical tubes to be measured for total volume and pH. Urine samples were then vortexed and two 1 mL aliquots were transferred to Eppendorf tubes for storage at −80 °C until analysis.

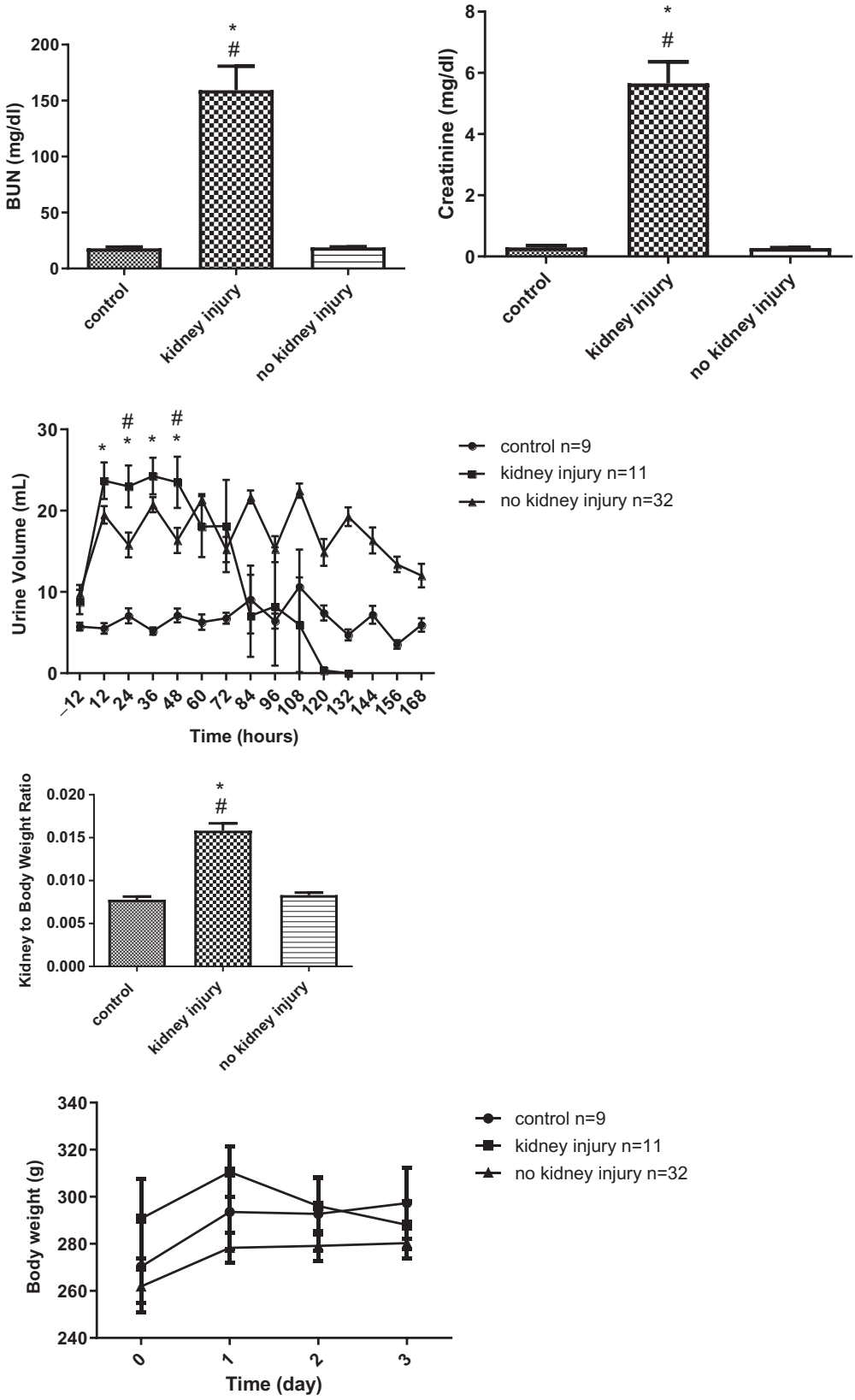

Figure 1. Subacute DEG oral administration produced kidney injury in rats without affecting body weight. Kidney injury was assessed by blood urea nitrogen (BUN) (A), plasma creatinine (B), urine volume (C), kidney to body weight ratios (D), and body weight over time across treatment groups (E). DEG doses ranged from 4 g/kg to 6 g/kg, administered once or twice daily. Data are represented as means ± SEM ($n = 9$ for controls, $n = 9$–11 for animals that developed kidney injury, $n = 32$ for animals that were administered DEG but did not develop kidney injury). Asterisk (*) indicates significant difference from control (one-way ANOVA followed by Bonferroni post hoc test, $p < .05$). Pound sign (#) indicates significant difference between DEG-treated animals that did and did not develop kidney injury (one-way ANOVA followed by Bonferroni post hoc test, $p < .05$).

DEG urine analysis

To measure DEG in urine, aliquots were filtered through 0.5 mL centrifugal filter units (10 kDa MW cutoff, Millipore) and prepared in a 1:20 dilution of deionized water and internal standard 1,3-propanediol, for injection into the gas chromatograph with flame ionization detector (Shimadzu GC-17A, Kyoto, Japan). The retention time of DEG with the Chromosorb 101 column was 9.6 min and the retention time of the internal standard was 5.1 min. The limit of quantitation was 0.125 mg/mL.

Blood collection and analysis

Animals were anesthetized at 168 h using isoflurane induction for CSF extraction, followed by sodium pentobarbital (50 mg/kg, i.p.) for tissue collection. Blood was collected from the abdominal aorta into heparinized 3 mL syringes and placed into microtainers with lithium heparin for plasma separation (BD, Franklin Lakes, NJ). The isolated plasma was stored at 4 °C until analysis for a comprehensive renal panel including BUN and creatinine, as well as glucose and various electrolytes (sodium [Na^+], potassium [K^+] and chloride [Cl^-]) by the Ochsner LSU Health – Shreveport Clinical Laboratory.

Tissue collection

After rats were anesthetized, kidneys, brain, and spinal cord were collected and weighed for histopathological analysis. One whole kidney, one hemisphere of brain, and the spinal cord were placed in an ethanol - dry ice bath and stored at −80 °C until further analysis. The remaining tissue was fixed in 10% neutral buffered formalin.

DGA levels in tissue

Kidney and brain tissue were analyzed for DGA content using high-performance liquid chromatographic (HPLC) analysis with a two-step solid-phase extraction protocol [22]. The method was modified according to previous optimization [19] following deproteinization, with the addition of KOH to neutralize the sample prior to solid-phase extraction.

CSF extraction for total protein analysis

This protocol is a modified technique for collecting cerebrospinal fluid in the cisterna magna without previous surgery [23,24]. The rat was anesthetized with isoflurane, the back of the neck and base of skull was shaved, and the rat was placed in a stereotaxic device with an isoflurane-oxygen nose-cone. A depression of the skull between the ears, or rhombus area between the occipital protuberance and the spine of the atlas, was located and marked for needle placement. A 23 g needle with attached catheter tubing was placed perpendicularly at the marked location and pushed through the dura mater. At approximately 5 mm, a sample was collected with minimal pullback pressure. If blood contamination occurred, the tubing was cut above site. The resulting clear CSF was placed into chilled Eppendorf tubes, centrifuged twice at 14,000 RPM for 10 min to remove stray red blood cells, and stored at 4 °C until analyzed for total protein content *via* a bicinchoninic acid assay.

Motor function tests

Motor function tests were conducted according to protocols previously described [25,26] before rats were placed into their metabolic chambers and on day 7 or sooner if the animal presented with symptoms of kidney injury. Rotarod (Rota-rod/RS; Letica Scientific Instruments, Barcelona, Spain) testing was administered by measuring the amount of time it takes for a rat to fall off a rotating rod that accelerates over time (latency to fall). The rod accelerated from 4 to 40 rpm over 2 min. The average of three trials for each rat was taken at each time point. Locomotor testing was conducted in an open field chamber with a photobeam activity-monitoring system (Truscan 2.0; Coulbourn Instruments, Whitehall, PA). Rats acclimated to a dark room for 30 min before testing began. Testing was carried out in the same dark room over a 30-min time period where the rat freely explored the chamber. The total horizontal distance traveled in the chamber during the 30-minute time period and the time the animal broke the vertical beam, indicative of normal rearing behavior, were calculated. Forearm grip strength was evaluated by placing the rat's forelimbs on a wire mesh attached to a 1 kg hanging scale (American Weight Scales, Inc., Norcross, GA). The scale was zeroed in the horizontal direction, and the rat's forelimbs were placed on the center of the mesh. The tail of the rat was gently pulled until the forelimbs released. The weight of release was measured 3 times and averaged. The hindlimb escape reflex, which qualitatively measured hind limb function, started by lifting the rat briefly by the tail 3 times and observing full hindlimb extension or splaying.

Statistics

Values in the text represent the group mean value ± SEM. Comparisons between multiple groups were performed using a one-way ANOVA with a Bonferroni *post hoc* analysis to determine treatment group differences. Two-way ANOVA was used for multiple comparisons, with Bonferroni's *post hoc* analysis used for significance. All analyses were performed using GraphPad Prism 5 or Prism 7.05 (La Jolla, Ca) for Windows. Tests were considered significant if $p < .05$.

Results

DEG subacute dosing led to DGA accumulation and acute kidney injury

Renal injury

Throughout the study, rats treated with DEG were monitored for changes in urine output as a presumptive measure of kidney dysfunction; a substantial decrease in urine volume (by 50% or more) in one 12 h collection period or signs of

distress such as decreased movement and grooming or decreased muscle tone resulted in: (1) the animal not being administered the next DEG dose; (2) and final motor function tests being conducted. Following the motor function testing, the animals were anesthetized and euthanized following tissue collection procedures. An increase in plasma BUN and creatinine values at the endpoint blood collection was used to validate that the animal was exhibiting kidney injury symptoms. Such animals were categorized as the "kidney injury" group for data analysis, while other treated animals were then categorized as the "no kidney injury" group. In total, 11 out of 43 animals treated with DEG developed AKI, with 9 out of the 11 being treated every 12 h (Table 1). One animal was euthanized on day 3 due to suspicion of kidney dysfunction, but did not have elevated renal biomarkers and was thus included in the no kidney injury group.

BUN and plasma creatinine values were significantly increased in animals that developed kidney injury compared to controls and to animals that were treated with DEG but did not develop kidney injury ($p < .05$) (Figure 1(A, B)). Urine volumes in DEG-treated animals were significantly increased from control at 12–48 h ($p < .05$) (Figure 1(C)). However, urine volume in animals with kidney injury started to decrease at 60 h and continued to decrease until they were anuric, or until the animals were euthanized. Animals without kidney injury continued to have significantly more urine output compared to control through 156 h except at 120 h ($p < .05$). DEG-treated animals that developed kidney injury had increased kidney-to-body-weight ratios (0.0158 ± 0.0009), compared to DEG-treated animals without kidney injury (0.0083 ± 0.0003) and to controls (0.0078 ± 0.0004) ($p < .05$) (Figure 1(D)). There was no difference in body weight among all groups through day 3 before animals started to be euthanized (Figure 1(E)).

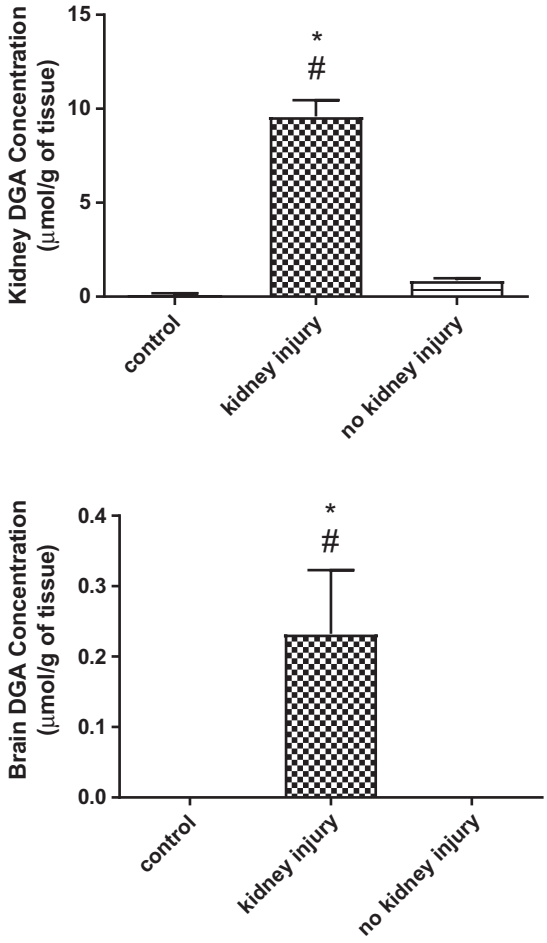

Figure 2. DGA concentrations in kidney (A) and brain (B) tissue at termination of study. Data are represented as means ± SEM ($n = 4$–5 for controls, $n = 5$–6 for animals that developed kidney injury, $n = 17$ for animals that were administered DEG but did not develop kidney injury). Asterisk (*) indicates significant difference from control (one-way ANOVA followed by Bonferroni post hoc test, $p < .05$). Pound sign (#) indicates significant difference between DEG-treated animals that did and did not develop kidney injury (one-way ANOVA followed by Bonferroni post hoc test, $p < .05$).

DGA accumulation in tissue

Kidney and brain tissue for DGA concentration analysis was collected after the 168 h time point in control animals or in animals that were DEG-treated that did not develop kidney injury. Animals that developed kidney injury had tissue collected from day 3 to day 6, depending on when they presented with symptoms. Animals that developed kidney injury had significantly increased DGA levels in the kidney compared to controls and to animals that did not develop kidney injury ($p < .05$) (Figure 2(A)). Kidney DGA levels in animals with kidney injury averaged 9.6 μmol/g, while animals that did not develop kidney injury averaged 0.9 μmol/g. Additionally, animals that developed kidney injury had significantly increased DGA levels in the brain compared to controls and to animals that did not develop kidney injury ($p < .05$) (Figure 2(B)). Brain DGA levels in animals with kidney injury averaged 0.233 μmol/g and animals that did not develop kidney injury averaged 0 μmol/g.

Metabolic acidosis

Endpoint plasma bicarbonate and anion gap values were used to assess the development of metabolic acidosis in DEG-treated animals. Animals that developed kidney injury had significantly decreased bicarbonate (1.83 mmol/$L \pm 0.31$) compared to controls (25.22 mmol/$L \pm 1.51$) and to animals that did not develop kidney injury (27.84 mmol/$L \pm 0.42$) ($p < .05$) (Figure 3(A)). The anion gap in animals with kidney injury was significantly increased (36.2 mmol/$L \pm 3.0$) compared to controls (11.9 mmol/$L \pm 2.0$) and to animals that did not develop kidney injury (10.7 mmol/$L \pm 0.55$) ($p < .05$) (Figure 3 (B)). As an index of acid exposure over time, urine pH significantly decreased in all DEG-treated animals compared to control from 12 to 48 h when animals with kidney injury needed to be euthanized ($p < .05$) (Figure 3(C)). Animals treated with DEG without kidney injury continued to have significantly more acidic urine compared to controls through 168 h ($p < .05$).

DEG concentrations in urine

DEG concentrations in urine were assessed every 12 h. DEG levels in animals that were dosed every 12 h did not show any discernable pattern in DEG accumulation (Figure 4(A)). Animals that were administered DEG every 24 h exhibited a

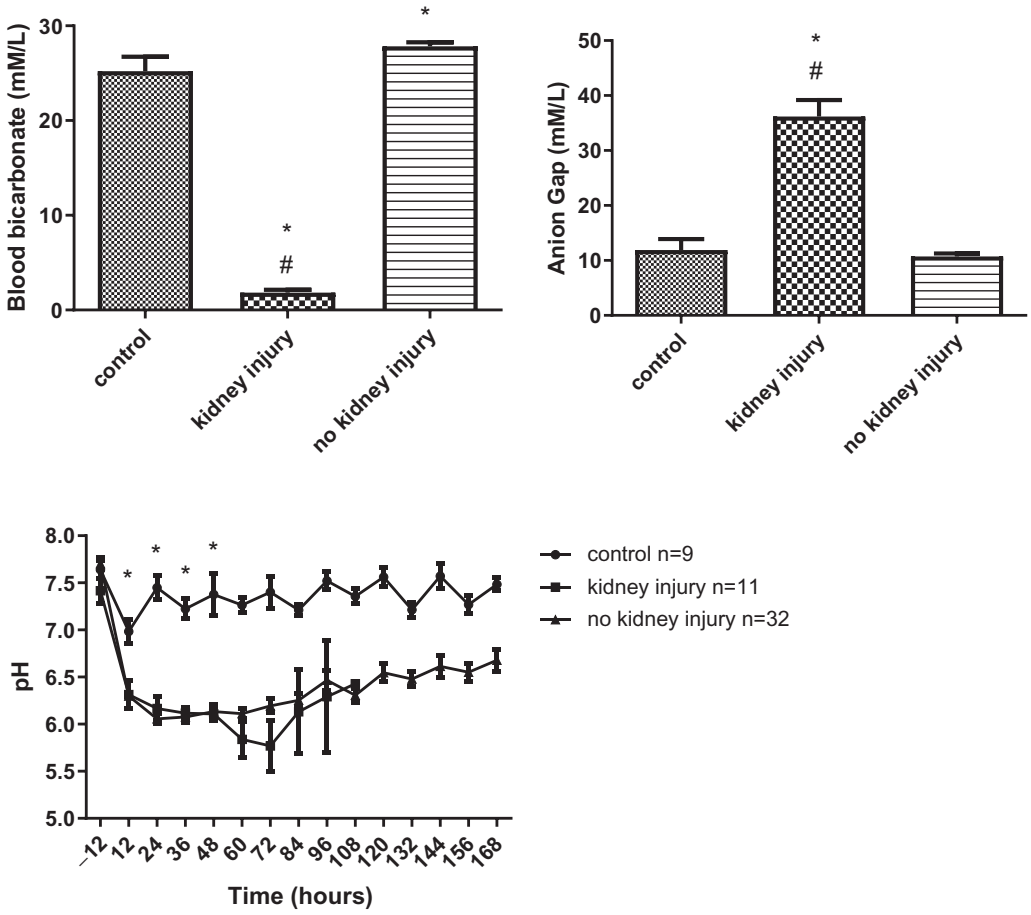

Figure 3. Subacute DEG oral administration produced metabolic acidosis in rats. Metabolic acidosis was assessed using blood bicarbonate at termination of study (A), anion gap at termination of study (B), and urine pH (C). Anion gap was calculated as $[(Na+ + K+) - (Cl^- + HCO^{3-})]$. Data are represented as means ± SEM ($n = 9$ for controls, $n = 6$–11 for animals that developed kidney injury, $n = 32$ for animals that were administered DEG but did not develop kidney injury). Asterisk (*) indicates significant difference from control (one-way ANOVA followed by Bonferroni post hoc test, $p < .05$). Pound sign (#) indicates significant difference between DEG-treated animals that did and did not develop kidney injury (one-way or two-way (urine pH and volume) ANOVA followed by Bonferroni post hoc test, $p < .05$).

peak and trough pattern where DEG levels increased at 12 h after each dose and decreased 12 h afterwards (Figure 4(B)). In these animals during the first 48 h only, there was a trend for dose-dependent levels, with the 6 g/kg group showing a slightly higher level of DEG compared to the 5 g/kg animals, which in turn was slightly higher than the 4 g/kg group. Overall, there was no difference in DEG levels in the urines of animals that did or did not develop kidney injury through 48 h (Figure 4(C)).

DEG subacute dosing produced neurotoxic effects

Cerebrospinal fluid protein content

Endpoint total protein in the cerebrospinal fluid was quantified as an indication of neurotoxic damage in animals receiving DEG administration. CSF protein was significantly increased in animals with kidney injury (2430 μg/mL ± 458) compared to controls (424 μg/mL ± 33) and to animals that did not develop kidney injury (559 μg/mL ± 56) ($p < .05$) (Figure 5).

Motor function

Neurological effects were evaluated using broad motor function tests to determine changes in limb strength and coordination. The total distance traveled during open field exploration was significantly decreased in all DEG-treated animals compared to control (12319 cm ± 884), with animals that developed kidney injury traveling an average of 3761 cm ± 1138 and animals without kidney injury traveling an average of 9998 cm ± 412 ($p < .05$) (Figure 6(A)). DEG-treated animals had decreased rearing time compared to control (598 $s \pm 58$), with animals that developed kidney injury averaging 110 $s \pm 34$ and animals without kidney injury averaging 435 $s \pm 29$ ($p < .05$) (Figure 6(B)). There was no difference in DEG-treated animals in distance spent in the center or edges of the open field chamber compared to control; all groups spent approximately half of their time between both zones of the chamber (Figure 6(C)). After DEG dose administration, forelimb grip strength was significantly decreased from baseline in animals that developed kidney injury (248 $g \pm 35$) compared to controls (390 $g \pm 14$) and to animals that did not develop kidney injury (369 $g \pm 16$) ($p < .05$) (Figure 6(D)). Latency to fall during rotarod acceleration did not significantly change in DEG-treated animals compared to control (Figure 6(E)). There was no change in hind limb splaying between DEG-treated animals and control; all animals expressed full hind limb expression after treatment with DEG or control.

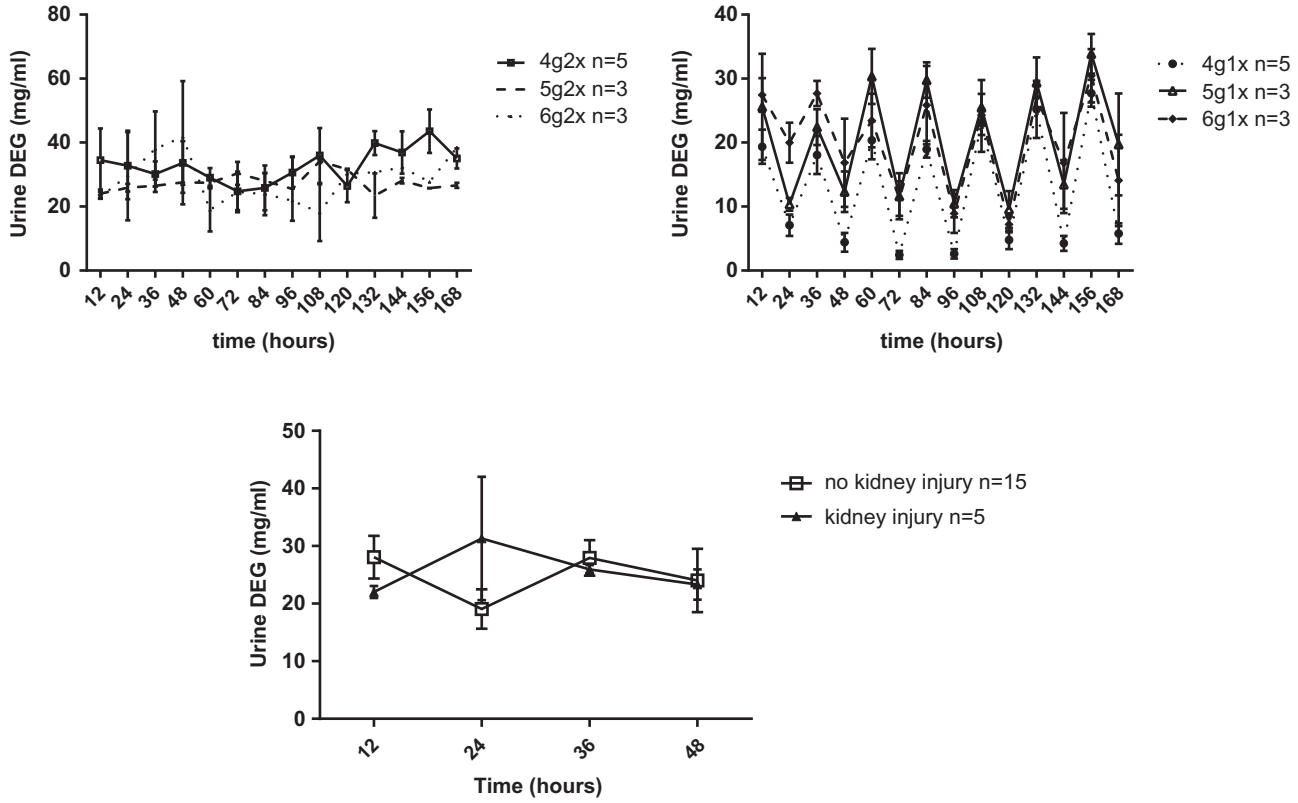

Figure 4. Urine DEG concentrations in rats treated with DEG every 12 h (A), every 24 h (B), and by establishment of kidney injury (C). Data are represented as means ± SEM ($n = 3$–15 per group). (two-way ANOVA followed by Bonferroni post hoc test, $p < .05$). Statistical analysis showed that there were no significant differences between DEG-treated animals that did and did not develop kidney injury.

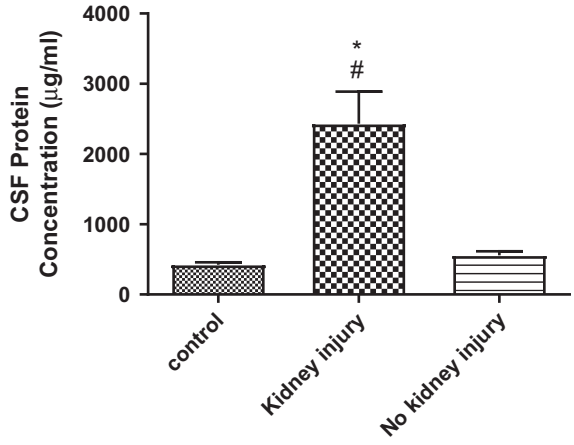

Figure 5. Total protein in cerebrospinal fluid of rats treated with DEG. Data are represented as means ± SEM ($n = 5$ for controls, $n = 7$ for animals that developed kidney injury, $n = 30$ for animals that were administered DEG but did not develop kidney injury). Asterisk (*) indicates significant difference from control (one-way ANOVA followed by Bonferroni post hoc test, $p < .05$). Pound sign (#) indicates significant difference between DEG-treated animals that did and did not develop kidney injury (one-way ANOVA followed by Bonferroni post hoc test, $p < .05$).

Discussion

The hallmark sign of DEG toxicity in humans as well as in animals is acute kidney injury, including increases in renal biomarkers like BUN and creatinine [1]. Normal serum creatinine values in rats range from 0.4 to 0.8 mg/dL and from 15 to 22 mg/dL for BUN [27], which reflect the values in our control and no AKI animals. The AKI animals averaged over 5 mg/dL for creatinine and 159 mg/dL for BUN, which both greatly exceed the normal ranges. Hence, AKI in these rats was established by values exceeding the normal historical ranges and also by the statistically significant increases over control values. Delayed neurological sequelae including loss of sensorimotor functions in peripheral limbs, and increased protein in the cerebrospinal fluid have also been documented in human DEG poisonings, particularly in the Panama epidemic [3]. The renal and hepatic effects have been determined to result from the metabolite DGA, as exemplified by tissue accumulation in target tissues of DEG-treated rats [17] and by the marked kidney and liver degeneration in animals directly treated with DGA [19]. This study recapitulated the AKI in a repeat-dose animal model like that observed in previous single-dose DEG studies. This study also characterized neurological signs and symptoms of DEG poisoning in animals that were similar to clinical presentations such as limb weakness, decreased or absent motor function, and increased total CSF protein. We also demonstrated a close connection between the kidney injury and the neuropathy, including the potential role of DGA. These results are similar to those in humans poisoned with DEG as reported in the Panama epidemic [3], where neurological toxicity was reported only in cases where there was marked increases in serum creatinine.

Variation of nephrotoxicity among doses in relation to DGA accumulation

In this study with repeated doses, only animals that developed kidney injury after DEG-administration exhibited

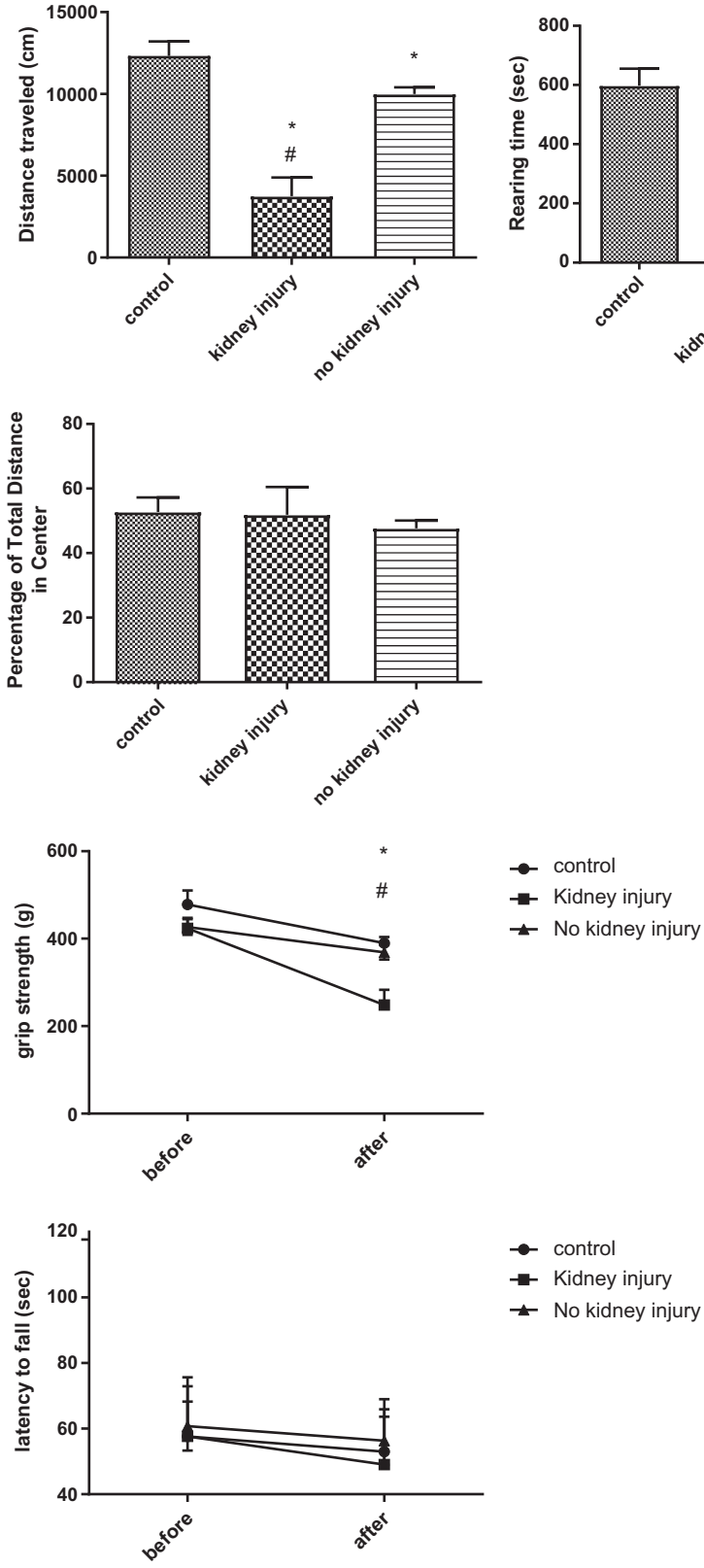

Figure 6. Motor function was altered in rats treated with DEG that developed kidney injury. Motor function was evaluated by open field total distance traveled (A), open field rearing (B), percentage of distance traveled in the center (C), forelimb grip strength (D), and latency to fall on an accelerating rotarod (E). Data are represented as means ± SEM ($n = 5$–9 for controls, $n = 5$–7 for animals that developed kidney injury, $n = 32$ for animals that were administered DEG but did not develop kidney injury). Asterisk (*) indicates significant difference from control (one-way (A, B, and C) or two-way (D and E) ANOVA followed by Bonferroni post hoc test, $p < .05$). Pound sign (#) indicates significant difference between DEG-treated animals that did and did not develop kidney injury (one-way (A, B and C) or two-way (D and E) ANOVA followed by Bonferroni post hoc test, $p < .05$).

significant accumulation of DGA in the kidney and brain. Interestingly, the doses that caused toxicity varied in concentration and frequency, with animals developing kidney injury at doses from 4 to 6 g/kg twice a day, or once a day with animals treated with 5–6 g/kg. Nine out of the 11 AKI animals were dosed twice a day, while only two of 16 animals treated once a day developed kidney injury, suggesting that frequency of administration (every 12 h) was important for development of toxicity. In general, there were many animals at all dose schedules that did not develop kidney injury, with only about 25% of the DEG-treated animals developing AKI. Despite similar doses of DEG, some animals accumulated DGA in tissues and developed kidney injury while others did not. This phenomenon has also been reported in a single dose DEG animal study, where only 3 out of the 6 high dose group (10 g/kg) developed severe kidney injury [15] and accumulation of DGA. Such variability in toxicity also occurs in humans, as has been reported in multiple epidemiological studies. In the sulfanilamide epidemic in the US in 1937, 353 people ingested the DEG-contaminated preparation, but only 260 were poisoned [28]. In a cohort study from Haiti of 49 ingestions, 17 subjects did not display symptoms or signs [29]. Lastly in the Panama epidemic, over a thousand people were estimated to have consumed the DEG-contaminated cough syrup, but only 119 cases of AKI were identified [30].

Despite the variability in toxicity among similarly-dosed rats, toxicity was only observed when there was accumulation of DGA within target tissues. Hence, this variability in DEG toxicity suggests some unevenness in the processing of DGA, either in its formation from DEG or its uptake and accumulation in tissues. The Wister-Han rat used in these studies is an outbred strain, or genetically diverse within the population, so DEG metabolism *via* alcohol dehydrogenase and aldehyde dehydrogenase, as well as a variability in DGA transporter activity could potentially affect the accumulation of DGA in target tissues [31,32]. Ethanol consumption studies in rats show that there is a low correlation between alcohol consumption and sensitivity to ethanol, and that genetic differences in alcohol and/or aldehyde dehydrogenase activities play a role in this difference just like in humans [33]. Because of the similarity of DGA's four carbon dicarboxylate structure with that of succinate [18], apical sodium dicarboxylate (NaDC)-1 and basolateral NaDC-3 transporters are suspected to be involved in DGA uptake into the proximal tubule cell. Because dicarboxylic acids are also effluxed from the proximal tubule cell in exchange for organic acids *via* the organic acid transporters (OATs), DGA could be effluxed by these same transporters. Individual variation in the activity of either the uptake or the efflux transporters could lead to differential accumulation of DGA by the proximal tubule cell and thus to individual sensitivity to DEG toxicity.

Importance of DGA accumulation in kidney injury

DGA accumulation in the kidney of injured rats, about 10 μmol/g, in this study was comparable to previous animal studies, with 5–17 μmol/g in rats treated with 10 g/kg DEG [17,22] and 5.6 μmol/g in rats treated with 300 mg/kg DGA [19]. The present animals received 3–5 days of DEG dosing before developing AKI, but they did not receive an acute DEG dose as high as those in the single acute dose studies (10 g/kg) [17,22]. However, the repeatedly dosed animals received more DEG overall, in a range of 15–60 g/kg, with median and mean doses to develop AKI being 30 and 33 g/kg. This amount of DEG was given over the span of 3–5 days, in which pharmacokinetic properties would affect the accumulation of DGA into target tissues. In literature, the LD_{50} for a single dose of DEG in rats ranges from 16 to 21 g/kg [34], so this study, albeit with multiple dosing, would represent the higher end of the dose spectrum. Possibly if we increased the dosage, either the amount or the frequency, we might get more consistent toxicity. However, we designed the study with repeated doses to elicit toxicity without overt fatality so as to be able to examine motor function tests. The human toxic dose is reported to be 0.36 g/kg in the Panama study [3] and $1-1.5$ g/kg in the Haiti study [29]. Although these doses are substantially lower than those that produce toxicity in rats (this study and [17,22]), the estimates of amounts ingested in these human epidemics are extremely arbitrary due to the likely inaccurate recall of consumption amounts and frequencies. While the dose-response of DEG-treated animals in the repeat dose paradigm was inconsistent and rats appear to be substantially less dose sensitive to DEG than humans, the kidney toxicity profile is very similar in rat studies and in human cases, indicating that the rat is a useful model for investigating mechanisms of nephrotoxicity and likely neurotoxicity.

CSF protein and DGA brain accumulation

In rats that developed AKI from DEG administration, we observed an increase in total CSF protein, similar to what was observed in human cases in Panama where 17 out of the 19 CSF samples had elevated protein levels [3]. An increase in protein in the CSF, suggestive of a demyelination occurring in the nervous system, is similar in appearance to conditions such as acute inflammatory demyelinating polyradiculoneuropathy or Guillain-Barré syndrome, both of which were included in the differential diagnosis during the 2006 Panama case study [3]. DGA has also been detected in the CSF of 7 out of 8 patient samples that developed neurotoxic effects from DEG-poisoning in the Panama outbreak [4]. This observation, along with the accumulation of DGA in the brain of DEG-treated rats in this study, suggest that an accumulation of the presumed toxic metabolite may play a role in the pathological and functional neurological changes that have been elucidated in this study. The primary proposed mechanism of these neurologic effects, based on nerve conduction studies by Sosa and coworkers, is a severe axonal sensorimotor neuropathy [3]. These symptoms are also comparable with other xenobiotic-induced peripheral neuropathies [3,35]. Some other suggested mechanisms of DEG neurotoxicity include transcellular fluid shifts, membrane destabilization through phospholipid or ion channel effects, acid–base derangements, or osmotic metabolite accumulation within cells [21], as well as alteration of neurofilament

transport or axonal swelling from demyelination [11]. Like in the kidney, DGA was also concentrated by an unknown mechanism into the brain. The presence of brain DGA correlated with the increased CSF protein and the decreased motor function. The scope of this study could not demonstrate the order in which nephrotoxicity and neurotoxicity occur, because rats were tested for neurotoxicity only at the end of the study or after the indications of renal dysfunction were present. Studies in which rats were examined at timed intervals for the presence of nephrotoxicity and neurotoxicity would be necessary to determine if one preceded the other. Nevertheless, these studies did observe that neurotoxicity was only present in rats that also had AKI suggesting a linkage.

Motor function

The results from the nerve conduction studies conducted in the Panama outbreak [3] were used as a guide in designing the battery of motor function tests performed in this study. 21 patients that had notable extremity weakness were selected for these nerve conduction studies, and, unexcitable motor and sensory responses, decreased motor and sensory amplitudes, and prolongation or absence of F waves were recorded in over 40% of these patients [3]. The forelimb grip strength test and rotarod performance test were utilized to evaluate similar parameters in limb function and coordination. The open field test, which assesses overall movement, and the amount of rearing, which assesses the ability of the rat to raise itself up from a plane, were also used for general limb movement and exploratory drive. The decreases in basic forelimb strength and in locomotor function of DEG-treated animals that developed AKI suggest DEG-induced functional neurological impairment in an animal model [3]. The reduction of open-field movement was found to occur in both the center and edge regions of the chamber indicating a lack of region preference and suggesting a generalized motor impairment rather than a reduced motivation. The lack of an effect on the rotarod performance test, a result that differs from the other motor function tests, may relate to this test measuring forced movement, balance and coordination with less relevance to limb function per se. Different brain regions are implicated in regulating balance and locomotion, such as the cerebellum and midbrain respectively, so an observed impairment in spontaneous locomotion may suggest a mechanism involving the alteration in the midbrain region [36,37]. Facial motor weakness, or bilateral "facial drooping" was commonly found in the Panama human studies, but we did not have an animal test to evaluate this neurologic sign. The relationship between AKI-development and motor dysfunction is probably not direct, but it could be an indication that enough DEG has been ingested to saturate the renal system and its ability to clear DGA. With reduced renal clearance, DGA would start accumulating in the CNS to possibly produce neurotoxicity.

Diuresis and acidosis

DEG is a diuretic, so it is expected that rats treated with DEG would show an increase in urine production as long as the kidneys continued to function normally [38]. In this study, animals treated with DEG had an increase in urine output compared to controls. Animals that developed kidney injury eventually showed decreased urine output, after kidney function began to be impaired. In general, AKI animals would be in anuria within 24 h from their last dose. Because DEG is primarily eliminated by excretion in the urine [1], the analysis of urinary DEG was used as a measure to understand its metabolism and correlation with metabolite accumulation. In general, urine DEG concentrations varied between 20 and 40 mg/mL, very high concentrations but expected based on previous studies indicating that urinary excretion of the unchanged form is a major route of elimination ($>$ 50% of the dose) [17]. In the animals treated once every 24 h, there was a consistent peak and trough pattern of DEG concentrations, while in those treated every 12 h, the urinary DEG remained somewhat level throughout. Interestingly, there were only small differences in the urinary DEG levels among the three dose levels. These results would suggest that the urinary elimination had reached its highest limit even by the dose of 4 g/kg, possibly indicating a saturable process [39]. Prior to the development of kidney injury (i.e., $<$ 48 h), there was no difference in DEG elimination between DEG-treated animals that developed kidney injury and those that did not, indicating that injury was not related to a change in renal elimination of DEG.

In DEG poisonings, metabolic acidosis is usually present in both patients and animal studies, often occurring before the later stages of toxicity [1,17]. Direct measures of acidosis, blood bicarbonate and anion gap, confirmed metabolic acidosis in animals that developed kidney injury and were comparable to the acidosis in previous studies. For example, DEG- treated animals with AKI in this study had an average blood bicarbonate of 1.8 mmol/L at 168 h compared to an acute DEG study with 5.6 mmol/L at 48 h (23–25 mmol/L in control rats). Possible explanations for this acidosis include accumulation of acidic metabolites, such as 2-HEAA, which has been implicated in past studies due to elevated concentrations found in the blood [15,17]. Mitochondrial dysfunction induced by DGA could also play a role in metabolic acidosis development, because a decrease in ATP production would increase anions such as lactate in the blood [19,40,41]. Urine pH showed a rapid and sustained decrease in all DEG-treated animals compared to control over the 7-day period. The decrease in urine pH throughout the study was probably related to the excretion of HEAA, since it is the major acid metabolite excreted in the urine [15], with only small amounts of DGA being excreted. This decrease was consistent with reduced urine pH in the high dose group in acute DEG animal studies. However unlike the consistent decrease in urine pH throughout this study, the urine pH of the later (24–36 h) time points in the acute studies recovered to control levels [17,22]. Presumably this difference reflects a time-limited increase in HEAA after a single DEG dose, while the

repeated doses of DEG likely produced a long term increase in HEAA excretion.

Study limitations

One of the main limitations to this study was the high variability in damage response among dose schedules, where the highest-dose group only produced 60% of the animals with AKI. One factor in this variance could be the inherent variability of the outbred Wistar strain. Also, this study was conducted with 9 sets of animals at a range of ages and weights; despite this variation, the mean weights and ages of the dose groups were similar, suggesting that this was not a confounding factor in the final analysis. The variability in the response of rats to DEG toxicity occurred after both single high dose and repeated dose administrations, but the toxicity only occurred in animals that showed tissue DGA accumulation. This latter observation suggests that there are unknown factors in the metabolism and/or transport of the metabolite DGA that vary among animals and that lead to its accumulation in the target tissues (kidney, liver and brain) and hence to toxicity. The inclusion of intermediate time points for renal biomarkers, such as blood collection through an in-dwelling jugular catheter, would have provided more insight to the pharmacokinetics and toxicity of a repeated-dose regimen. While we established that AKI must develop in animals in order to detect neurological changes, motor function tests throughout the study would have given us a chronological picture in correlating renal damage with the subsequent neural effects. Alternatively, animals could be terminated at intermediate time points to observe time-dependent changes in kidney and neurological function. Additionally, the animals could be further dosed beyond the 7-day dosing schedule until an animal develops nephrotoxicity or neurotoxicity. It was observed in the Panama outbreak that protein in the CSF was elevated in 13 patients before the onset of overt neurological signs, so intermediate CSF collection would have allowed us to study CSF protein as a possible early indicator of more severe neurotoxicity [3]. Lastly, the motor function tests conducted were a global measurement of limb movement and coordination. For example, we were not able to observe bilateral facial paresis or "facial drooping" in rats, a specific neurological effect seen in many DEG human cases. While the tests we employed for motor dysfunction are commonly used for these parameters, more sensitive tests such as nerve conduction studies that have been performed in clinical settings would paint a more complete neurotoxicity picture during DEG-administration.

Conclusion

This repeated-dose study of DEG in an animal model recapitulated nephrotoxicity and established neurotoxic effects. Kidney injury biomarkers were markedly increased in DEG-treated animals that developed early signs of decreased urine output and lethargy, confirming that these animals had developed acute kidney injury. Most animals that developed kidney injury were administered a dose every 12 h for at least 3–4 days, which is representative of a typical clinical presentation of DEG-poisonings. DGA accumulated in the kidney tissue of animals at levels comparable to previous acute DEG and DGA studies, but only in the animals that had kidney injury. DGA also accumulated in the brain only in animals that developed AKI and neurological injury. Urine DEG concentrations were generally similar in animals treated with different doses, suggesting a saturation of the renal elimination of DEG. CSF total protein was significantly increased only in animals with kidney injury, corresponding to human case studies. There were marked motor function decreases in animals that developed AKI, including forelimb strength and locomotor movement. We have established this neurotoxicity model of DEG to elucidate mechanisms of neurotoxicity and how this may be related to the primary nephrotoxicity. Overall, this expanded understanding of DEG's toxicity could pave the way for better clinical interventions in patients with DEG poisoning.

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