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Reduction in Rat Oocyte Fertilizability Mediated by *S*-(1, 2-dichlorovinyl)-L-cysteine: A Trichloroethylene Metabolite Produced by the Glutathione Conjugation Pathway

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Abstract Trichloroethylene (TCE), a commonly used industrial degreasing solvent and environmental toxicant, reduces rat oocyte fertilizability by an incompletely understood mechanism. Previous evidence implicated cytochrome P450 dependent oxidation of TCE. The current study investigated a second pathway, glutathione conjugation using *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC), a mutagenic and cytotoxic TCE-metabolite. In vitro exposure of oocytes and in vivo exposure of females to DCVC significantly reduced oocyte fertilizability (63% vs. 26%; $p < 0.005$ and 60% vs. 36%; $p < 0.005$, respectively). Reduced fertilizability of oocytes following in vivo TCE exposure may be mediated partially by the glutathione conjugation pathway.

Keywords Trichloroethylene · Oocyte fertilizability · Ovary · Reproduction

Trichloroethylene (TCE) is a chlorinated hydrocarbon commonly used as an industrial solvent for degreasing metal parts and as an ingredient in consumer products, such as adhesives and spot removers. Although most of the TCE used in the United States is released into the atmosphere as vapor from degreasing operations, trichloroethylene can contaminate surface waters via direct discharges or enter groundwater through leaching from disposal operations and Superfund sites (Scott and Cogliano 2000). Typical routes of TCE exposure include inhalation, transdermal absorption, and oral ingestion. Most TCE toxicity is dependent on bioactivation, which occurs by: (1) cytochrome P450 dependent oxidation or (2) conjugation with glutathione (Lash et al. 2000). The first step in glutathione conjugation of TCE is catalyzed by glutathione S-transferases (GSTs), a group of enzymes found in almost every mammalian species and in most organs. Since the ovary contains some of the same isoforms of GST as the kidney, an organ which bioactivates TCE by glutathione conjugation, bioactivation of TCE within the ovary by glutathione conjugation is reasonable (Cummings et al. 2000; Rahilly et al. 1991; Rozell et al. 1993; Toft et al. 1997). The initial metabolite formed by glutathione conjugation of TCE is *S*-(1,2-dichlorovinyl) glutathione (DCVG). The DCVG can be further metabolized into *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC), a potential cytotoxic compound, via hydrolysis reactions with γ-glutamyltransferase and an additional enzyme, such as cysteinylglycine dipeptidase (Cummings et al. 2000; Goeptar et al. 1995).

Reduced fertility of oocytes is observed following in vivo exposure of female rats to TCE, although TCE itself is not a potent cytotoxicant (Berger and Horner 2003; Wu and Berger 2007). To further explore potential female reproductive toxicity by ovarian glutathione conjugation, oocyte fertilizability was assessed following in vitro exposure of rat oocytes and in vivo exposure of female rats to DCVC. This study provides evidence that exposure to DCVC is cytotoxic to oocytes and glutathione conjugation of TCE may contribute to reduced oocyte fertilizability following TCE exposure.

Materials and Methods

Simonson albino rats, a Sprague–Dawley derived strain, from the Department of Animal Science breeding colony at

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the University of California, Davis were used for this study. Females were between 28 and 45 days old, and male rats were approximately 100 days old. All animals were housed in a temperature (70 ± 2°F) and humidity (40–70%) controlled facility under a 14L: 10D light cycle. Rats were fed Purina Formulab 5008 rat chow (St. Louis, MO, USA) ad libitum. After weaning (21 days of age) and before TCE exposure, rats had ad libitum access to deionized water. The University of California, Davis Animal Use and Care Administrative Advisory Committee approved all animal use. The DCVC was a generous gift from Dr. James L. Stevens (Eli Lilly and Company, Indianapolis, IN, USA). All other chemicals were reagent grade or tissue culture tested. Primary antibodies were goat anti-GST Ya, goat anti-GST Yb (Oxford Biomedical Research; Oxford, MI, USA) and rabbit anti-dinitrophenyl (DNP) (Sigma-Aldrich; St. Louis, MO, USA). The secondary antibodies were peroxidase-conjugated donkey anti-goat IgG and peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA, USA).

Fertilizability of oocytes following in vitro exposure of oocytes to DCVC was evaluated in four replicates. Oocytes were obtained from five untreated females in each replicate as previously described (Berger and Horner 2003; Wu and Berger 2007). The pool of zona-free oocytes was divided into two groups (approximately 30 oocytes/group in each replicate). Control oocytes were preincubated for 4 h at 37°C, 5% CO_2, in HEPES-TALP (94.19 mM NaCl, 4.75 mM KCl, 1.71 mM CaCl2, 1.17 mM KH2PO4, 1.17 mM MgSO4, 25.1 mM NaHCO3, 26 mM Na lactate, 0.5 mM Na pyruvate, 5.5 mM glucose, 50 μg gentamycin sulfate/ml, 4 mg bovine serum albumin (BSA)/mL, with 20 mM [4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid (HEPES)). Oocytes in the treated group were preincubated in 5 mM DCVC in HEPES-TALP (concentration of DCVC was estimated from previous in vivo exposures to TCE and assuming 50% of TCE metabolized by the glutathione conjugation pathway). Oocytes were then rinsed three times in HEPES-TALP prior to incubation with capacitated rat sperm from untreated males. Following gamete coincubation, the number of fertilized oocytes, and the number of unfertilized oocytes with bound sperm were determined after staining nuclei with Hoechst 33342.

Fertilizability of oocytes following in vivo exposure of female rats to DCVC was evaluated in four replicates. Females (two per replicate) received a single intraperitoneal (ip) injection of 25 mg DCVC/kg body weight (DuTeaux et al. 2003) approximately 16–18 h before oocyte retrieval for the IVF assay. Control females received equivalent volumes of sterile saline. Sperm from untreated males was preincubated to capacitate; a single male provided semen for oocytes from DCVC-exposed and control females in each replicate.

The α and μ forms of GST were immunolocalized in ovaries from control and TCE-exposed females (2 week exposure, three replicates) (Wu and Berger 2007). Ovaries were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4), embedded, and 5 μm paraffin sections cut. Glutathione S-transferase α and μ expression were quantitatively assessed using Western blots. Each replicate consisted of ovaries from rats treated with TCE for 2 days, TCE for 3 days, and vehicle for 3 days. Four replicates were evaluated (total of 12 females). Kidneys from TCE-treated rats served as a positive control. Tissues were individually homogenized for approximately 2 min. The suspension was centrifuged at 10,000 × *g* for 20 min and supernate was collected and stored at −20°C. Protein concentration was assessed using the BCA protein assay. Ovarian protein samples were solubilized in a final concentration of 0.9% SDS (w/v), boiled for 2 min, separated on a 10% SDS-polyacrylamide gel, and electroblotted onto 0.45 μm polyvinylidene fluoride (PVDF) membrane. Membranes were blocked, incubated with goat anti-GST Ya (α), goat anti-GST Yb (μ) antibodies or normal goat serum as a negative control and visualized by chemiluminesence following incubation with peroxidase-conjugated donkey anti-goat IgG. Immunoreactive bands were quantified by densitometry using Image Quant software (Molecular Dynamics, Sunnyvale, CA). Oxidative stress in ovaries was evaluated following DCVC exposure (and vehicle exposure) in four replicates as previously described (Wu and Berger 2007).

In vitro fertilization data, body weights, and densitometry values were subjected to analysis of variance (ANOVA) using SAS (SAS Statistical Programs, Cary, NC, USA) with treatment as a fixed factor and replicate a random factor. The percentage of oocytes fertilized and percentage of oocytes with sperm bound were weighted by the number of oocytes evaluated, and were analyzed before and after transformation (arc sine square root) to improve normality. *p* values associated with the transformed data are presented. The effect of DCVC on final body weight was evaluated using initial body weights as a covariate. Densitometry values used to quantify expression of GST α and μ from each replicate were adjusted for protein loading by dividing GST α and μ densitometry values by total protein densitometry values from blots stained with Coomassie blue.

Results and Discussion

In vitro exposure of oocytes to DCVC for 4 h reduced the fertilizability of zona-free oocytes compared with those incubated in medium alone (26% vs. 63%; $p < 0.005$, SEM = 3, Fig. 1). Similarly, in vivo exposure of females

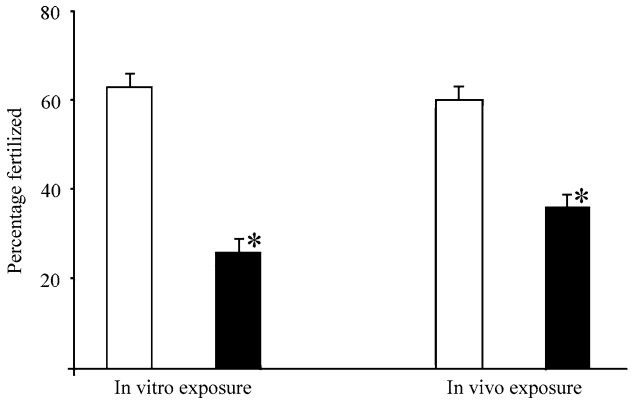

Fig. 1 In vitro and in vivo exposure of oocytes to DCVC (dark bars) significantly reduced the ability of oocytes to be fertilized in vitro by rat sperm. Bars represent least squares means from four replicates; 321 oocytes evaluated after in vitro exposure and 407 oocytes evaluated after in vivo exposure. * indicates $p < 0.005$ compared with the vehicle control (open bars)

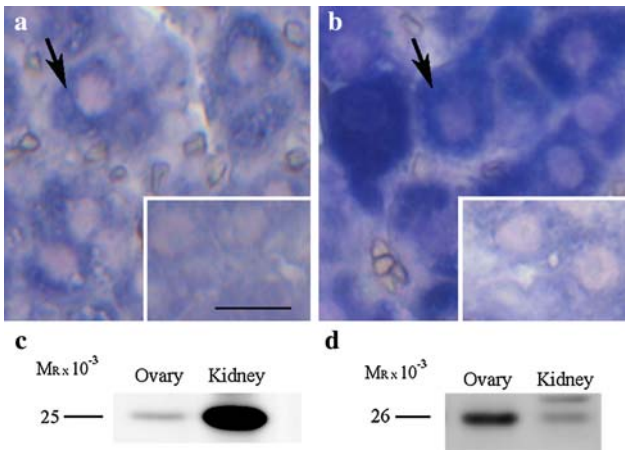

Fig. 2 Immunohistochemical detection of GST α (**a**) and μ (**b**) in rat ovarian tissue. Picture representative of three replicates; bar represent 50 μm. Ovarian granulosa cells are prominently labeled blue indicating the presence of GST (arrow). Insets demonstrate that tissue incubated with normal goat serum (negative control) was not labeled. Each picture is representative of three replicates. Western blots demonstrate GST α (**c**) and μ (**d**) in ovarian and kidney homogenates and that the GST antibodies recognize the appropriate M_r band in ovarian tissues. Each blot is representative of four replicates

to DCVC reduced oocyte fertilizability (36% vs. 60%; $p < 0.005$, SEM = 3, Fig. 1). The ability of oocytes to bind sperm from normal males was reduced by in vitro exposure of oocytes to DCVC (46% of oocytes bound sperm vs. 88%; $p < 0.05$, SEM = 4) but not by in vivo exposure of females to DCVC (91% vs. 98%; $p = 0.1261$, SEM = 3). Similar to TCE exposures, in vivo exposure to DCVC did not appear to affect the general health of females as assessed by body weights (mean final body weight of 89.2 g for DCVC-treated animals vs. 92.5 g for controls, $p = 0.30$, SEM = 3). These observations suggest ovarian metabolism of TCE by glutathione conjugation may contribute to the reduced oocyte fertilizability following TCE exposure.

Consistent with the glutathione conjugation pathway, the enzyme GST is present in the rat ovary but precise localization in the rat ovary was previously unreported (Toft et al. 1997). In the current study, GST α and μ isoforms, the most common isoforms of GST in the rat ovary, were clearly present in the granulosa cells (Fig. 2a, b). Negative controls, sections incubated with normal goat serum instead of goat anti-GST α and goat anti-GST μ, had no detectable labeling (Fig. 2a, b insets). This is similar to their reported presence in granulosa (and as well as theca) cells in human and bovine ovaries (Rabahi et al. 1999; Rahilly et al. 1991; Tiltman and Haffajee 1999). Similar to the report for the bovine, no labeling for GST was detectable in oocytes. Western blot analysis of ovarian homogenates confirmed that the GST antibodies recognized the appropriate M_r protein (Fig. 2c, d). Consistent with ovarian metabolism of TCE by glutathione conjugation, γ-glutamyltransferase, another enzyme involved in this pathway, was previously immunolocalized to the granulosa cells in the rat ovary (Rutenburg et al. 1969). Since the granulosa cells interact intimately with the growing oocyte, this localization is consistent with the observed effect on oocyte fertilizability (Sugiura and Eppig 2005).

Any differences in GST protein expression following 2 or 3 day exposure to TCE compared with controls were not detectable by immunohistochemistry or by Western blot analysis. Previously, no significant differences in RNA expression were detected after 1 or 5 days of exposure (Wu and Berger 2008). Microarray analyses also indicated expression of γ-glutamyltransferase in the rat ovary was unaltered by in vivo exposure to TCE for 1 and 5 days (Wu and Berger 2008). Changes may be below detection limits or toxicant metabolism may not instigate changes in RNA expression or protein levels (Bartosiewicz et al. 2001).

Ovarian homogenates from DCVC-treated and vehicle control rats had similar labeling of protein carbonyls, an indicator of protein oxidation. Mean densitometry values were 3.94 for samples from DCVC-treated rats and 3.86 for samples from vehicle control rats (arbitrary units; SEM = 0.26; $p > 0.75$; n = 4 per group). This observation contrasts with our previous observations indicating significantly increased protein oxidation following TCE exposures (Wu and Berger 2007) and suggests large increases in protein oxidation are not a characteristic of the glutathione conjugation pathway in the ovary.

The DCVC induced reduction in oocyte fertilizability, presence of GST α and μ in rat granulosa cells, and previous studies which demonstrated females exposed to TCE

had reduced oocyte fertilizability (Berger and Horner 2003; Wu and Berger 2007), suggest metabolic production of DCVC from TCE may be partially responsible for the reduction in oocyte fertilizability. The current study in conjunction with the previous study suggests ovarian metabolism of TCE and oocyte toxicity may result from both the glutathione conjugation pathway and from increased ovarian protein oxidation resulting from cytochrome P450 dependent metabolism.

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