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Diethylene glycol-induced toxicities show marked threshold dose response in rats

Greg M. Landry^a, Cody L. Dunning^a, Fleurette Abreo^b, Brian Latimer^a, Elysse Orchard^{a,c}, and Kenneth E. McMartin^{a,*}

^aDepartment of Pharmacology, Toxicology, & Neuroscience, Louisiana State University Health Sciences Center, Shreveport, LA, United States

^bDepartment of Pathology, Louisiana State University Health Sciences Center, Shreveport, LA, United States

^cDivision of Animal Resources, Louisiana State University Health Sciences Center, Shreveport, LA, United States

Abstract

Diethylene glycol (DEG) exposure poses risks to human health because of widespread industrial use and accidental exposures from contaminated products. To enhance the understanding of the mechanistic role of metabolites in DEG toxicity, this study used a dose response paradigm to determine a rat model that would best mimic DEG exposure in humans. Wistar and Fischer-344 (F-344) rats were treated by oral gavage with 0, 2, 5, or 10 g/kg DEG and blood, kidney and liver tissues were collected at 48 h. Both rat strains treated with 10 g/kg DEG had equivalent degrees of metabolic acidosis, renal toxicity (increased BUN and creatinine and cortical necrosis) and liver toxicity (increased serum enzyme levels, centrilobular necrosis and severe glycogen depletion). There was no liver or kidney toxicity at the lower DEG doses (2 and 5 g/kg) regardless of strain, demonstrating a steep threshold dose response. Kidney diglycolic acid (DGA), the presumed nephrotoxic metabolite of DEG, was markedly elevated in both rat strains administered 10 g/kg DEG, but no DGA was present at 2 or 5 g/kg, asserting its necessary role in DEG-induced toxicity. These results indicate that mechanistically in order to produce toxicity, metabolism to and significant target organ accumulation of DGA are required and that both strains would be useful for DEG risk assessments.

Keywords

Diglycolic acid; Animal model; Risk assessment; Nephrotoxicity; Hepatotoxicity

*Corresponding author at: Department of Pharmacology, Toxicology, & Neuroscience, Louisiana State University Health Sciences Center, Shreveport, LA, 1501 Kings Highway, Shreveport, LA 71105-3932, United States. Fax: +1 318 675 7857.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

Introduction

Diethylene glycol (DEG; CAS RN 111-46-6) is primarily used as an industrial chemical, but is also found in certain consumer products, such as brake fluid and antifreeze, thereby allowing for possible consumer exposure (Marraffa et al., 2008). DEG has recently been involved in several mass epidemics of renal failure and death world-wide (O'Brien et al., 1998; Schier et al., 2013). DEG poisoning clinically manifests in metabolic acidosis, hepatotoxicity, renal failure, and peripheral neuropathy, with the hallmark being acute renal failure involving proximal tubule cell necrosis and cortical degeneration (Schep et al., 2009). The metabolic pathway for DEG has been elucidated at toxic dose levels in male Wistar rats (Besenhofer et al., 2010, 2011). DEG is metabolized by alcohol and aldehyde dehydrogenases to two primary metabolites, 2-hydroxyethoxyacetic acid (2-HEAA) and diglycolic acid (DGA). This metabolism has been shown to be necessary for the target damage to the kidney (Besenhofer et al., 2010). In addition, DGA is the only metabolite causing necrotic cell death in human proximal tubule cells in vitro (Landry et al., 2011), with no effects being seen with the parent DEG or with 2-HEAA. Supporting this finding, DGA concentrations in kidney tissues of Wistar rats given toxic DEG doses were 100-fold higher than in the blood (Besenhofer et al., 2011). Taken together these results suggest that DEG kidney toxicity is a result of the metabolite, DGA, and not the parent compound.

The dose relationships among the amount of DEG exposure, the DGA accumulation and the resulting toxicity have not been well established. Reports in the literature suggest a difference between the minimal toxic dose of DEG in humans and the apparent toxic dose in rats. Calvery and Klumpp calculated that the smallest lethal dose in adults who ingested the DEG-containing elixir of sulfanilamide during the 1937 Elixir of Sulfanilamide disaster to be approximately 1.1 mL DEG/kg (1.2 g/kg body weight) (Calvery and Klumpp, 1939). O'Brien et al. reported that children in Haiti who presented with acute renal failure from DEG poisoning had an estimated mean ingested dose of 1.34 mL DEG/kg (1.5 g/kg), ranging from 0.2 to 4.4 mL/kg (O'Brien et al., 1998). However, in a similar case in Argentina the estimated lethal dose for humans was between 0.014 and 0.17 g/kg, much lower than what had been previously reported in Haiti and in the U.S. (Ferrari and Giannuzzi, 2005). In the Panama epidemic, the ingested dose to produce renal failure was estimated as 0.36 g/kg (Sosa et al., 2014). Therefore, estimates on the DEG dose associated with lethality in humans vary widely with the minimum value being 0.014 g/kg and the maximum being 1.8 g/kg (Schier et al., 2011). Such variability could result from the usual inter-human variability, but also from the normally poor quality of exposure data in acute human poisonings or from co-exposure to other substances in these poisonings. Nevertheless, these studies suggest that the dose producing renal toxicity in humans is substantially less than the acute dose that produces renal toxicity in rats. For example, a dose of 2 g/kg in Wistar rats produces no toxicity, but would be considered a severely toxic and nearly lethal dose in humans (Besenhofer et al., 2010, 2011; Schier et al., 2011).

One possible explanation for a human-rat species difference could be a rat strain difference in sensitivity to DEG. As one important and related example, male Wistar rats have been shown to have increased sensitivity to ethylene glycol (EG)-induced nephrotoxicity, about double that of male F-344 rats (Cruzan et al., 2004). EG-treated Wistar rats have increased

renal calcium oxalate crystal retention, as well as higher plasma oxalate levels (Li and McMartin, 2009; Corley et al., 2008; Li et al., 2010). In fact, the most recent DEG studies were done in male Wistar rats specifically to maximize the potential for a role of EG (calcium oxalate) in mediating the renal toxicity of DEG. Although these studies demonstrated that metabolite accumulation was necessary for DEG toxicity (Besenhofer et al, 2010, 2011), EG (oxalate) accumulation was minimal and is now considered to be irrelevant for DEG toxicity. Although studies assessing the toxicity, pharmacokinetics, and biotransformation of DEG have been done using a variety of species including dogs, cats, mice, and rats (Winek et al., 1978; Lenk et al., 1989; Freundt and Weis, 1989; Wiener and Richardson, 1989; Mathews et al., 1991; Durand et al., 1976; Hebert et al., 1978; Harris, 1949), whether there is a sensitivity difference between Wistar and F-344 rats in renal toxicity or in DGA accumulation is yet unknown. Hence, this study was primarily designed to provide insight into an appropriate rat model by using a DEG dose response paradigm (0, 2, 5, or 10 g/kg) that covers the range of no to severe toxicity in Wistar rats (Besenhofer et al., 2010). In addition, the study provides key mechanistic insight by relating the magnitude of DGA tissue retention to the presence of toxic effects. The study provides information about rat strain differences to assist future risk assessments regarding DEG exposure and toxicity in humans.

Materials and methods

Materials

DEG for gavage was provided by Shell Chemical LP (Houston TX) and analyzed for purity by gas chromatography (GC). The DEG contained DEG (99.78%), EG (0.05%), and triethylene glycol (0.08%).

Animal protocol for strain comparison studies in vivo

Male Wistar and Fischer-344 rats (Harlan, Indianapolis, IN) were each randomly placed into one of four treatment groups with four rats per group per strain, including a 0 g/kg control group, which received water by gavage, and three treatment groups, which received a single dose of either 2 g/kg, 5 g/kg, or 10 g/kg DEG by gavage. The overall study was conducted as a sum of two separate experiments in which rats of both strains at about 12 wk of age were used in one phase and at about 22 wk of age in the other phase. In each phase, all doses and both strains were utilized—no apparent difference in response at the various doses between the two ages was noted and therefore results were pooled to diminish any variance. At 6 h, one 2 g/kg Wistar rat expired due to gavage trauma bringing the 2 g/kg Wistar group to an *n* of 3 instead of 4.

All rats were fasted with free access to water for 12 h prior to gavage administration. Following gavage at time 0, animals were housed in metabolic cages for 48 h for urine collection. Throughout the time course of the experiment, animals were monitored for behavioral signs indicative of morbidity, such as decreased food or water intake or decreased response to stimuli. Standard conditions of humidity, temperature ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$), and light (12:12 h light–dark) were maintained in the animal room, and all rats were allowed free access to food (normal rat chow) and water after dosing.

Urine collection and analysis

Urine was collected in iced tubes at timed intervals up to 48 h. Metabolic cages were rinsed with water between collections. Immediately after collection, the urine samples were vortexed and the volume and pH were recorded. The urine was allowed to settle for 30 min on ice, and then one to two 1 mL aliquots of clean urine were transferred to microtubes and stored at -80°C until needed.

Blood collection and analysis

At 48 h, the animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and blood was drawn from the inferior vena cava into heparinized tubes. Heparinized whole blood was analyzed for pH, pCO_2 , and pO_2 by a blood gas analyzer, which also calculated blood bicarbonate concentrations. The remaining blood was transferred to separator tubes (BD, Franklin Lakes, NJ) to isolate plasma. Plasmas were analyzed for a basic metabolic panel, including markers of renal function (urea nitrogen [BUN] and creatinine), liver function (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]), glucose, and electrolytes (sodium [Na^+], potassium [K^+], chloride [Cl^-], and calcium [Ca^{2+}] by the Louisiana State University Health Sciences Center-Shreveport Clinical Laboratory.

Determination of DGA concentrations in kidney tissues

Kidney tissue was analyzed for DGA content by HPLC by adapting a method initially developed for plasma citric acid levels (Gu et al., 2008). Samples of kidney tissue (~0.2 g) were homogenized in 800 μL of 100 mmol/L potassium phosphate buffer, pH 7.4, containing 1.15% KCl. To a 190 μL aliquot of homogenate, 10 μL of sodium citrate (80 mmol/L in water) was added as internal standard. The homogenates were deproteinized using 200 μL of perchloric acid (15%). The resulting supernatant was first filtered through 0.5 mL centrifugal filter units (10 kDa MW cutoff, Millipore) and subjected to a two-step solid phase extraction (SPE) protocol to remove interfering peaks. 400 μL of filtered supernatant was applied to the donor side of a C18 SPE column (Varian, Palo Alto, CA) that had been conditioned with methanol and 0.02 M sulfuric acid. The sample was eluted by centrifugation and the resulting eluent was then applied to an SAX SPE column (Varian) that had been conditioned with methanol and 8 mmol/L sulfuric acid. The second column was washed once with distilled water, and then DGA and citrate were eluted in 1 mL of 8 mmol/L sulfuric acid. HPLC separation (50 μL injection) was performed on a Phalanx C18 5 μm analytical column (250 mm $\times$ 4.6 mm, Higgins Analytical) with a C18 guard column (Supelco). The mobile phase consisted of 20 mmol/L sulfuric acid (pH 2.0, using 1 mol/L ammonia) containing 1% acetonitrile and was pumped at a flow rate of 1 mL/min for 18.5 min. DGA and citrate were detected at a wavelength of 210 nm and displayed retention times of 8.2 and 11.3 min, respectively. Data analysis and chromatogram processing was performed by Beckman Gold software (version 8.10). The limit of quantitation of DGA by this method was 1.05 $\mu\text{mol/g}$.

Histology studies

At 48 h, kidneys and whole liver were collected and weighed for further histopathological analysis. For each tissue, a 1 mm slice was fixed in 10% neutral buffered formalin. Four

micrometer sections were cut, embedded, and stained (hematoxylin and eosin) by the LSUHSC-S Department of Cell Biology and Anatomy. Tissues were examined with light microscopy by the LSUHSC-S Department of Pathology to visualize early pathophysiological changes as well as necrosis and/or apoptosis. The observer (F.A.) was blinded as to the animal treatment. Additionally, 4 μm sections of formalin-fixed liver tissue were cut and stained with periodic acid-Schiff (PAS), and examined with light microscopy to detect for glycogen depletion (Myers et al., 2008).

Statistics

Values in the text represent the group mean value $\pm$ SEM. All analyses were performed using Graphpad Prism 5 for Windows. Tests were considered significant if $p < 0.05$.

Results

Animal observations

Animals did not exhibit any gross behavioral signs of morbidity throughout the 48 h post-dosing period. However by 36 h, the 10 g/kg DEG-treated rats in both strains were oliguric (< 2 mL in 12 h) (Fig. 1A and B). At 48 h, rats were anesthetized for blood collection and tissue procurement.

Metabolic acidosis

Metabolic acidosis was assessed using the parameters of blood pH, bicarbonate, CO_2 and the calculated anion gap (Table 1). At 48 h, blood pH, bicarbonate, and CO_2 in the 10 g/kg dose groups were significantly decreased from 0 g/kg controls without a difference between the strains. In contrast, there was no decrease in these parameters at the 2 and 5 g/kg doses in either strain (Table 1). Plasma was also analyzed for electrolyte concentrations (sodium, potassium, and chloride) and no parameters were altered by DEG in either strain or at any of the doses (data not shown). The anion gap for each rat was then calculated using the formula, $([\text{Na}^+ + \text{K}^+] - [\text{Cl}^- + \text{HCO}_3^-])$. DEG-treated rats showed a two-fold significant increase ($p < 0.05$) in the anion gap at the highest dose, with no difference between strains. No increases in anion gap were observed at the 2 and 5 g/kg doses (Table 1). Treatment with DEG did not affect plasma calcium or glucose concentrations nor did it statistically change lactate levels (data not shown). Urine pH in DEG-treated Wistar and F-344 rats was significantly lower from 6 to 36 h (Fig. 1C) and from 6 to 24 h (Fig. 1D), respectively in the 10 g/kg dose groups compared to controls. From 6 to 24 h, there was no difference in urine pH between strains. Wistar rat urine pH in the 2 and 5 g/kg dose groups was significantly decreased at 6, 12, and 24 h and in the 5 g/kg group at 36 h (Fig. 1C). This contrasts to the F-344 rat urine pH, whereby the only significant decrease was in the 10 g/kg treated animals (Fig. 1D). Urine pH in the 2 and 5 g/kg dose groups returned to normal by 48 h. Urine pH was not measureable at 48 h in the 10 g/kg DEG-treated rats due to the oliguria or anuria.

Renal injury

Since DEG has been shown to have dose-dependent diuretic effects (Lenk et al., 1989), urine volumes were measured from both rat strains at each time point. Wistar rats showed

significant diuresis at 6 and 12 h after 10 g/kg DEG and at 6 h in the 5 g/kg dose group (Fig. 1A). F-344 rats showed significant diuresis at 6 and 12 h after both 5 and 10 g/kg DEG, with animals in the 10 g/kg dose group having significant diuresis until 24 h (Fig. 1B). Both strains receiving 10 g/kg DEG were oliguric by 36 h (significantly lower urine volumes than controls) and had become an-uric by 48 h. Additionally at the high dose only, DEG-treated F-344 and Wistar rat kidneys appeared large, swollen, and weighed substantially more than the control kidneys.

Renal injury was assessed primarily using BUN and creatinine levels as well as kidney to body weight ratios. At 48 h after dosing, both BUN (Fig. 2A) and creatinine (Fig. 2B) were significantly increased in 10 g/kg DEG-treated rats compared to controls, with no difference between strains. No increases were observed at the 2 or 5 g/kg doses in either strain. In addition, both rat strains treated with 10 g/kg DEG, but not those at lower doses, had significantly higher kidney to body weight ratios than control rats (Fig. 2C).

DGA levels in the kidney

Kidney levels of DGA, the nephrotoxic metabolite of DEG, were determined in tissue samples of both Wistar and F-344 rats collected at 48 h. An increase in the kidney levels of DGA was detected only in the 10 g/kg-treated animals and observed in both strains (Fig. 3). If 1 g of kidney tissue is approximately equivalent to 1 mL, then the Wistar kidney DGA concentrations were approximately 17 mmol/L, and F-344 concentrations were approximately 10 mmol/L. Both strains had undetectable DGA tissue concentrations in the 2 and 5 g/kg DEG dose groups.

Liver injury

To assess liver injury, AST and ALT levels were measured in the plasma of control and DEG treated animals. At 48 h, AST and ALT levels were significantly elevated in both rat strains, but only in animals treated with 10 g/kg DEG, with no elevation in animals treated with 2 or 5 g/kg (Table 2). There was no difference between strains. Other markers of liver injury including albumin, alkaline phosphatase, and bilirubin were not different between the treatment groups or strains (data not shown). Liver to body weight ratios were not significantly increased in any of the DEG dose groups regardless of elevated plasma enzymes, strain, or DEG dose (Table 2).

Kidney and liver histology

Major renal pathological abnormalities in both rat strains treated with 10 g/kg DEG consisted of marked vacuolar degeneration of the proximal convoluted tubules with multifocal necrotic cell death and destruction of the majority of the proximal convoluted tubule cellular architecture (Fig. 4 and Table 3). Renal histology at the lower DEG doses (2 and 5 g/kg) showed minor changes including focal vacuolization and tubular simplification, whereby the tubular lumen appeared wider in only a few localized areas; similar effects were noted in controls (Fig. 4, Table 3). At the lower DEG doses, there was no strain difference and no necrosis noted. DEG produced marked renal necrotic damage only at the 10 g/kg dose regardless of rat strain, with only minor, non-necrotic effects at 2 and 5 g/kg, indicating

a threshold dose effect as opposed to a linear, no threshold increase in DEG-induced renal injury (Table 3).

Liver injury at the low doses in both strains was characterized by minor cytoplasmic reticulation coalescing to form minor microvesicular fatty changes; similar effects were also seen in a majority of the control rats from both strains (Fig. 5 and Table 3). However, both rat strains treated with 10 g/kg DEG showed marked hepatocellular changes characterized as substantial vacuolization and edema with centrilobular necrosis (Table 3). Vascular congestion was noted in all groups and in both rat strains. A threshold dose effect was thus observed with DEG-induced liver injury because vacuolization and necrosis were not noted until 10 g/kg.

To determine whether DEG exposure produces glycogen depletion, liver slices from DEG-treated animals were stained using periodic acid-Schiff staining to detect for the presence of glycogen (Fig. 6). The results showed little to no glycogen depletion in either rat strain at the lower doses of DEG (2 and 5 g/kg) (Fig. 6 and Table 4). Severe and marked glycogen depletion occurred in the livers of both rat strains treated with 10 g/kg DEG when compared to control animals (Fig. 6).

Discussion

Our previous studies have characterized the toxicity of DEG in Wistar rats and have shown the key role of metabolism in eliciting the toxicity. The present study has advanced beyond those findings by showing the strong threshold dose response in both Wistar and F-344 rats, where DEG toxicity was seen only in the 10 g/kg dose groups and no adverse effects were noted at 5 g/kg. This steep threshold was observed for the kidney toxicity, the liver toxicity and the metabolic acidosis. In addition, these results add to existing mechanistic information regarding DEG toxicity by showing that metabolism to and target organ accumulation of DGA is required to produce toxicity—DGA was detected in the kidneys of 10 g/kg-treated animals and only these animals showed significant renal toxicity in terms of metabolic parameters and histology. Overall this study has concluded that both strains would be appropriate models to conduct DEG mechanistic studies or risk assessments.

DEG toxicity is characterized by severe damage to the kidneys, specifically proximal tubule cell necrosis and cortical degeneration, and by moderate damage to the liver, characterized by mild steatosis (Besenhofer et al., 2010; Schep et al., 2009). Most studies investigating DEG-induced target organ toxicity have been completed in three rat strains, Wistar, F-344, and Sprague Dawley, but no direct inter-strain comparisons have been done. Most recent studies have used the Wistar strain because of the known strain difference in ethylene glycol (EG) toxicity (Cruzan et al., 2004), and the historical idea that the renal damage from DEG might result from the metabolism to EG, which then would produce the renal damage. This rat strain difference was initially demonstrated with a chronic low-dose EG administration, where F344 rats required twice as much EG to elicit renal damage (Cruzan et al., 2004) and where EG exposure produced calcium oxalate monohydrate (COM) crystal retention only in Wistar rats (Li et al., 2010). Although recent studies have shown that EG and oxalate do not have significant roles in the renal damage from DEG (Besenhofer et al., 2010; Besenhofer et

al., 2011), the present studies were needed to elucidate if a strain difference also exists with DEG toxicity. In contrast to the situation with EG, both strains appeared equally sensitive to the kidney damage produced by DEG, with toxic effects observed only in the 10 g/kg dose group, as shown by similar increases in the renal parameters (BUN, creatinine, and kidney to body weight ratios). In addition, histopathology revealed marked vacuolar degeneration of the proximal convoluted tubules and necrotic cell death in the kidneys of both 10 g/kg DEG-treated rat strains and the damage appeared to be of equal severity. At the 2 and 5 g/kg doses, no changes in renal function were noted and only minor increases in vacuolization were present in both strains; the minor pathology noted in these groups did not approach the magnitude of vacuolar degeneration observed in the 10 g/kg DEG animals.

Previous studies have suggested that the nephrotoxic metabolite of DEG is DGA, which leads in vitro to ATP depletion, reactive oxygen species production, succinate dehydrogenase inhibition, and ultimately necrotic proximal tubule cell death (Landry et al., 2011, 2013). Also, DGA is markedly accumulated in liver and kidney, as compared to the plasma DGA levels, of DEG-intoxicated rats (Besenhofer et al., 2011). The importance of DGA in the toxicity of DEG has recently been confirmed in human cases in the Panama epidemic, where the presence of elevated DGA concentrations in the serum and urine had the strongest association with case status (odds ratio > 999), compared to DEG and its other possible metabolites (2-HEAA, oxalate, glycolate, EG); also, the absolute concentrations of DGA in the serum and urine were markedly higher than any other metabolite (Schier et al., 2013). The present study provides convincing evidence that the key step in the mechanism of toxicity of DEG is the formation of DGA and its retention in tissues. As noted in this dose-response assessment, only rats in the high dose group showed an increase in kidney tissue DGA content and this same group was the only dose group that showed marked tissue damage and diminished renal function. The kidney DGA levels in this study were roughly 10–15 times higher than the limit of quantitation (Fig. 3) and were higher than the mean concentration (~5 mmol/L) reported previously in Wistar rats at the 10 g/kg dose (Besenhofer et al., 2011). In that experiment, there were animals that reached a kidney DGA concentration of ~13 mmol/L, similar to what is reported in this study. Although kidney DGA levels looked higher in Wistar rats than in F-344 rats at 10 g/kg, these differences were not statistically significant, suggesting that Wistar and F-344 rats similarly metabolize DEG to DGA.

Metabolic acidosis is one of the major characteristics of DEG poisoning in humans and animals (Alfred et al., 2005; Hebert et al., 1978; Heilmar et al., 1993), as noted by an increased anion gap and decreased blood bicarbonate and pH levels. 2-HEAA has been shown to be the acid metabolite responsible for inducing the metabolic acidosis (Besenhofer et al., 2010, 2011). Blood lactate concentrations were not measured in that study, but there was no increase in lactate in the present study indicating the minimal role of lactic acidosis in the overall acidosis. Our results show that Wistar and F-344 rat strains had similarly decreased blood pH and bicarbonate as well as similar anion gaps, only at the 10 g/kg dose, indicating the same degree of metabolic acidosis in both strains. Because none of these parameters were changed at the 2 and 5 g/kg doses, these data support a marked accumulation of an acidic species in the blood at the 10 g/kg DEG dose, most likely 2-HEAA (Besenhofer et al., 2011). Although there were no differences in blood parameters

related to acidosis between strains, there were minor differences between strains in the changes in urine pH. For example, Wistar rats showed significant decreases in urine pH up to 24 h at the 2 and 5 g/kg doses, with recovery by 48 h, whereas in F-344 rats, urine pH values in the 2 and 5 g/kg dose groups were not statistically different from controls. Additionally, urine pH was significantly decreased from 6 to 36 h in Wistar rats treated with 10 g/kg DEG, whereas in F-344 rats urine pH was decreased only from 6 to 24 h. A presumable explanation is that there is a larger amount of an acidic metabolite excreted in the urine of Wistar rats after DEG doses, even though there is no difference in blood metabolite levels between Wistar and F-344 rats. These results would appear to indicate a somewhat greater excretion of 2-HEAA at the lower doses and for longer periods in Wistar rats, but this would need to be confirmed analytically.

DEG has been shown to produce osmotic diuresis in laboratory animals (Lenk et al., 1989; Besenhofer et al., 2010) and in humans (Calvery and Klumpp, 1939), with a linear relationship between DEG dose and volume of urine produced (Lenk et al., 1989). In our studies, both strains exhibited similarly increased urine volumes after DEG administration at the 5 and 10 g/kg doses with diuresis in both strains peaking at roughly 3-fold higher than controls at 6–12 h; urine volume was not increased at 2 g/kg in either strain. The general pattern of urine production was similar in the two strains. DEG induced diuresis is most likely due to its hygroscopic nature (Laug et al., 1939), and DEG functions like many other alcohols producing diuretic effects upon initial consumption.

In addition to severe renal injury, DEG-induced toxic effects on the liver have also been observed (Schep et al., 2009). Pathology reports in this study showed that there was slight cytoplasmic reticulation in both rat strains at 0, 2, and 5 g/kg DEG doses. Minor cytoplasmic reticulation and fatty changes observed in control rats indicate that a level of fatty change and reticulation is normal in both F-344 and Wistar rat strains. Liver pathology appeared to dramatically progress to cytoplasmic vacuolization and centrilobular necrosis at the 10 g/kg DEG dose, with equal severity in both strains. Additionally, AST and ALT levels were significantly elevated to the same magnitude (6-fold for AST and 2-fold for ALT) in both strains at the 10 g/kg dose, with no increase at the lower doses in either strain. These data support the hypothesis of a threshold between the 5 and 10 g/kg doses for hepatotoxicity.

Periodic-acid Schiff staining revealed a dramatic loss of hepatic glycogen as the DEG dose increased past 5 g/kg, with severe glycogen depletion at 10 g/kg DEG regardless of strain, and essentially no depletion at 2 and 5 g/kg DEG. Histological examination of livers from acetaminophen-treated animals, as well as from starved animals, reveals a substantial glycogen depletion that results from severe glutathione (GSH) depletion (Hinson et al., 1983). Glycogen depletion occurred initially in the centrilobular region and progressed outward, results similar to that observed in high dose DEG intoxication in our studies. Severe glycogen depletion has also been reported in livers of animals administered GSH-depleting agents, where the GSH deficiency induces glycogen breakdown by effectively altering the GSH/GSSG ratio to then stimulate glycogenolysis (Braun et al., 1996). It is likely that the severe glycogen depletion seen in animals administered 10 g/kg DEG is a result of marked GSH depletion related to the capability of DGA to induce significant increases in reactive oxygen species leading to marked alterations in cellular redox status

(Landry et al., 2013). The increased glycogenolysis and subsequent glycogen breakdown would be expected to mobilize glucose, thereby increasing circulating blood glucose levels. However, glycogenolysis-induced increases in blood glucose levels peak at approximately 2.5 h, and regress back to normal levels by 4 h, when maximum glycogen depletion is observed histologically (Hinson et al., 1983). In previous DEG studies, blood glucose levels were not initially measured until 4 h, and then were not elevated (Besenhofer et al., 2010). In the current study, blood was collected at termination (48 h) to provide endpoint metabolic data, and showed no significant hyper-glycemia (data not shown). Therefore, increases in blood glucose likely could have peaked early after DEG administration, but were undetectable at 4 h and 48 h, even with severe glycogen depletion being detectable histologically at 48 h post-termination.

In conclusion, this study has added important new information regarding the mechanisms involved in DEG toxicity. These experiments have demonstrated that DEG-induced kidney and liver toxicity and metabolic acidosis depict a steep threshold dose response; whereby, essentially no toxicity was observed at the lower 2 and 5 g/kg doses, but toxicity was substantial and severe at 10 g/kg dose. The threshold response is likely explained by the data showing that DGA was not detected in the kidney tissue of DEG-treated animals until the dose reached 10 g/kg, which perfectly mirrored the dose at which indications of toxicity were noted. Thus, these results support the mechanistic findings that DGA is the nephrotoxic metabolite of DEG and that it causes the same degree of renal damage regardless of rat strain. Also, this study has shown that 10 g/kg DEG-induced liver damage manifested as severe glycogen depletion and centrilobular necrosis, and that it also followed a threshold response, much like the kidney toxicity, regardless of rat strain. As such, these studies clearly demonstrated that Wistar and F-344 rats show equal sensitivity to DEG-induced toxicity, so either strain could be used in mechanistic studies or for health risk assessments related to DEG.

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List of Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
BUN	blood urea nitrogen
DEG	diethylene glycol

DGA	diglycolic acid
EG	ethylene glycol
HEAA	hydroxyethoxyacetic acid
GSH	glutathione

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Fig. 1.

Urine volume is increased in both F-344 and Wistar rat strains at 5 and 10 g/kg DEG doses (A and B). Dose and strain-dependent decreases in urine pH in DEG-treated Wistar and F-344 rats (C and D). Data are represented as means $\pm$ SEM ($n = 3$ for Wistar (2 g/kg); $n = 4$ for all other groups). Asterisk (*) indicates significant difference from control as determined by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$.

Fig. 2.

Treatment with DEG at 10 g/kg, but not at 2 or 5 g/kg, produces kidney injury at 48 h in both Wistar and F-344 rats as assessed by blood urea nitrogen (BUN) (A), plasma creatinine (B), and kidney to body weight ratios (C). Data are represented as means $\pm$ SEM ($n = 3$ for Wistar (2 g/kg); $n = 4$ for all other groups). Asterisk (*) indicates significant difference from control as determined by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$. There were no significant differences between the strains.

Fig. 3.

DGA concentrations at 48 h in kidney tissue of Wistar and F-344 rats treated with DEG show no significant differences between the strains. Data are represented as means $\pm$ SEM ($n = 3$ for Wistar (2 g/kg); $n = 4$ for all other groups). The dotted line indicates the limit of quantitation for the DGA method. Asterisk (*) indicates significant difference from control as determined by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$.

Fig. 4. Representative hematoxylin and eosin images at 48 h of cortical renal tissue from both Wistar and F-344 rat strains for each of the treatment groups show little to no damage for rats treated with 0, 2 or 5 g/kg DEG, but severe kidney injury for rats treated with 10 g/kg DEG. Magnification $\times 100$ for all images.

Fig. 5. Representative hematoxylin and eosin images at 48 h of liver tissue from both Wistar and F-344 rat strains for each of the treatment groups show little to no damage for rats treated with 0, 2 or 5 g/kg DEG, but substantial liver injury for rats treated with 10 g/kg DEG. Magnification $\times 100$ for all images.

Fig. 6.

Histochemistry images for periodic acid-Schiff/hematoxylin staining from representative animals. Both Wistar and F-344 rats show normal liver glycogen staining in the 0, 2, and 5 g/kg DEG dose groups. 10 g/kg DEG causes severe liver glycogen depletion in both strains by 48 h. Magnification was 100 $\times$ for all images.

Table 1

Markers of metabolic acidosis.

Parameter	F-344	Wistar
CO ₂ (mmol/L)		
0 g/kg	25.2 ± 0.6	24.8 ± 1.9
2 g/kg	25.2 ± 1.8	24.3 ± 1.8
5 g/kg	25.8 ± 0.8	25.0 ± 1.2
10 g/kg	4.2 ± 0.8*	2.1 ± 2.0*
Blood pH		
0 g/kg	7.33 ± 0.05	7.35 ± 0.03
2 g/kg	7.33 ± 0.02	7.37 ± 0.01
5 g/kg	7.35 ± 0.02	7.36 ± 0.01
10 g/kg	6.92 ± 0.05*	6.81 ± 0.11*
Bicarbonate (mmol/L)		
0 g/kg	23.5 ± 1.6	23.2 ± 0.7
2 g/kg	24.4 ± 0.6	23.8 ± 0.7
5 g/kg	25.0 ± 0.7	25.7 ± 0.6
10 g/kg	6.8 ± 0.7*	5.6 ± 1.8*
Anion gap		
0 g/kg	23.8 ± 2.7	22.5 ± 0.3
2 g/kg	23.7 ± 1.8	20.6 ± 0.8
5 g/kg	21.4 ± 1.0	19.9 ± 1.6
10 g/kg	47.5 ± 2.5*	47.8 ± 2.4*

Parameters determined from blood collected at 48 h post-treatment. Data are represented as means ± SEM ($n = 3$ for Wistar (2 g/kg); $n = 4$ for all other groups). Asterisk (*) indicates significant difference from 0 g/kg control as determined by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$. There were no significant differences between strains.

Table 2

Markers of liver injury.

Parameter	F-344	Wistar
AST (U/L)		
0 g/kg	96.0 ± 19.0	95.2 ± 18.5
2 g/kg	88.2 ± 15.3	57.7 ± 29.2
5 g/kg	77.8 ± 8.0	100.8 ± 5.7
10 g/kg	617.5 ± 39.7*	589.2 ± 78.8*
ALT (U/L)		
0 g/kg	73.2 ± 23.2	56.8 ± 10.4
2 g/kg	47.2 ± 3.9	48.0 ± 3.6
5 g/kg	52.8 ± 2.0	48.0 ± 5.6
10 g/kg	155.8 ± 14.6*	206.8 ± 89.0*
Liver:BW ratio		
0 g/kg	0.0336 ± 0.0020	0.0357 ± 0.0048
2 g/kg	0.0335 ± 0.0024	0.0336 ± 0.0016
5 g/kg	0.0354 ± 0.0021	0.0396 ± 0.0041
10 g/kg	0.0347 ± 0.0009	0.0403 ± 0.0028

Parameters determined from samples collected at 48 h post-treatment. Data are represented as means ± SEM ($n = 3$ for Wistar (2 g/kg); $n = 4$ for all other groups). Asterisk (*) indicates significant difference from 0 g/kg control as determined by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$. There were no differences between strains or in other markers of liver function including total bilirubin and alkaline phosphatase.

Table 3

Kidney and liver tissue injury in DEG treated rats.

Data are represented as number of rats assigned to each of the above categories out of the total number of rats from each strain ($n = 3$ for Wistar (2 g/kg); $n = 4$ for all other groups); PCT, proximal convoluted tubule.

Kidney			Liver									
Focal vacuolization and tubular simplification			Marked PCT vacuolar degeneration		PCT necrosis		Cytoplasmic reticulation		Cytoplasmic vacuolization		Centrilobular necrosis	
Rat strain:	W	F	W	F	W	F	W	F	W	F	W	F
Treatment group												
0 g/kg	1	3	0	0	0	0	3	3	0	0	0	0
2 g/kg	1	4	0	0	0	0	1	4	0	0	0	0
5 g/kg	1	4	0	0	0	0	3	3	0	0	0	0
10 g/kg	0	0	4	4	4	4	0	1	3	3	4	4

Table 4

Periodic acid-Schiff staining for liver glycogen detection in DEG treated rats.

Rat strain:	Wistar	F-344	Wistar	F-344
Treatment group	++++		0	
0 g/kg	4	4	0	0
2 g/kg	3	4	0	0
5 g/kg	4	4	0	0
10 g/kg	0	0	4	4

Data are represented as number of rats from each strain assigned to 1 of 2 following categories: (++++) normal glycogen staining or (0) no glycogen staining with $n = 3$ for Wistar (2 g/kg) and $n = 4$ for all other groups.