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Drug Metabolism and Disposition

Effects of Multiple Doses of Dichloroacetate on GSTZ1 Expression and Activity in Liver and Extrahepatic Tissues of Young and Adult Rats

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Running title:

Effect of DCA Doses on GSTZ1 Expression and Activity in Rats

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Abbreviations: DCA, dichloroacetate; EPNPP, 1,2-epoxy-3-(4-nitrophenoxy)propane; GSH, glutathione; GSTZ1, glutathione transferase zeta 1; MAA, maleylacetoacetate; MA, maleylacetone; MAAI, maleylacetoacetate isomerase; S.D., standard deviation;

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Abstract

GSTZ1, expressed in liver and several extrahepatic tissues, catalyzes dechlorination of dichloroacetate (DCA) to glyoxylate. DCA inactivates GSTZ1, leading to auto-inhibition of its metabolism. DCA is an investigational drug for treating several congenital and acquired disorders of mitochondrial energy metabolism, including cancer. The main adverse effect of DCA, reversible peripheral neuropathy, is more common in adults treated long-term than in children, who metabolize DCA more quickly after multiple doses. One dose of DCA to Sprague Dawley rats reduced GSTZ1 expression and activity more in liver than extrahepatic tissues, however the effects of multiple doses of DCA that mimic its therapeutic use have not been studied. Here, we examined the expression and activity of GSTZ1 in cytosol and mitochondria of liver, kidney, heart, and brain 24 hours after completion of 8-days oral dosing of 100 mg/kg/day sodium DCA to juvenile and adult Sprague Dawley rats. Activity was measured with DCA and with 1,2-epoxy-3-(4-nitrophenoxy)propane (EPNPP), reported to be a GSTZ1-selective substrate. In DCA-treated rats, liver retained higher expression and activity of GSTZ1 with DCA than other tissues, irrespective of rodent age. DCA-treated juvenile rats retained more GSTZ1 activity with DCA than adults. Consistent with this finding, there was less measurable DCA in tissues of juvenile than adult rats. DCA-treated rats retained activity with EPNPP, despite losing over 98% of GSTZ1 protein. These data provide insight into the differences between children and adults in DCA elimination under a therapeutic regimen and confirm that the liver contributes more to DCA metabolism than other tissues.

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Significance Statement

DCA is one of few drugs exhibiting higher clearance from children than adults, following repeated doses, for reasons that are unclear. We hypothesized that juveniles retain more GSTZ1 than adults in tissues following multiple DCA doses, and found this was the case for liver and kidney, with rat as a model to assess GSTZ1 protein expression and activity with DCA. Although EPNPP was reported to be a selective GSTZ1 substrate, its activity was not reduced in concert with GSTZ1 protein.

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Introduction

Glutathione transferase zeta 1 (GSTZ1)/maleylacetoacetate isomerase (MAAI) is a cytosolic and mitochondrial enzyme with a critical role in tyrosine catabolism, catalyzing the *trans*-isomerization of maleylacetoacetate (MAA) and maleylacetone (MA) into fumarylacetoacetate (FAA) and fumarylacetone (FA), respectively (Blackburn *et al.*, 1998; Li *et al.*, 2011). While the conversion of MAA and MA is an important physiological function of the enzyme, GSTZ1 also catalyzes the glutathione-dependent dehalogenation of dichloroacetate (DCA) to glyoxylate (Tong *et al.*, 1998). The GSTZ1-catalyzed conversion of DCA to glyoxylate represents the primary metabolic route for DCA. GSTZ1 is the only enzyme known to metabolize DCA to glyoxylate. However, secondary metabolites derived from the formation of glyoxylate have also been identified, including carbon dioxide, glycine, and oxalate (Gonzalez-Leon *et al.*, 1997; James *et al.*, 1998, 2017).

DCA is a by-product from municipal drinking water chlorination, present at concentrations as high as 160 µg/L in some municipalities (Miller and Uden, 1983; Jia *et al.*, 2006; Stacpoole, 2011). High doses of DCA administered to inbred strains of rodents demonstrate hepatocellular carcinogenicity in experimental animals (Deangelo *et al.*, 1991; DeAngelo, 1996), but carcinogenic effects in humans have not been observed (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2014). Notably, DCA is an investigational drug for the treatment of cancer and other diseases of mitochondrial dysfunction via its inhibition of pyruvate dehydrogenase kinase and stimulation of the mitochondrial pyruvate dehydrogenase complex (Kankotia and Stacpoole, 2014; James and Stacpoole, 2016; James *et al.*, 2017). In addition, DCA is currently utilized in a phase 3 clinical trial for treatment in children with congenital pyruvate dehydrogenase complex deficiency (NCT02616484, ClinicalTrials.gov) (Stacpoole *et al.*, 2018). This underscores the important therapeutic applications of DCA in humans.

The pharmacology and toxicity of DCA are influenced, in part, by subject age (Shroads *et al.*, 2008). For example, the chronic use of DCA in adults can lead to development of reversible peripheral

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neuropathy (Kaufmann *et al.*, 2006; Stacpoole *et al.*, 2019). However this effect is seldom clinically symptomatically observed in children (Stacpoole, 2011; Abdelmalak *et al.*, 2013), who metabolize DCA more quickly after multiple doses than adults (Shroads *et al.*, 2008; Mangal *et al.*, 2018). In people, DCA metabolism is independent of sex (Stacpoole *et al.*, 1998). DCA is a mechanism-based inactivator of GSTZ1 (Anderson *et al.*, 1999; Tzeng *et al.*, 2000), and repetitive DCA dosing results in the auto-inhibition of DCA metabolism (Li *et al.*, 2008). Tissue accumulation of DCA, MAA, and MA, due to GSTZ1 degradation, may be a mechanism responsible for DCA toxicity, as MA and MAA are capable of forming covalent modifications to proteins (Cornett *et al.*, 1999; Lantum, Liebler, *et al.*, 2002).

Although liver is the main site of GSTZ1 expression and activity, extrahepatic tissues also express GSTZ1 (Board *et al.*, 1997; Lantum, Baggs, *et al.*, 2002; Jahn *et al.*, 2018). Therefore, elimination and disposition of DCA can be influenced by extrahepatic metabolism. It was shown that a single dose of a therapeutic concentration of DCA to female rats reduced GSTZ1 expression and activity in liver to a greater extent than in kidney, brain, and heart (Jahn *et al.*, 2018), such that DCA metabolism was more prominent in these extrahepatic tissues than liver after the single dose. DCA is given on a daily basis under therapeutic conditions, however, the role of extrahepatic tissues in metabolism of DCA, MAA, and MA following repeated doses of DCA that mimic therapy has not been studied. We hypothesized that extrahepatic tissues contribute to the GSTZ1-catalyzed metabolism of DCA after multiple doses of the drug. Moreover, given the influence of subject age on DCA pharmacology, we also hypothesized that children retain a greater capacity for the GSTZ1-catalyzed metabolism of DCA. This study examines the effect of repetitive DCA dosing on the expression and activity of GSTZ1 in tissues of juvenile and adult Sprague Dawley rats, which have been used previously to model the effects of DCA exposure in relation to neuropathy, a side-effect of DCA therapy (Calcutt *et al.*, 2009).

Measuring GSTZ1 activity with its established substrates, DCA or MA, is expensive and time-consuming, therefore we were interested to read reports that 1,2-epoxy-3-(4-nitrophenoxy)propane

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(EPNPP) was a GSTZ1-selective substrate in two species of monkeys (Uno *et al.*, 2013, 2020). Measuring activity with EPNPP is inexpensive and rapid (Habig *et al.*, 1974), so we utilized this substrate as well as DCA to monitor activity in control and DCA-treated rats, as well as with recombinant rat and human GSTZ1.

Materials and Methods

Chemicals, Animals, and Reagents. Four-week and 52-week male and female Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and approved for use by the University of Florida Institutional Animal Care and Use Committee. A custom polyclonal rabbit antibody to the full-length rat GSTZ1 protein (NCBI accession#: NM_001109445.1) was produced by Cocalico Custom Antibody (Reamstown, PA) and isolated from serum at 4°C using the Protein A IgG Purification Kit (Thermo Scientific, Waltham, MA). TransBlot® Turbo nitrocellulose RTA Transfer Kit, TGX Stain-free FastCast 12% Acrylamide kit, Clarity™ Western ECL Substrate reagent, 10X Tris/Glycine/SDS Buffer, Blotting-grade blocker nonfat dry milk, and Precision Plus Protein™ Dual Color Standards were purchased from Bio-Rad Laboratories (Hercules, CA). We obtained HEPES, sodium chloride, sucrose, dithiothreitol, glutathione (GSH), glycerol, potassium chloride, boron trifluoride-methanol solution, 2-keto-hexanoic acid (GCMS internal standard) and anti-rabbit HRP secondary antibody from Sigma Aldrich (St Louis, MO). The Pierce™ BCA Protein Assay Kit was purchased from ThermoFisher Scientific (Waltham, MA). EPNPP was purchased from AK Scientific (Union City, CA). We purchased clinical grade sodium DCA from TCI America (Portland, OR) and analyzed it by GC/MS to verify that it was >99% pure and did not contain mono- or tri-chloroacetate. Sodium acetate was purchased from Tocris Bioscience (Minneapolis, MN) and [^{14}C]-DCA from American Radiolabeled Chemicals (St. Louis, MO). Radioactive samples were analyzed with a Beckman model LS-6500 liquid scintillation counter using EcoLume liquid scintillation cocktail (MP Biomedicals, Solon, OH). Immunoblots were visualized using the ChemiDoc™

Imaging System from Bio-rad Laboratories (Hercules, CA). All other reagents were purchased from commercial suppliers.

Rat dosing and tissue collection. Adult female (n = 16), adult male (n = 4), juvenile female (n = 8), and juvenile male rats (n =10) were dosed with sodium DCA (100 mg/kg) or sodium acetate (100 mg/kg) by oral gavage daily for 8 consecutive days in groups of 4 to 8 per treatment. The mean ± S.D. of initial body weights (grams) for adult female, adult male, juvenile female, and juvenile male rats were 413 ± 54, 768 ± 89, 83 ± 6, and 89 ± 9, respectively. Because of the rapid growth in juvenile rats, dosing was adjusted to the body weight determined each day of the treatment period. The weight of the adult rats did not change over the dosing period. On the 9th day the animals were sacrificed and the liver, kidney, heart, and brain organs were collected and used to prepare cytosolic and mitochondrial subcellular fractionations as previously described (Li *et al.*, 2011). We selected the rat DCA dose of 100 mg/kg because it was calculated to be therapeutically similar to the human dose of 25 mg/kg/day after species scaling (Boxenbaum, 1980), and sodium acetate was chosen as control since it is a similar molecule to DCA that was previously shown to have no effect on GSTZ1 expression or activity (Jahn *et al.*, 2018; Smeltz *et al.*, 2019). Four and 52 week-old rats were used to model children or adults around the ages of 2 and 30 years, respectively (Quinn, 2005), and a treatment period of 8 days was chosen as studies in humans and rats show a steady state reduction in GSTZ1 is achieved between 5 and 7 days (Schultz and Sylvester, 2001; Saghir and Schultz, 2005; Shroads *et al.*, 2012).

Recombinant rat and human GSTZ1. Expressed human and rat GSTZ1 were prepared and purified as described in previous work (Li *et al.*, 2012; Smeltz *et al.*, 2019).

GSTZ1 protein expression. GSTZ1 protein expression was assessed by Western blot using a ratio standard curve constructed from purified recombinant rat GSTZ1 and a cytosolic protein reference sample (Supplemental Fig. 1). Known amounts of cytosolic and mitochondrial protein were denatured for 5 minutes at 100°C in 1X sample buffer (Alfa Aesar, Havervill, MA) containing 20 mM HEPES, 40 mM

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NaCl, 2 mM GSH, and 2 mM DTT. The proteins were separated by SDS-PAGE, applying more protein per lane for samples derived from DCA-treated animals to ensure the GSTZ1 levels were within the linear range of the standard curve. Subsequently, the proteins were transferred to nitrocellulose membranes using the Trans-blot™ Turbo Transfer System (Bio-rad Laboratories, Hercules, CA) and then blocked for 1 hour at room temperature in 5% milk prepared in TBST (tris-buffered saline containing 0.05% Tween 20). The membranes were probed overnight at 4°C using a rat GSTZ1 antibody dilution of 1:10,000 in 5% milk. On the following morning and at room temperature, the membranes were washed in TBST for 30 minutes (buffer changed every 5 minutes), incubated for 1 hour in secondary anti-rabbit HRP antibody (1:5000, prepared in 5% milk), and washed again in TBST as described above. The membranes were incubated in Clarity ECL substrate prepared from 1 part luminol/enhancer solution, 1 part peroxide solution, and 5 parts water. GSTZ1 bands were visualized using the ChemiDoc system (Bio-rad Laboratories, Hercules, CA), where the chemiluminescent signal for each sample was normalized against the cytosolic protein reference sample from the standard curve. We show representative immunoblots of cytosolic GSTZ1 protein in liver, kidney, heart, and brain of acetate- and DCA-treated young female rats in Supplemental Figs. 2 to 5. The expression of GSTZ1 (ng GSTZ1/μg protein) is reported as the mean ± standard deviation of duplicate determinations.

GSTZ1 activity assays. Subcellular fractions from liver and extrahepatic tissues were examined for GSTZ1 activity by measuring the conversion of [^{14}C]-DCA to [^{14}C]-glyoxylate as previously described (Li *et al.*, 2011; Smeltz *et al.*, 2019). Each 100 μL reaction was performed at pH 7.6 and contained 100 mM HEPES, 0.2 mM [^{14}C]-DCA, and either 1 mM GSH (cytosol), 5 mM GSH (liver mitochondria), or 10 mM GSH (kidney mitochondria). Since GSH is consumed by gamma-glutamyl transpeptidase activity in rat kidney mitochondria (Jahn *et al.*, 2018), we utilized 10 mM GSH in the current work to maintain GSH saturation for all kidney mitochondria incubations. The reactions were initiated at 37°C by the addition of dialyzed protein (0.1 to 1.0 mg) for 10 to 30 minutes for liver samples and up to 120 minutes for

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extrahepatic tissue samples. Incubations were then quenched with methanol and examined for [^{14}C]-glyoxylate by the HPLC method (Li *et al.*, 2011). To determine the contribution of each organ to DCA metabolism, we calculated the total activity per organ (nmol glyoxylate formed/min/whole tissue) from the specific activity in cytosol and mitochondria (nmol glyoxylate formed/min/mg protein), the protein yield of each subcellular fraction (mg protein/gram tissue), and the total weight of the organ (grams). The total activity per organ was then normalized to the rodent's weight (nmol glyoxylate formed/min/kg rat) and is expressed as the mean ± standard deviation of duplicate determinations. For liver and kidney cytosol, we also calculated activity per gram tissue and per whole tissue.

Activity assay with EPNPP. Liver cytosol fractions from acetate- and DCA-treated young female rats were examined for activity using EPNPP as substrate. EPNPP was also examined as substrate with purified recombinant rat and human GSTZ1. Briefly, each reaction was performed at 37°C and contained 10 mM potassium phosphate pH 7.4, 1 mM GSH, and 1 mM EPNPP in a final reaction volume of 3.0 mL. The reactions were initiated by the addition of rat liver cytosol (0.5 mg) or purified rat or human GSTZ1 (0.3 mg) for 5 minutes, where formation of the glutathione conjugate of EPNPP at 360 nm was recorded. Calculation of specific activity (nmol/min/mg protein) was based on 0.5 $mM^{-1}cm^{-}1$ as the molar extinction coefficient of the GSH conjugate of EPNPP (Habig *et al.*, 1974).

Comparison of GSTZ1 protein sequences between species: The amino acid sequences of human, rat, and marmoset GSTZ1 were compared for similarity using BLAST (National Center for Biotechnology Information). The protein sequences were obtained from the following UniPortKB accession identifiers: O43708 (MAAI_HUMAN), P57113 (MAAI_RAT), F7A4H9 (F7A4H9_CALJA), and U3AQX4 (U3AQX4_CALJA).

Quantitation of DCA: To determine DCA concentrations, sections of whole tissue (0.1 – 0.3 g) from liver, kidney, heart, and brain of control- and DCA-treated rats were homogenized in 12% boron-trifluoride methanol solution (1 mL) to derivatize DCA to the methyl ester by heating the samples at

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115°C for 15 minutes then cooling to 4°C. Methyl-DCA was extracted into methylene chloride for subsequent analysis by GC-MS (Yan *et al.*, 1997). GC-MS analysis was performed using an Agilent® GC 7890B (column: Thermo TG WAXMS, 30 m x 0.25 mm x 0.25 μm), Agilent 5977B mass spectrometer with Agilent 7693 autosampler. Quantitation was performed using m/z 59 for a fragment of methyl DCA versus m/z 57 for the most abundant fragment of methylated internal standard, 2-ketohexanoic acid and MSD ChemStation software (Agilent).

Data Analysis. Data transformation and determination of means, standard deviation, and statistical significance were performed using GraphPad Prism 6 software (San Diego, CA). Group comparisons within subcellular fractions used either the one-way ANOVA (corrected for multiple comparisons by the Tukey method) or a two-tailed Student's t-test (corrected for multiplicity by the Holm-Sidak method). Statistical significance was defined as $p < 0.05$.

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Results

Cytosolic and mitochondrial GSTZ1 protein expression is reduced after eight daily DCA doses in rats. Data were obtained using 16 adult females (8 per group), 4 adult control (acetate-treated) males, 10 young males (4 DCA-treated, 6 acetate-treated), and 8 young female rats (4 per group). Additional control, acetate-treated, young males were used because of high variability of control values in the 4 used initially. As seen in Fig. 1, repetitive DCA dosing significantly reduced the expression of cytosolic GSTZ1 in liver and extrahepatic tissues of young and adult rats. Of the tissues studied, liver retained the highest expression in adults, at 0.030 ± 0.026 ng GSTZ1/μg protein (± S.D., Supplemental Table 1). Similarly, liver retained the highest expression of cytosolic GSTZ1 in DCA-treated juveniles (Supplemental Table 2). In controls, the expression of cytosolic GSTZ1 was similar in young and adult females (1.0 ± 0.4 and 1.1 ± 0.1 ng GSTZ1/μg protein, respectively). In male control rats dosed with 100 mg/kg sodium acetate, GSTZ1 protein expression showed high individual variability both in juveniles and adults. Following 8-day dosing with 100 mg/kg sodium acetate, adult male rats demonstrated 1.05 ± 0.52 ng cytosolic GSTZ1/μg protein in liver, and juvenile males had 0.70 ± 0.49 ng GSTZ1/μg protein. These control levels were not significantly different from those in females (Supplemental Tables 1 and 2).

As with cytosolic GSTZ1, mitochondrial GSTZ1 was significantly reduced in rat tissues after 8-day dosing with DCA (Fig. 2). Adult rat kidney retained the highest expression at 0.048 ± 0.019 ng GSTZ1/μg protein (Supplemental Table 3). The hepatic expression of mitochondrial GSTZ1 in DCA-treated adults was 0.020 ± 0.008, whereas levels in heart and brain were much lower (0.0013 ± 0.0017 and 0.0012 ± 0.0010, respectively). These findings were a likely indication that juveniles would also express very low levels of mitochondrial GSTZ1 after multiple DCA dosages. Our investigation of mitochondrial GSTZ1 expression in young rats was limited to liver and kidney tissue, in part because the small size of brain and heart from the young rats made it difficult to isolate sufficient mitochondria to study. These results,

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summarized in Supplemental Table 4, show mitochondrial GSTZ1 was more highly retained in liver after DCA dosing in juveniles. There were no gender-dependent variations in mitochondrial GSTZ1 expression for either age group.

Repetitive DCA dosing in rats decreases cytosolic and mitochondrial GSTZ1 activity with DCA in extrahepatic tissues to a greater extent than liver. We measured the activity of GSTZ1 in mitochondrial and cytosolic matrices from rat liver and some extrahepatic tissues. In general, there was strong correlation between GSTZ1 protein expression and GSTZ1 activity. The average activity in liver cytosol of adult male and female controls was 2.20 ± 0.47 and 2.89 ± 0.54 nmol glyoxylate/min/mg protein (± S.D.), respectively. After DCA dosing in adult females, GSTZ1 activity in hepatic cytosol was reduced over 90% to 0.026 ± 0.008 nmol/min/mg. Nevertheless, liver retained the highest activity of cytosolic GSTZ1 in DCA-treated adult rats, as activity in extrahepatic tissues was 0.002 nmol/min/mg or lower (Supplemental Table 5). The trend was similar in juvenile rats. In liver cytosol, young male and female controls demonstrated GSTZ1 activities of 1.83 ± 0.81 and 1.95 ± 0.48 nmol/min/mg, respectively, whereas the activity decreased to 0.034 ± 0.007 and 0.040 ± 0.011 nmol/min/mg in males and females dosed with DCA (Supplemental Table 6). There was low activity in kidney cytosol of DCA-treated juveniles (0.004 ± 0.001 for males, 0.005 ± 0.003 for females); rates in heart and brain cytosol were not determined as they were expected to be at the limit of detection with the low amount of tissue we had. Additionally, interrogation of mitochondrial GSTZ1 activity was limited to liver and kidney fractions. Liver retained the highest activity of mitochondrial GSTZ1 in DCA-treated adults with an average rate of 0.005 ± 0.008 (vs kidney rate of 0.0007 ± 0.0003 nmol/min/mg, Supplemental Table 7). The remaining rates for juvenile controls are summarized in Supplemental Table 8. The total cytosolic and mitochondrial activity in tissues studied are summarized on a whole rat basis in Tables 1 and 2, respectively, for adult and juvenile rats. For further comparisons, we calculated the cytosolic activities

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remaining in DCA-treated adult and juvenile liver and kidney on a per gram tissue and per whole organ basis, as shown in Figure 3.

Proportion of liver to body weight is higher in juvenile rats than adults. The percent weight of the liver (g) relative to total body weight (g) was calculated for each rodent. Adult males and females demonstrated liver to body weight percentages of 3.49 ± 0.21 and 3.56 ± 0.48 (± S.D.), respectively, whereas the values in juvenile male and female rats were significantly higher ($p < 0.0001$) at 6.08 ± 0.63 and 5.94 ± 0.80. The body and liver weights from each rat group are summarized in Supplemental Table 9.

Activity with EPNPP did not change with DCA treatment. We examined liver cytosol from acetate- and DCA-treated rats to determine if the loss of GSTZ1 protein by repetitive DCA dosages would show an effect on activity with EPNPP. Additionally, we examined whether the conjugation of EPNPP was catalyzed by the human or rat GSTZ1 protein. Our studies showed the activity with EPNPP in DCA-treated young females (131.6 ± 39.7 nmol/min/mg, ± S.D.) was not significantly different from activity in controls (132.8 ± 38.1 nmol/min/mg, $p = 0.97$). Furthermore, incubations of EPNPP with purified recombinant rat or human GSTZ1 did not show an increase in absorbance at 360 nm after 5 minutes (data not shown), nor could a reliable rate be obtained when the incubation time was extended to 10 minutes (data not shown). To confirm catalytic activity of the recombinant enzymes, we incubated purified rat GSTZ1 and human GSTZ1C with [^{14}C]-DCA using the assay conditions described for GSTZ1 activity in Materials and Methods. We found the activities for rat GSTZ1 and human GSTZ1C were 598 and 65 nmol glyoxylate formed/min/mg, respectively.

After 8-day DCA dosing, DCA concentrations in adult rat tissues are higher than in juvenile rats. DCA was detected in whole tissues of young and adult rats 24 h after eight daily doses of 100 mg/kg DCA. As seen in Table 3, DCA concentrations in all adult tissues were significantly higher than in juveniles. No differences in tissue concentrations between male and female juveniles were observed.

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Discussion

GSTZ1 has a critical physiological role in tyrosine catabolism, though the expression and activity of GSTZ1 are also important for the disposition and elimination of DCA, as GSTZ1 is the only enzyme known to metabolize DCA. Much attention has been given to DCA in recent years because of its therapeutic application in cancer and other metabolic diseases, in which there is dysfunction of the pyruvate dehydrogenase complex/pyruvate dehydrogenase kinase axis and subsequent perturbation of mitochondrial bioenergetics (James *et al.*, 2017). However, the chronic effects of DCA administration vary among individuals. Diversity in GSTZ1 haplotype is known to affect the pharmacokinetics of DCA (Board and Anders, 2011; Li *et al.*, 2012; Zhong *et al.*, 2014; Tian *et al.*, 2019). Other variables contributing to differential responses to DCA are age (Kaufmann *et al.*, 2006; Stacpoole, 2011; Shroads *et al.*, 2012; Abdelmalak *et al.*, 2013) and tissue chloride concentrations, as chloride slows the inactivation of GSTZ1 by DCA (Zhong *et al.*, 2014; Jahn *et al.*, 2018; Smeltz *et al.*, 2019).

The distribution and activity of GSTZ1 in rodent tissues has been studied previously (Lantum, Baggs, *et al.*, 2002). Recently, we reported that a single "therapeutic" dose of DCA to rats reduced the activity and expression of GSTZ1, but the effect in extrahepatic tissues was weaker than in liver (Jahn *et al.*, 2018). Given this observation, we hypothesized that extrahepatic tissues contribute more to DCA metabolism than liver after multiple doses of DCA. We tested this hypothesis in the current work by dosing Sprague Dawley rats with 100 mg/kg DCA for 8 days. Additionally, we hypothesized that juveniles retain a higher capacity for the GSTZ1-catalyzed metabolism of DCA. This premise was formulated based on age-related effects of chronic DCA use in humans and the larger liver to body weight ratio in children (Noda *et al.*, 1997; Shroads *et al.*, 2008).

GSTZ1 was detected in cytosolic and mitochondrial subcellular fractions from acetate- and DCA-treated rats, though the expression and activity was significantly lower in fractions from the DCA group. There was a strong correlation between expression and activity of GSTZ1 for each sample, which has

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been described previously (Jahn *et al.*, 2018; Smeltz *et al.*, 2019). Of the tissues examined, liver retained the highest expression and activity of GSTZ1 irrespective of rodent age or gender. Indeed, the total activity per organ from cytosolic and mitochondrial GSTZ1 (Tables 1 and 2, respectively) show that extrahepatic tissues have no greater contribution to DCA metabolism than liver after multiple drug doses. Furthermore, considering the higher protein yield and larger size of the liver compared with those of extrahepatic tissues, our findings demonstrate that most DCA metabolism occurs in the liver, even after repeated drug exposure. These data are consistent with human studies, which show no sex difference in DCA kinetics (Stacpoole *et al.*, 1998), as well as with a clinical study demonstrating that during the anhepatic phase of liver transplantation, DCA plasma clearance was undetectable (Shangraw and Fisher, 1996).

Of note, there was a greater reduction of total cytosolic GSTZ1 activity in liver and kidney of DCA-treated adults than in juveniles. The total activity in liver cytosol was 113 ± 37 and 115 ± 33 nmol glyoxylate/min/kg rat, respectively, for juvenile male and females, whereas the remaining activity in liver cytosol of 52-week female rats was 44.7 ± 13.4 nmol glyoxylate/min/kg rat (Tables 1 and 2). Here, the total activity retained in liver cytosol of adults was 2.6-fold lower than the levels in juveniles ($p < 0.001$). Comparison of the activity in kidney cytosol revealed there were also lower levels of cytosolic GSTZ1 in adults ($p < 0.001$). To ensure that our calculation of remaining activity on a per kg rat basis did not skew the results, we also calculated the activity remaining in liver and kidney cytosol after DCA dosing per g tissue and per whole organ: these results are shown in Figure 3. Although the remaining activity varied slightly with calculation method, the conclusion that juveniles retain more activity following repeat DCA doses did not change. The cytoplasm is the major subcellular location of GSTZ1, and these findings suggest that juveniles have a greater capacity for DCA metabolism in cytosolic matrices after multiple doses of DCA. Given the greater proportion of liver to body weight in juveniles, the hepatic metabolism of DCA is predicted to be augmented within this age group. This is consistent

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with the higher clearance of DCA observed in young populations (Shroads *et al.*, 2008; Mangal *et al.*, 2018), as children may retain a higher capacity for the GSTZ1-catalyzed metabolism of DCA, due to relative liver size.

There is recent evidence that macaque and marmoset GSTZ1 catalyze the conjugation of glutathione to EPNPP (Uno *et al.*, 2013, 2020). Prior to these reports, the biotransformation of haloacetic acids and *trans*-isomerization of MAA and MA were the only reactions described in literature to be catalyzed by GSTZ1 (Tong *et al.*, 1998; Board *et al.*, 2001). Our results, showing no decrease in activity with EPNPP in liver cytosol of DCA-treated rats, reveal that EPNPP is not a substrate for the rat GSTZ1. Furthermore, no activity with EPNPP was found with expressed human or rat GSTZ1 proteins. One explanation for the difference in EPNPP activity between species could relate to differences in protein sequence. Human GSTZ1 and marmoset GSTZ1 share 90% amino acid sequence according to recent reports (Uno *et al.*, 2020). Our analysis indicated the percent identity between human and marmoset GSTZ1 was slightly lower (88%) and that the sequences differed by the presence of a motif (^{124}VSKDLRE^{130}) in marmoset GSTZ1 that is absent in both human and rat GSTZ1. Therefore, inclusion of the VSKDLRE motif in marmoset GSTZ1 may allow for the productive binding and catalytic turnover of EPNPP. This might explain the observed discrepancy in GSTZ1 activity with EPNPP between species.

The DCA concentrations in tissues from younger rats were much lower than those in adults (Table 3), reflective of the higher total ability of young rats to metabolize DCA after multiple doses. Significant changes were not observed when comparing concentrations between tissues from the same rodent group. This was a surprising observation, given the higher metabolism of DCA in liver matrices demonstrated in the current work. One explanation for this observation is the ADME properties of DCA, which readily crosses membranes and is both water and lipid soluble. Studies in male Sprague Dawley rats show radioactivity from [^{14}C] is widely distributed in liver, kidney, heart, brain, spleen, lung, and muscle after dosing with [^{14}C]-DCA. (James *et al.*, 1998). Female Sprague Dawley rats given a single oral

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dose of DCA, 100 mg/kg, and sacrificed at different times after the dose did not show any age-related differences in absorption, as similar concentrations were found in liver at 15 minutes after the dose in juvenile and adult rats (Smeltz et al., 2019). Since repetitive DCA dosing results in the auto-inhibition of DCA metabolism, the unchanged DCA can be absorbed and evenly distributed to tissues. This is reflected in the current work by the presence of DCA in brain, heart, kidney, and liver organs 24 h after the last dose.

In summary, this was the first study to examine the contribution of extrahepatic tissues to DCA metabolism following repetitive dosages of a therapeutic concentration of DCA. Repetitive DCA dosing markedly reduced GSTZ1 in liver, kidney, heart, and brain, although the greatest expression and activity levels were retained in the liver. In general, the influence of repetitive DCA dosing on the contributions of liver and extrahepatic tissues to the GSTZ1-catalyzed metabolism of DCA was not affected by age or gender, although the retention of total GSTZ1 activity in liver and kidney cytosol was higher in 4-week-old rats than 52-week rats. Lastly, the lower DCA concentrations in tissues of juvenile rats were a reflection of the larger liver size and higher clearance of DCA observed in younger populations.

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Authorship Contributions:

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Conducted experiments: Squirewell, Smeltz, Rowland-Faux, Horne, James

Performed data analysis: Squirewell, James, Rowland-Faux, Horne

Wrote or contributed to writing of the manuscript: Squirewell, Smeltz, Rowland-Faux, Horne, Stacpoole, James

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Footnote

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Figure Legends

Fig 1. Cytosolic GSTZ1 protein expression in liver, kidney, heart, and brain of (A) adult female and (B) juvenile male and female rats administered 8 daily oral doses of 100 mg/kg sodium acetate (control) or 100 mg/kg sodium DCA. Male and female sexes are denoted by (M) and (F), respectively. Data are means ± standard deviations; n = 8 per group (adults), 4 per group (juvenile females), 6 for control and 4 for DCA-treated juvenile males. Statistical significance: ****$p < 0.0001$, *** $p < 0.001$, **$p < 0.01$, *$p < 0.05$

Fig 2. Expression of mitochondrial GSTZ1 in liver, kidney, heart, and brain of (A) adult female and (B) juvenile male and female rats given 8 daily doses of 100 mg/kg sodium acetate (control) or 100 mg/kg sodium DCA. Male and female sexes are denoted by (M) and (F), respectively. Data are means ± standard deviations; n = 8 per group (adults) and 4 or 6 per group (juveniles). Statistical significance: ****$p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, *$p < 0.05$.

Fig 3. Cytosolic GSTZ1 activity with DCA remaining in juvenile and adult liver (top panels) and kidney (bottom panels) of individual DCA-treated rats, expressed as percent mean control values. Values from females are shown in red and from males in purple. Data is presented per g tissue (left panels), per whole liver or kidney (middle panels) and per kg rat (right panels). Adult activities were significantly lower than juvenile values as indicated by the p values. The data shown in the right panels is also presented in Tables 1 and 2.

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TABLE 1

Total GSTZ1 activity per whole tissue in adult female rats given 8 daily oral doses of 100 mg/kg sodium acetate (control) or 100 mg/kg sodium DCA, normalized to body weight. Values are mean ± standard deviation of duplicate determinations; n = 8 (cytosol, controls), 4 to 8 (cytosol, DCA-treated), 8 (mitochondria, controls), or 2 to 8 (mitochondria, DCA-treated). ND, not determined.

Tissue	**Adult Cytosolic GSTZ1** *(nmol glyoxylate/min/kg rat)*		**Adult Mitochondrial GSTZ1** *(nmol glyoxylate/min/kg rat)*	
	Control	DCA	Control	DCA
Liver[a]	4036 ± 1006	44.7 ± 13.4****	376 ± 105	6.5 ± 9.6****
Kidney	71.5 ± 22.1	0.48 ± 0.12****	3.8 ± 0.9	0.06 ± 0.03***
Brain	4.0 ± 1.0	0.031 ± 0.008****	ND	ND
Heart	1.4 ± 0.6	0.020 ± 0.004***	ND	ND

[a]Total activity in cytosol was calculated based on the cytosolic yield recovered experimentally. We used a mitochondrial yield of 30 mg/g liver to correct for the loss of mitochondria during isolation (Fleischer *et al.*, 1979). The asterisks denote statistical significance when compared to activity within the same tissue of controls: ****$p < 0.0001$, *** $p < 0.001$.

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TABLE 2

Total GSTZ1 activity per whole tissue in juvenile rats given 8 daily oral doses of 100 mg/kg sodium acetate (control) or 100 mg/kg sodium DCA, normalized to body weight. Values are mean ± standard deviation of duplicate determinations; n = 4 per group. ND, not determined.

Tissue	**Juvenile females (Cytosolic GSTZ1)** *(nmol glyoxylate/min/kg rat)*		**Juvenile males (Cytosolic GSTZ1)** *(nmol glyoxylate/min/kg rat)*	
	Control	DCA	Control	DCA
Liver[a]	3947 ± 1462	115 ± 33***	4103 ± 2684	113 ± 37***
Kidney	67.6 ± 19.3	1.4 ± 1.0***	81.0 ± 29.6	1.8 ± 0.3***
Brain	2.6 ± 0.4	ND	1.4 ± 1.4	ND
Heart	1.9 ± 0.7	ND	1.1 ± 1.6	ND

[a]Total activity in cytosol was calculated based on the cytosolic yield recovered experimentally. The asterisks denote statistical significance when compared to activity within the same tissue of controls: ***$p < 0.001$.

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TABLE 3

DCA concentrations (µg DCA/g tissue) in 4-wk and 52-wk old rats given 8 daily oral doses of 100 mg/kg sodium DCA. Values are mean ± standard deviation. ND, not determined.

Tissue	**52-wk female (n = 8)**	**4-wk female (n = 4)**	**4-wk male (n = 4)**
Liver	47.11 ± 27.13	10.06 ± 6.59*	11.11 ± 4.28*
Kidney	34.97 ± 13.77	16.42 ± 9.85*	11.94 ± 4.90*
Brain	20.44 ± 7.27	8.32 ± 5.03*	8.47 ± 2.26*
Heart	48.49 ± 20.03	ND	ND

DCA concentrations in liver, kidney and brain were significantly different between age groups by ANOVA, $p = 0.011$ (liver), 0.0095 (kidney) and 0.005 (brain). Multiple comparisons showed concentrations in young rats were significantly lower than adults * $p < 0.05$.

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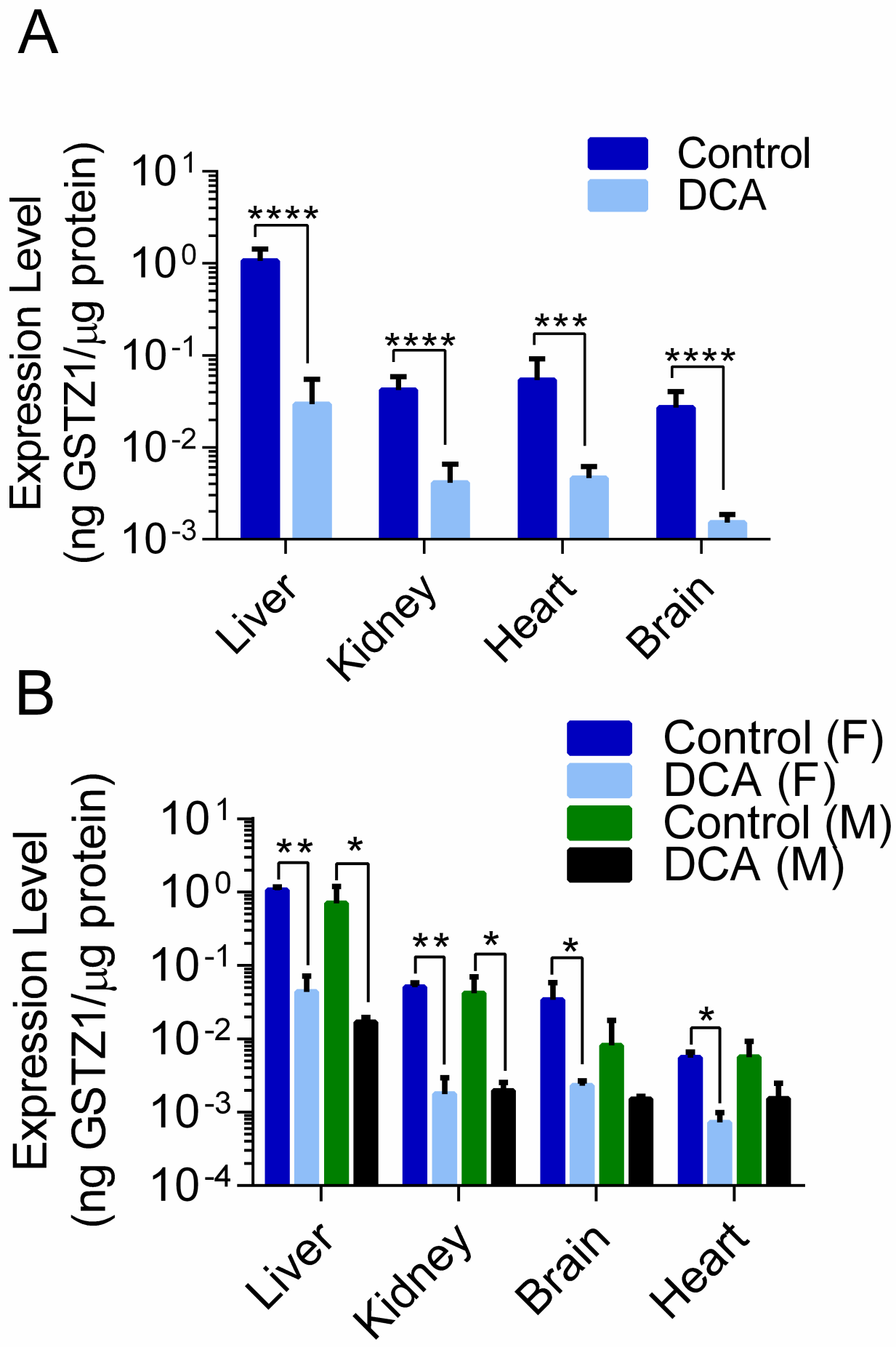

Figure 1

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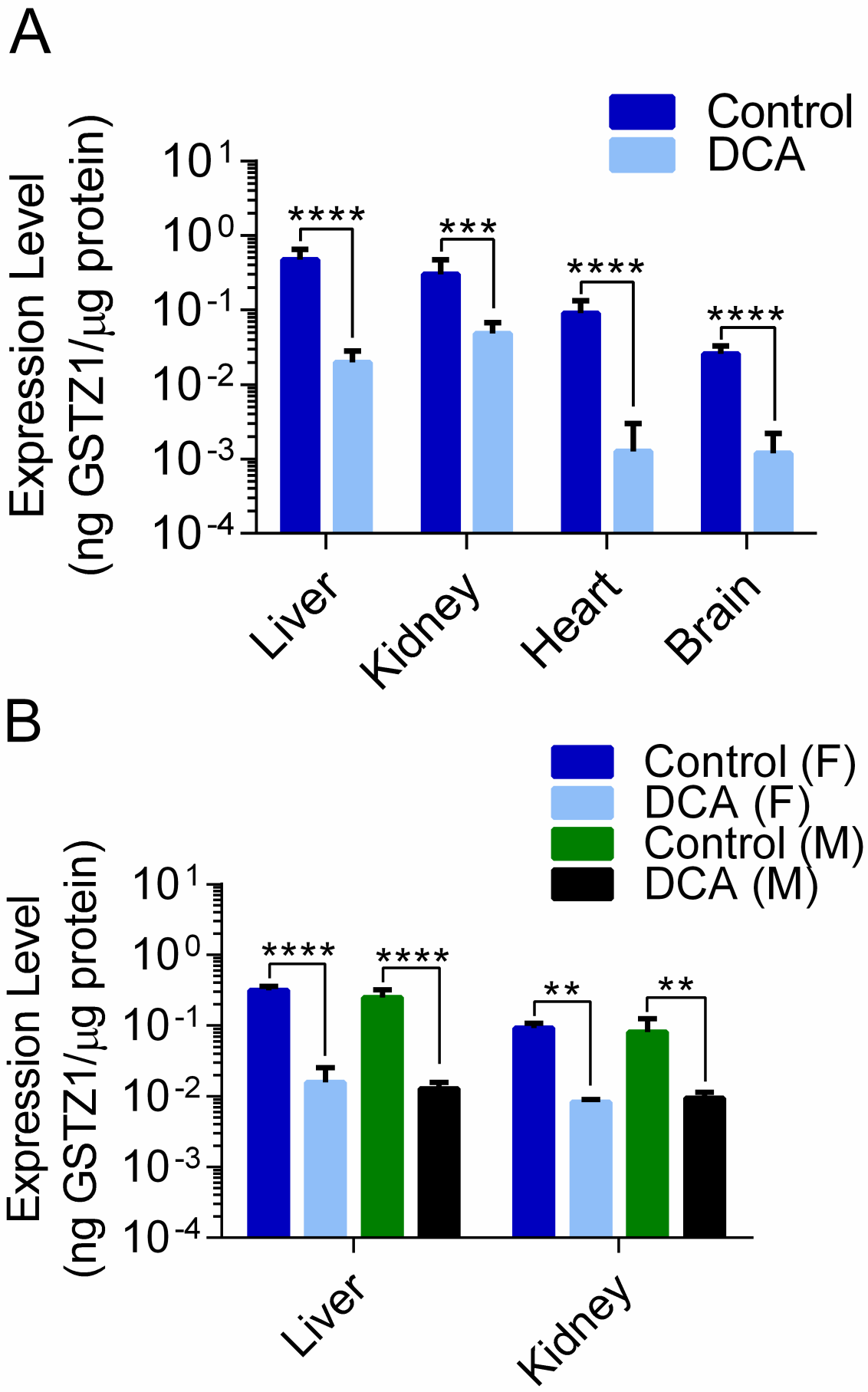

Figure 2

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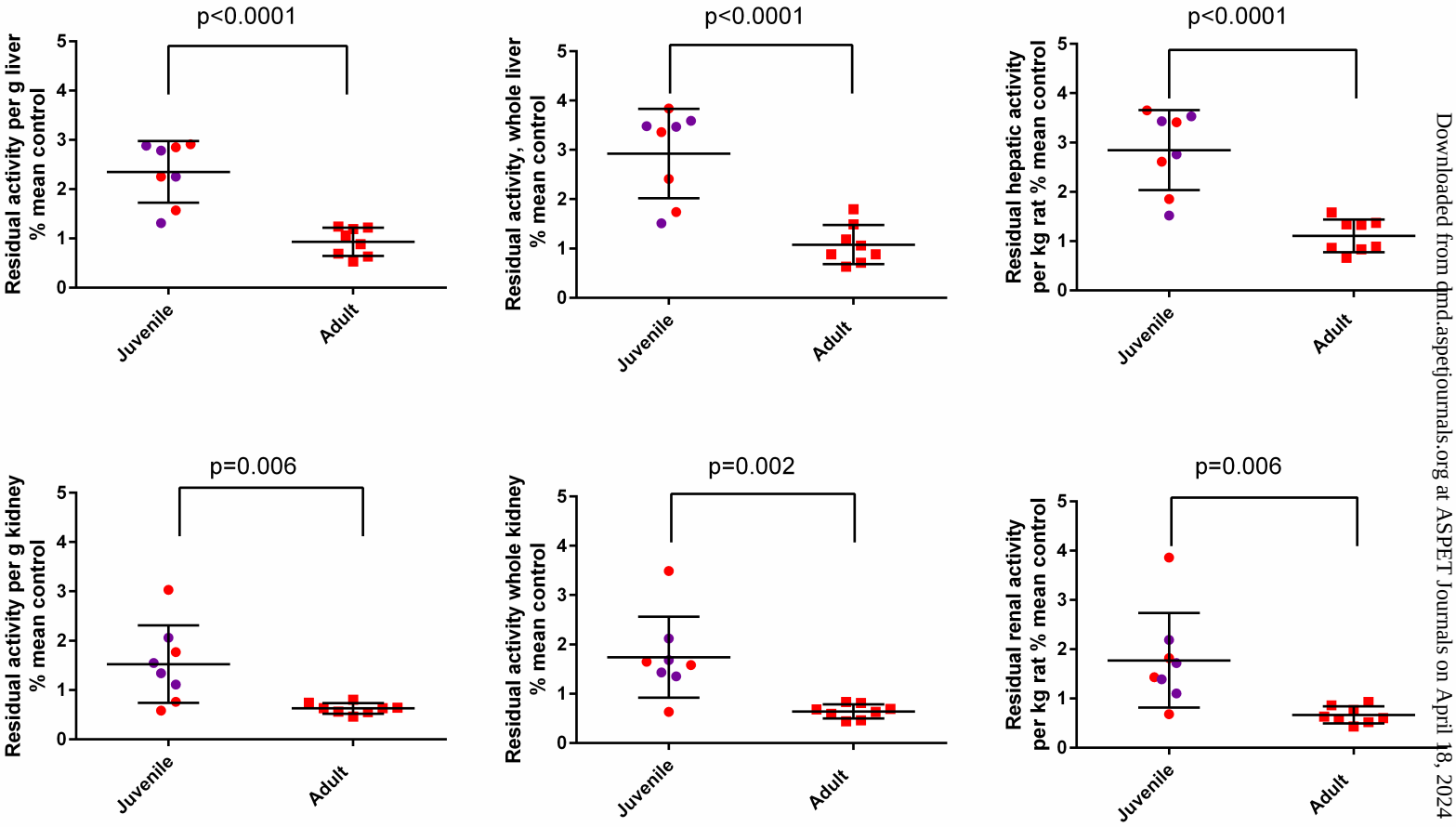

Figure 3