Comparison of diglycolic acid exposure to human proximal tubule cells in vitro and rat kidneys in vivo

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ABSTRACT

Diglycolic acid (DGA) is present in trace amounts in our food supply and is classified as an indirect food additive linked with the primary GRAS food additive carboxymethyl cellulose (CMC). Carboxymethyl starches are used as a filler/binder excipient in dietary supplement tablets and a thickening ingredient in many other processed foods. We sought to utilize the human proximal tubule HK-2 cell line as an in vitro cellular model system to evaluate its acute nephrotoxicity of DGA. We found that DGA was indeed toxic to HK-2 cells in all in vitro assays in our study, including a highly sensitive Luminex assay that measures levels of an in vitro biomarker of kidney-specific toxicity, Kidney Injury Molecule 1 (KIM-1). Interestingly, in vitro KIM-1 levels also correlated with in vivo KIM-1 levels in urine collected from rats treated with DGA by daily oral gavage. The use of in vitro and in vivo models towards understanding the effectiveness of an established in vitro system to predict in vivo outcomes would be particularly useful in rapidly screening compounds that are suspected to be unsafe to consumers. The merit of the HK-2 cell model in predicting human toxicity and accelerating the process of food toxicant screening would be especially important for regulatory purposes. Overall, our study not only revealed the value of HK-2 in vitro cell model for nephrotoxicity evaluation, but also uncovered some of the mechanistic aspects of the human proximal tubule injury that DGA may cause.

1. Introduction

Diglycolic acid (DGA) is present in the U.S. food supply as an indirect additive from the use of carboxymethyl cellulose (CMC). CMCs are used as binding agents in dietary supplement tablets and thickeners in other processed food products [1]. Typical food products found to contain DGA include frozen dairy products, cake baking mixes, syrups, and glazes. It is the chemical process of CMC synthesis in which condensing glycolic acid with monochloroacetic acid produces DGA as a reaction byproduct [2]. DGA remains in the final CMC reaction product as an impurity that cannot be easily removed [3].

The DGA exposure level to consumers is currently unknown. Actual data on exposure levels are lacking and will require comprehensive analytical studies using large sample sizes of numerous types and brands of food products. DGA is suspected to be a renal toxicant based on the toxicity of diethylene glycol (DEG) and its metabolism into DGA [4,5]. Reports by others [6,7] have also indicated that DGA is a renal toxicant based on in vitro and rat in vivo research with diethylene glycol, whose metabolism in vivo generates DGA. Research by Landry et al. [7] has indicated that inhibition of a key citric acid cycle enzyme, succinate dehydrogenase, can occur, along with a decrease in cellular respiration and an increase in reactive oxygen species (ROS) following DGA exposure in human proximal tubule cells. Excess ROS levels can lead to a number of kidney-related pathologies, including DNA damage, protein modification, and lipid peroxidation [8–10]. A single case report of accidental ingestion of DGA by a human also demonstrated the potency of DGA in causing kidney damage [11].

Given the potential for DGA to induce kidney damage, we sought to carry out two main goals. First, to evaluate the effect of its direct exposure of human kidney cells using the in vitro model cell line HK-2. This cell line carries many phenotypic and functional characteristics of primary renal cells and has been shown to be a useful model for in vitro evaluation of several renal toxicants [12–14]. We used several in vitro techniques to capture multiple facets of DGA-induced nephrotoxicity.
including both cellular and mitochondrial toxicity, as well as Luminex detection of the FDA-approved biomarker of nephrotoxicity Kidney Injury Molecule-1 (KIM-1) [15]. Expression of KIM-1 has been shown to become the most highly upregulated protein in settings where proximal tubule cells are injured. This biomarker may also play a role in the regeneration of proximal tubules following injury [16].

As our second goal, we sought to determine whether our in vitro model correlated with a rat in vivo model of DGA toxicology. As a subset of a recent in vivo study [17], we measured the same kidney-specific biomarker measured in the HK-2 system in the urine of rats exposed to DGA. Our in vitro and in vivo findings yielded strikingly similar findings. Extending our in vitro assessments to the in vivo setting is a highly valuable to the FDA and to industry. Understanding the value of in vitro modeling to predict in vivo outcomes for future compounds of interest can help accelerate the pace of toxicology research.

2. Materials and methods

2.1. Cell culture and treatments

HK-2 cells were grown in keratinocyte-SFM media supplemented with 5% FBS, recombinant human EGF, and bovine pituitary extract (Invitrogen, Carlsbad, CA) at 37 °C in a 5% CO₂ humidified atmosphere, following similar culture conditions to when this cell line was established [18]. Viable cells were counted by Trypan blue dye (Invitrogen) exclusion via hemocytometer and seeded at a density of 2 × 10⁴ cells per 100 ul per well in 96-well plates (Corning, New York, NY). Per- phrotoxicant (positive control) treatment solutions of Diglycolic acid (Sigma, St. Louis, MO), nephroprotectant (negative control) valproic acid (Sigma) were made by weighing out their powders, dissolving them in cell culture media and diluting this mixture with media to achieve serial dilutions ranging from 0 mM to 10 mM. Chemically treated cells were incubated in triplicate for 24 h.

2.2. Cytotoxicity assay

CellTiter-Glo Cell Viability Assay (Promega, Madison, WI) was used to determine the cytotoxicity of DGA relative to cisplatin and valproic acid. This luminescence-based method establishes cellular levels of ATP, which is directly proportional to cell viability. Following manufacturer’s instructions, cells were treated in black-wall, clear bottom 96-well plates and when ready for the assay, plates were equilibrated to room temperature for 30 min, during which time water in the outer wells were replaced with 100 ul of treatment or media only controls. CellTiter-Glo working solution (100 ul) was added to each well. Plates were put on an orbital shaker for 2 min to induce cell lysis and then incubated for an additional 10 min before being read on an OMG Fluorostar Omega plate reader (BMG LABTECH, Ortenberg, Germany) to determine the levels of luminescence emitted from each well.

2.3. Reactive oxygen species assay

The levels of reactive oxygen species (ROS) were determined using the ROS-Glo H₂O₂ luminescence-based detection system (Promega) and data were normalized to cell viability. Cells treated with DGA, cisplatin, or valproic acid were incubated with H₂O₂ substrate for the remaining 5 h of their 24-h treatment at 37 °C in a 5% CO₂ humidified atmosphere. The Detection Reagent was added and samples were incubated at room temperature for at least 20 min. Luminescence was read on an BMG Fluorostar Omega plate reader.

2.4. Mitochondrial membrane potential assay

The ratiometric dye JC-10 (Enzo, Farmingdale, NY) was utilized to evaluate changes in mitochondrial membrane potential (MMP). A stock solution of 400 uM JC-10 was made by mixing JC-10 lyophilized powder with 1 ml of HBSS. A working solution of JC-10 was then made by diluting the stock solution by 20-fold for a final concentration of 20 uM. Treated cells were labeled with dye for 2 h, washed twice in HBSS and then overlaid with 100 ul of HBSS. Plates were read using an BMG Fluorostar Omega plate reader to measure emission at 520 and 590 nm following excitation at 485 nm. Background auto-fluorescence levels from treatments or media alone were insignificant.

2.5. Luminex biomarker assay

Culture supernatants from cells treated in vitro for 24 h with DGA, cisplatin, and valproic acid at doses of 0–10 mM were evaluated for levels of Kidney Injury-1 (KIM-1) using the Human Kidney Toxicity kit (Bio-Rad, Hercules, CA). For comparison, urine samples from an in vivo study [17] were also tested for KIM-1 levels. All animals from which urine samples were collected were maintained and handled under IA- CUC-approved protocols. As described in detail in Sprando et al. [17], urine samples were collected from female Sprague Dawley rats treated daily by oral gavage with DGA at doses of 0, 0.3, 1.0, 3.0, 10.0, 30.0, 100.0 and 300.0 mg/kg of body weight (bw) for 28 days (or sooner, if euthanasia was deemed necessary). Following the manufacturer’s protocol, all samples were blocked in their plates with 10 ul of blocking reagent for 1 h and washed two times using the Bioplex plate washer (Bio-Rad). Sample, standard, and control volumes of 30 ul were added to their pre-designated wells and incubated for 1 h. Plates were washed and 30 ul of detection antibody were added for a final incubation of 30 min. Plates were placed on an orbital shaker for 2 min to induce cell lysis and then incubated for an additional 10 min before being read using a Luminex 200 instrument (Bio-Rad). Biomarker expression levels were normalized to cell viability.

2.6. Statistics

Software programs Microsoft Excel and Prism (GraphPad, San Diego, CA) were used to perform data calculations and analyses, respectively. Student t-tests or 2-way ANOVAs were used to determine whether dose-matched DGA effects in our described assays were statistically significant from the effects of control compounds (valproic acid and cisplatin) at P values less than 0.05, as indicated.

3. Results

3.1. Exposure of kidney cells to DGA in vitro is associated with significant cell death

To investigate the effect of DGA on kidney cell viability, we exposed human proximal tubule cells, HK-2, to increasing doses of DGA and compared these effects to exposing HK-2 cells to control compounds: cisplatin and valproic acid (positive and negative controls, respectively). The cell viability assay we employed measures ATP levels since ATP production decreases as cells undergo cell death. As shown in Fig. 1, we found that whereas our negative control, valproic acid, did not induce measurable cell death between the treatment concentrations of 0–10 mM, our positive control, cisplatin, exhibited a rapid decrease in ATP production beginning at the low exposure concentration of 0.5 mM after 24 h of treatment, as expected. Interestingly, DGA started out having an innocuous effect between the treatment concentrations of 0–2.5 mM, but proceeded towards cytotoxicity at 5 mM; cell viability was significantly lower than that of valproic acid-treated cells (P < 0.05), but higher than that of cisplatin-treated cells (P < 0.05).

As the DGA concentration was increased, cell viability of DGA-treated continued to decrease sharply at concentrations to levels similar to
cisplatin-treated cells. Further analysis of these results yielded lethal concentration 50 (LC50) values for DGA to be 4.0 mM, which is intermediary to that of cisplatin (0.4 mM) and valproic acid (41.7 mM). In an effort to verify these results, we used an alternative technique to determine if DGA was inducing HK-2 cell death, as opposed to interfering with the chemical aspect of the ATP assay. Calcein AM is a well-accepted method of cell viability detection which relies of the ability of live cells to cleave non-fluorescent Calcein AM dye into a fluorescent molecule. As shown in Supplementary Fig. S1, increasing levels of DGA and cisplatin both decrease the levels of live cells, confirming our ATP assay findings.

3.2. DGA induces dose-dependent mitochondrial toxicity in HK-2 cells

To investigate the involvement of mitochondria in the cytotoxic effect of DGA, we used two main approaches. First, we investigated the extent to which the mitochondrial populations lost their membrane potential (MMP) in response to DGA exposure relative to valproic acid or cisplatin exposure. A widely accepted method utilizes the dye JC-10, which selectively enters membrane-compromised mitochondria and fluoresces at 520 nm, while JC-10 dye molecules that are excluded from intact mitochondria fluoresce at 590 nm. Staining HK-2 cells with JC-10 after 24 h of treatment with DGA, cisplatin, or valproic acid showed that DGA could indeed exert mitochondrial toxicity onto HK-2 cells (Fig. 2a). Below the concentration of 5 mM, the ratio of mitochondria with lost vs. maintained MMP remained low following exposure to valproic acid and DGA. At the treatment concentration of 5 mM or above, this ratio significantly increased (P < 0.05) in DGA-treated cells, but not valproic acid-treated cells. By contrast, the relative levels of mitochondrial populations whose MMP were lost actually increased starting from 0.05 mM of cisplatin. In excess of 5 mM of treatment, DGA and cisplatin achieved indistinguishable levels of compromised to intact mitochondria. We used the alternative approach of staining DGA-treated HK-2 cells with resazurin in order to help corroborate our findings. As shown in Supplementary Fig. S2, the mitochondrial reductase activity that resazurin detects shows decreasing mitochondrial activity as DGA treatment concentration increases.

To explore the reason for the observed mitochondrial toxicity associated with DGA, we next determined whether elevations in reactive oxygen species (ROS) were taking place in treated HK-2 cells. The oxidative stress associated with such elevations could help account for mitotoxicity. As shown in Fig. 2b, we determined that not only did elevations in ROS levels take place in DGA-treated HK-2 cells, but the trend of this elevation took place at the exact treatment concentrations that we measured in our investigation of MMP loss shown in Fig. 2a. Specifically, DGA treatment induced a spike in ROS production at concentrations of 5 mM and above relative to valproic acid (P < 0.05). By contrast, valproic acid produced no measurable change in ROS levels between the whole range of 0–10 mM while cisplatin treatment was associated with potent ROS release starting at 0.5 mM treatment reaching levels of DGA-induced ROS production above 5 mM.

3.3. Kidney-specific toxicity determination of DGA in vitro and in vivo

We used the highly sensitive LumineX technique of measuring kidney-specific damage to HK-2 cells using the biomarker Kidney Injury Molecule-1 (KIM-1). KIM-1 is an FDA-qualified biomarker for nephrotoxicity as it is expressed at increasing levels by proximal tubule cells as cellular damage takes place. In our in vitro cellular system, we found that exposing HK-2 cells for 24 h to DGA induced a measurable increase in KIM-1 expression that became statistically significant beyond the 5 mM exposure dose (P < 0.05) as shown in Fig. 3. Our negative control valproic acid did not cause an elevated KIM-1 levels, but negative control cisplatin was associated with a rise in KIM-1 starting from 0.05 mM treatment concentration. Interestingly, KIM-1 induction by DGA appeared to exceed that of cisplatin at the high doses of 7.5 mM and 10 mM (P < 0.05).

In an effort to corroborate our in vitro findings, we sought to compare our results to the levels of KIM-1 in urine samples collected from rats that had been treated with increasing doses of DGA. As described in a separate manuscript [17], rats were orally gavaged daily with a range of increasing concentrations of DGA solutions or vehicle control solution for up to 28 days. As shown in Fig. 4a, urine samples were tested for KIM-1 by LumineX after 2, 4, 8, 16, and 22 days post-exposure. DGA induced its expression in the highest dose cohort of animals (300 mg/kg bw) after merely 2 days of exposure to DGA. The animals in this cohort stopped urinating by Day 4 post-DGA exposure and needed to be euthanized by Day 5, thereby preventing longer-term urine samples to be assayed for KIM-1 levels. However, the cohort of animals that received 100 mg/kg bw did survive beyond the 22 day post-exposure time point and produced enough urine for KIM-1 level determination. We found that KIM-1 expression significantly increased after 4 days of DGA exposure (100 mg/kg bw) relative to vehicle control (P < 0.05) and that this level decreased over time. The remaining groups of animals receiving 30 mg/kg bw DGA or less did not exhibit any significant changes in KIM-1 expression (Fig. 4a).

In order to further investigate the kinetics of KIM-1 elevation in the 300 mg/kg bw dose cohort, a second rat study was performed where animals were either orally gavaged daily with vehicle control solution or 300 mg/kg bw of DGA [17; manuscript under review]. Urine samples were collected from animals after 1, 2, 3, or 4 days post-DGA administration and assayed for KIM-1 levels. As shown in Fig. 4b, the increase in KIM-1 began after just 2 days post-DGA exposure and continued to increase until reaching statistical significance at Day 3 post-DGA exposure (P < 0.05). As expected, the lack of urine output at Day 4 post-DGA prevented urinary KIM-1 evaluation.

4. Discussion

Identifying potential toxicity related to food additives is an important part of ensuring the safety of the U.S. food supply. Although CMCs themselves may have limited toxicity, their unavoidable contamination by their byproduct DGA raises the question of its safety.
Until recently, evidence pointing to kidney toxicity by DGA remained sparse; one human case of fatal poisoning [11] and two reports of DEG causing renal toxicity due to its metabolite DGA [4,5] were published. New interests in DGA resurfaced with the publication of recent in vitro and in vivo research showing that DEG is nephrotoxic on account of its liver-derived metabolite, DGA [6,7]. As part of a larger effort to directly examine the safety of DGA for the FDA, we began with an in vitro model system using HK-2 cell line. We selected the HK-2 cell line as our cellular in vitro model for its ability to closely mimic both morphological and metabolic features of primary human proximal tubule cells [18]. HK-2 cells express epithelial cell biomarkers and brush border enzymes that are specific to proximal tubules [18]. Although we did not evaluate the effects of DGA on primary proximal tubule cells from human donors, several other investigators have successfully used the HK-2 cell line to show that it can reliably and accurately model in vivo injury when exposed to well-established nephrotoxins, including cisplatin, further supporting its use as a cellular in vitro model [13,19,20]. The data we generated using cisplatin and valproic acid as our positive and negative controls, respectively, in this study are consistent with the findings of other researchers using HK-2 cells [21,13]. Having both a positive and negative control allowed us to gauge the toxicity of DGA from two points of view.

Our study using the HK-2 in vitro model revealed four main findings. First, HK-2 exposure to DGA was acutely and dose-dependently cytotoxic at a level that was intermediary between the innocuous valproic acid and the highly toxic cisplatin. Second, this apparent cytotoxicity correlates with our evidence of oxidative stress mechanisms involving over-production of reactive oxygen species and loss of mitochondrial integrity. Indeed, high ROS production can lead to mitochondrial damage [22], which in turn can prevent cellular respiration for maintained viability. Whether the correlation between cytotoxicity and increased ROS levels is causative remains to be fully investigated. Reports by Landry et al. [23] and Conrad et al. [30] show that the reduction of DGA-induced ROS by antioxidants does not prevent mitochondria from becoming dysfunctional, suggesting that ROS elevations may simply be a secondary effect. The third major finding of our study was that DGA cytotoxicity elicited strong KIM-1 expression. The sensitive detection Luminex technique we used captured the production of this important biomarker, in response to increasing doses of DGA treatment. Finally, this biomarker was also increased in our in vivo system, specifically in

![Diagram](image-url)
the two highest dose animal cohorts within a short time of initiating their exposure to DGA. The elevation of KIM-1 specifically reflect proximal tubule damage in vivo [24,25], as well as damaged HK-2 cells [12–14].

Our comparison of DGA nephrotoxicity in vitro to in vivo is a unique and important aspect of this study. The two systems complement each other well and validate the idea that in vitro systems can be used for preliminary extrapolation of an in vivo outcome. The in vitro approach is relatively rapid and inexpensive and reveals key mechanistic details, but leaves doubt regarding its utility in predicting in vivo models. Herein, we address this issue by first establishing that DGA is nephrotoxic in vitro and using Lumimex biomarker detection to relate the in vitro to in vivo findings. That the two systems were consistent in indicating DGA nephrotoxicity not only validates HK-2 cells as a useful predictor for the in vivo results, but calls for more research to be done to compare the predictability of HK-2 cells in predicting the nephrotoxicity of other compounds on proximal tubule cells.

Although the nephrotoxicity of DGA was evidently revealed by our two model systems, it does not seem clear whether it is possible to accurately conclude exactly how toxic DGA is in vivo based on our in vitro data. Exposing HK-2 cells to DGA treatment solutions of varying strengths offers a sense of how toxic DGA is relative to our chosen control compounds. However, the question of how these concentrations translate to in vivo dosing remains unanswered. Quantitative analyses of DGA levels in the kidneys of rats treated with DGA or even DEG like those performed in Robinson et al. [31] and Landry et al. [26], respectively, would assist in helping to establish how in vivo dosing translates to renal exposure and hence give a better understanding of the in vivo state. Additional studies would be also be needed to better understand how ingested DGA can translate to in vivo blood levels and how these components enter the blood and get filtered through the glomerulus for further exposure to the proximal tubules.

This gap in understanding the translation of in vitro to in vivo dosing is further complicated by the notion that DGA is a notoriously difficult byproduct to extract out of CMC products [3]. This difficulty implies that if DGA is present in food as a contaminant of a food additive, then it raises the question of how readily would it be released in vivo following ingestion. DGA is present in food at very low concentrations [27] and that it likely takes very high doses of DGA to induce the toxicity we observed in vivo [17]. If DGA remains bound to CMC molecules, then the question of how would its nephrotoxicity profile change becomes less relevant. In vivo studies using DGA mixed into animal feed, for example, would help address this question. Further research towards understanding what the actual levels of exposure are to U.S. consumers is also critical to gaining a better perspective of how realistic it would be for an average consumer to be exposed to effective doses of DGA that would cause proximal tubule cell damage.

Ultimately, the balance between safety and risks associated with DGA will depend on many factors, including the long-term vs. short-term exposure to DGA, the concentration and frequency of exposure,
and the reversibility of any chronic or acute kidney damage. It should be noted that although our study focused on the effects of DGA on renal systems, the potential for DGA to target other organ systems, exists. As such, we have also investigated the toxicity of direct exposure of DGA on the liver (manuscript submitted) and the heart [28]. Other publications have also demonstrated DGA-induced liver toxicity in animal models (Robinson et al., 2017,[17]). In humans, the aforementioned cases report of accidental ingestion of DGA includes a description of its injurious effects on kidneys, liver and brain [11]. Moreover, the mass-population DEG poisoning in Panama also led to damage in these organs [29]. Of note, studies demonstrating neuronal toxic effects by DGA have yet to be performed.

Taken together, the results of our study support the idea that DGA is nephrotoxic in a dose-dependent manner and may exert its effects through a mitochondrial mechanism. Our collective data highlights the utility of the HK-2 in vitro system for the purpose of predicting in vivo toxicity outcomes. This study also underscores the value of performing in vitro research in parallel with in vivo research. As more data is gathered in such comparative studies, quantitative meta-analyses will become feasible to help determine the predictability of specific in vitro systems for animal and even human outcomes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2017.06.011.

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