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

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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.

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

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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 at 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 °C ± 2 °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 × 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 μmol/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).