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Reactive Oxygen Stimulation of Interleukin-6 Release in the Human Trophoblast Cell Line HTR-8/SVneo by the Trichlorethylene Metabolite *S*-(1,2-Dichloro)-L-Cysteine[1]

Iman Hassan,[3,5] Anjana M. Kumar,[5] Hae-Ryung Park,[4,5] Lawrence H. Lash,[6] and Rita Loch-Caruso[2,5]

[5]*Department of Environmental Health Sciences, University of Michigan, Ann Arbor, Michigan*
[6]*Department of Pharmacology, Wayne State University School of Medicine, Detroit, Michigan*

ABSTRACT

Trichloroethylene (TCE) is a common environmental pollutant associated with adverse reproductive outcomes in humans. TCE intoxication occurs primarily through its biotransformation to bioactive metabolites, including *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC). TCE induces oxidative stress and inflammation in the liver and kidney. Although the placenta is capable of xenobiotic metabolism and oxidative stress and inflammation in placenta have been associated with adverse pregnancy outcomes, TCE toxicity in the placenta remains poorly understood. We determined the effects of DCVC by using the human extravillous trophoblast cell line HTR-8/SVneo. Exposure to 10 and 20 μM DCVC for 10 h increased reactive oxygen species (ROS) as measured by carboxydichlorofluorescein fluorescence. Moreover, 10 and 20 μM DCVC increased mRNA expression and release of interleukin-6 (IL-6) after 24-h exposure, and these responses were inhibited by the cysteine conjugate beta-lyase inhibitor aminooxyacetic acid and by treatments with antioxidants (alpha-tocopherol and deferoxamine), suggesting that DCVC-stimulated IL-6 release in HTR-8/SVneo cells is dependent on beta-lyase metabolic activation and increased generation of ROS. HTR-8/SVneo cells exhibited decreased mitochondrial membrane potential at 5, 10, and 20 μM DCVC at 5, 10, and 24 h, showing that DCVC induces mitochondrial dysfunction in HTR-8/Svneo cells. The present study demonstrates that DCVC stimulated ROS generation in the human placental cell line HTR-8/SVneo and provides new evidence of mechanistic linkage between DCVC-stimulated ROS and increase in proinflammatory cytokine IL-6. Because abnormal activation of cytokines can disrupt trophoblast functions necessary for placental development and successful pregnancy, follow-up investigations relating these findings to physiologic outcomes are warranted.

cytokines, DCVC, HTR-8/SVneo cells, human placental cells, oxidative stress, reactive oxygen species, S-(1,2-dichlorovinyl)-L-cysteine, trichloroethylene, trophoblasts

INTRODUCTION

Trichloroethylene (TCE) is a chlorinated industrial solvent used as a metal degreaser and additive in paint removers and adhesives [1]. Although, the amount of TCE released into the environment has decreased from >57 million pounds in 1988 to approximately 2.4 million pounds in 2010, approximately 3 million people are exposed annually in the United States to TCE through environmental and occupational exposures [1]. In 2014, TCE was reclassified by the International Agency for Research on Cancer (IARC) as a ''known human carcinogen'' and by the National Toxicology Program (NTP) as ''reasonably anticipated to be a human carcinogen'' [2, 3].

TCE is also a reproductive and developmental toxicant [4]. Exposure to TCE during pregnancy has been associated with adverse birth outcomes including increased risk of miscarriages, cardiac malformations, neural tube defects, and cleft palate [5]. Additionally, Forand et al. [6], in a recent retrospective cohort study of 1440 live births among New York residents, found significant associations between exposure to TCE and decreased birth weight. Animal studies have also shown that exposure to TCE during pregnancy increases fetal loss, alters glucose metabolism in the developing brain, and delays organ and bone development [7–10].

TCE toxicity is primarily dependent on being metabolized, which is tissue-, species-, and sex-dependent [5, 11]. TCE is metabolized by two separate pathways [11]. The major pathway is oxidative metabolism in the liver by cytochrome P450 enzymes to an epoxide and then to chloral hydrate, which is further metabolized to trichloroethanol or trichloroacetate. Trichloroacetate can be further oxidized to dichloroacetate and eventually excreted in the urine [11]. The second pathway is through conjugation with glutathione (GSH) in the liver and kidney by glutathione *S*-transferase to *S*-(1,2-dichlorovinyl)-glutathione (DCVG), which is subsequently biotransformed in the kidney by γ-glutamyl transpeptidase to *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC), and then by cysteine conjugate β-lyase to a transient metabolite that rearranges to generate more toxic species [11]. In addition, DCVC undergoes *N*-acetylation and is excreted in the urine as NAcDCVC or the sulfoxide NAcDCVCS [11].

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[2]Correspondence: Rita Loch-Caruso, Department of Environmental Health Sciences, University of Michigan, 1415 Washington Heights, Ann Arbor, MI 48109-2029. E-mail: rlc@umich.edu
[3]Current address: Toxicity Assessment Division, U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Research Triangle Park, NC, 27711.
[4]Current address: Program in Molecular and Integrative Physiological Sciences, Departments of Environment Health, and Genetics and Complex Diseases, Harvard T.H. Chan School of Public Health, Boston, MA, 02115.

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Perturbation of mitochondrial calcium homeostasis is an early effect of DCVC exposure and a key step in DCVC toxicity in the kidney [12]. DCVC stimulates formation of reactive oxygen species (ROS) in human kidney proximal tubular cells in vitro, which is blocked by treatment with antioxidants [13]. Additionally, in vivo exposure to DCVC in mice increases levels of proinflammatory cytokines, including interleukin-6 (IL-6) in blood plasma, and stimulates increased mRNA expression of tumor necrosis factor-α (TNF-α), IL-6, and cyclooxygenase-2 in the kidney [14].

Increased release of proinflammatory cytokines plays a critical role in placentation and parturition [15]. Specifically, increased concentrations of the proinflammatory cytokines IL-6, IL-1β, IL-8, and TNF-α in the gestational compartment are associated with adverse birth outcomes in humans [16]. Moreover, chemokines such as IL-8 stimulate activation and recruitment of leukocytes to the extraplacental membranes, resulting in chorioamnionitis, an inflammatory condition commonly associated with preterm birth and low birth weight [17, 18]. Studies of lipopolysaccharides (LPS), highly immunogenic components of the outer membrane of gram-negative bacteria, suggest mechanistic linkage between infection, proinflammatory responses, and oxidative stress in gestational tissues. Specifically, exposure of human fetal membranes to LPS leads to oxidative stress with increased production of 8-isoprostanes [15]. Moreover, the antioxidant *N*-acetylcysteine prevents LPS-stimulated parturition in mice as well as LPS-induced proinflammatory cytokine release from extraembryonic membranes in vitro [15].

Biomarkers of inflammation and oxidative stress are associated with pathophysiology of pregnancy, including preterm labor, preeclampsia, and intrauterine growth restriction (IUGR) [19–22]. Additionally, increased levels of biomarkers of inflammation and oxidative stress, including 8-isoprostane, IL-6, TNF-α, and C-reactive protein, were found in plasma of women with preeclampsia [23]. Together, these findings suggest that oxidative stress mediates at least some proinflammatory responses in gestational tissues with relevance to adverse pregnancy outcomes. Although exposure to TCE and its metabolite DCVC stimulates oxidative stress responses in the kidney, their ability to do so in the placenta has not been previously explored. In the present study, we investigated the effects of DCVC on ROS-mediated stimulation of IL-6 in the human extravillous trophoblast cell line HTR-8/SVneo.

MATERIALS AND METHODS

Chemicals and Reagents

DCVC was synthesized by the University of Michigan Medicinal Chemistry Core according to procedures described by McKinney et al. [24]. Purity (98.7%) was determined by high-performance liquid chromatography analysis and identity was confirmed by proton nuclear magnetic resonance spectroscopy. Phosphate-buffered saline (PBS), Hanks balanced salt solution (HBSS), 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCF-DA), Hoechst 33342 dye, and 0.25% trypsin were purchased from Invitrogen Life Technologies. Aminooxyacetic acid (AOAA), *tert*-butyl hydroperoxide, (±)-α-tocopherol, and deferoxamine mesylate (DFO) were purchased from Sigma-Aldrich. RPMI 1640 culture medium with L-glutamine and without phenol red, 10 000 U/ml penicillin/10 000 μg/ml streptomycin (P/S) solution and fetal bovine serum (FBS) were from Gibco. LPS from *Salmonella enterica* serovar Typhimurium was purchased from List Biological Laboratories, Inc. The 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) mitochondrial membrane potential (MMP) assay was purchased as a kit from Cayman Chemical. Bongkrekic acid (BkA) was purchased from Calbiochem.

Cell Culture and Treatment

HTR-8/SVneo cells were kindly provided by Dr. Charles H. Graham (Queen's University, Kingston, ON, Canada). The HTR-8/SVneo cell line was derived from first-trimester human cytotrophoblast cells and immortalized with simian virus 40 large T antigen [25]. HTR-8/SVneo cells were cultured as previously described [26]. Briefly, cells were cultured between passages 73 and 85 in RPMI 1640 medium supplemented with 10% FBS and 1% P/S at 37°C in a 5% CO_2 humidified atmosphere. Cells were maintained in growth medium with 10% serum prior to and during experiments to ensure optimal cell growth, as shown by Graham et al. [25]. Cells were grown to 70%–80% confluence at 24 h after subculture before the experiment was started. A stock solution of 1 mM DCVC was prepared in PBS and stored at −20°C. Prior to each experiment, DCVC stock solution was quickly thawed in a 37°C water bath and then diluted in RPMI 1640 medium with 10% FBS and 1% P/S to final exposure concentrations of 5–50 μM DCVC. Because data for exposures of human placental cells to DCVC were lacking, DCVC concentrations for the current study were selected based on the lower range of concentrations used in studies of kidney cells [12, 27].

Cytotoxicity

Cytotoxicity was assayed using a MultiTox-Glo multiplex cytotoxicity kit for live/dead cell protease activities, following the manufacturer's directions (Promega). The assay detects viable cells by using the cell-permeable substrate glycyl-phenylalanyl-aminoflourocoumarin (GF-AFC), which is cleaved by intracellular proteases to yield the fluorescent product aminoflourocoumarin (AFC). Cell death was detected using the cell-impermeable protease substrate alanyl-alanyl-phenylalanyl-aminoluciferin (AAF-Glo), which is cleaved by extracellular proteases following cell membrane compromise. Briefly, HTR-8/SVneo cells were seeded at a density of 10 000 cells per well in a 96-well, white, clear-bottom plate, incubated for 24 h at 37°C, and then exposed to 10, 20, or 50 μM DCVC for 5, 10, and 24 h. GF-AFC was added to the wells, and fluorescence was measured using a SpectraMax M2e Multi-Mode microplate reader (Molecular Devices); fluorescence was correlated to the number of viable cells. AAF-Glo was then added to the wells, and luminescence was quantified using the Glomax Multi Plus detection system (Promega); luminescence was proportional to the number of dead cells.

Cytotoxicity was also assessed by detection of changes in ATP, using the CellTiter-Glo luminescent cell viability assay kit (Promega) according to the manufacturer's protocol. HTR-8/SVneo cells were seeded at a density of 10 000 cells per well and cultured for 24 h in a 96-well clear bottom white plate. The cells were then treated for 24 h with 5, 10, or 20 μM DCVC or untreated (controls). Cell titer Glo reagent was added to cells and incubated in the dark at room temperature for 30 min, and luminescence was measured using the Glomax Multi Plus detection system (Promega). The luminescence signal is proportional to the amount of ATP as an index of cell number.

Cellular Generation of Reactive Oxygen Species

A modification of the dichlorofluorescein (DCF) assay was used to assess DCVC-stimulated generation of oxidant chemical species in HTR-8/SVneo cells [26]. Cells were seeded at a density of 30 000 cells per well in a 96-well black clear-bottom plate. After a 24-h incubation, cells were treated with 10 or 20 μM DCVC for 10 h. Treatment medium was then removed, cells were rinsed twice with HBSS, and cells were incubated with 100 μM carboxy-H_2DCF-DA in HBSS for 1 h at 37°C. The concentration of 100 μM carboxy-H_2DCF-DA was chosen based on prior studies with HTR-8/SVneo cells [26]. Cleavage of the acetate groups from carboxy-H_2DCF-DA by intracellular esterases generates a product that forms the fluorescent molecule carboxy-DCF upon oxidation. After loading cells with the probe, cells were washed twice with HBSS. Fresh HBSS was added back to the cells. Fluorescence was measured immediately, using a SpectraMax M2e Multi-Mode microplate reader (Molecular Devices) at wavelengths of 492 nm excitation and 522 nm emission.

Stimulation of intracellular ROS generation was also visualized by microscopic detection of carboxy-DCF fluorescence. HTR-8/SVneo cells were seeded at a density of 400 000 cells per well in a 6-well plate. Cells were cultured for 24 h and then exposed for 10 h to 10 μM DCVC, the lowest DCVC concentration and shortest exposure duration at which we detected significantly increased carboxy-DCF fluorescence, as measured by spectrofluorometry. *tert*-Butyl hydroperoxide (25 μM), a prototypical chemical oxidant used to generate intracellular ROS [26, 28], was included as positive control. Treatment medium was removed, and then cells were rinsed with HBSS and subsequently incubated at 37°C with 100 μM carboxy-H_2DCF-DA in HBSS for 1 h. The HBSS containing carboxy-H_2DCF-DA was removed, cells were rinsed with HBSS, and then the nuclei were counterstained with 5 μg/ml Hoechst 33342 for

5 min. Using an EVOS digital inverted fluorescence microscope (ThermoFisher Scientific), intracellular carboxy-DCF fluorescence was visualized at 470 nm excitation and 525 nm emission, and Hoechst 33342 stain was visualized at 360 nm excitation and 447 nm emission. Five images were taken, 1 in each of the 4 quadrants and 1 in the middle of the well. Equivalent adjustment for brightness and contrast was applied to each image, using ImageJ software (National Institutes of Health).

Intracellular Glutathione Assessment

The effect of DCVC on GSH levels was measured in HTR-8/SVneo cells by using the commercial GSH-Glo glutathione assay (Promega) following the manufacturer's protocol. This assay is based on glutathione *S*-transferase catalysis of a luciferin derivative to luciferin in the presence of GSH, coupled with luciferase to generate a luminescent signal. Briefly, cells were cultured at a density of 10 000 cells/well in a 96-well clear-bottom, white plate for 24 h and then treated with 10, 20, or 50 μM DCVC for 24 h. Treatment medium was removed, and then the cells were washed with PBS. Aliquots of 100 μl of prepared GSH-Glo reagent were added to each well. The plate was briefly placed on a plate shaker for 2 min for mixing. The plate was removed and incubated at room temperature for 30 min. Aliquots of 100 μl of reconstituted luciferin detection agent were then added to each well. The plate was again placed on a plate shaker for 2 min. The plate was removed from the shaker and incubated at room temperature for 15 min. Luminescence was read using a Glomax Multi Plus detection system (Promega).

DCVC-Stimulated IL-6 Release and IL6 mRNA Expression

Effects of DCVC on cellular release of IL-6 and *IL6* mRNA expression were measured in HTR-8/SVneo cell cultures. Cells were seeded at a density of 50 000 cells/well in 24-well plates, cultured for 24 h, and then treated with 5, 10, or 20 μM DCVC or untreated (control). Cells were exposed to DCVC for 10 or 24 h in the IL-6 release experiment and for 24 h in the *IL6* mRNA expression experiment. LPS (100 ng/ml) was included as a positive control in the IL-6 release experiment. The concentration of IL-6 in cell culture medium was quantified using ELISA (R&D Systems), and mRNA abundance was quantified using quantitative real-time PCR (qRT-PCR).

RNA Extraction and Real-Time qPCR

After treatment, HTR-8/SVneo cells were lysed using RNA lysis buffer (Qiagen). Cell lysates were collected and homogenized using QIAshredder (Qiagen), and homogenates were pooled. The mRNA was then extracted using RNeasy Plus mini-kit (Qiagen) following the manufacturer's protocol. The RNA extraction method we used included multiple genomic DNA elimination steps. Following RNA isolations, our excitation-to-emission ratios, 260:280 nm absorbance, were above 1.8. We also included controls in our PCR reaction to check for genomic DNA contamination. Aliquots of 1 μg of mRNA were used for cDNA synthesis, using the RT2 First Strand kit (Qiagen) following the manufacturer's protocol. Real-time qPCR chain reactions were performed using 12.5 μl of RT^{2}SYBR Green qPCR Master Mix, 1 μl of gene-specific primer target (*TXNRD*1, *GLRX*2, and *IL6*), 4 μl of cDNA template, and 7.5 μl of nuclease-free H_2O in a total volume of 25 μl. Samples were analyzed using a CFX96 real-time PCR detection system (Bio-Rad Laboratories). The housekeeping gene beta-2-microglobulin was used as the reference gene. We performed qRT-PCR with an initial denaturation step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and then 5 s at 60°C. The threshold cycle, $\Delta\Delta C_t$, method [29] was used to quantify and normalize the signal intensities for the target gene to the housekeeping gene signal, using CFX manager software (Bio-Rad Laboratories). The mRNA levels were represented as fold-changes relative to nontreatment controls. All samples were analyzed in triplicate.

Modulation of DCVC-Stimulated IL-6 Responses

Various treatments were used to explore the mechanism by which DCVC stimulated cellular release of IL-6. HTR-8/SVneo cell cultures were set up as described for the IL-6 release concentration-dependent and time-course experiments. To investigate the role of ROS, HTR-8/SVneo cells were co-treated for 24 h with 10 or 20 μM DCVC and 50 μM (±)-α-tocopherol, a peroxyl radical scavenger, or were pretreated for 1 h with 1 mM DFO, an iron chelator, prior to exposure to 10 or 20 μM DCVC. We used 1 mM DFO because this concentration blocks DCVC-induced oxidative stress in rabbit renal proximal tubular cells [30] and is not cytotoxic to HTR-8/SVneo cells [26]. IL-6 protein concentration in culture medium were assayed by ELISA and changes in mRNA expression were assessed by qRT-PCR. To investigate the role of mitochondrial dysfunction in DCVC stimulation of IL-6 release, HTR-8/SVneo cells were pretreated for 1 h with 10 μM BkA, an inhibitor of the mitochondrial permeability transition pore [31]. To evaluate the role of cysteine conjugate β-lyase in DCVC-stimulated IL-6 release, HTR-8/SVneo cells were pretreated for 1 h with 1 mM AOAA, a β-lyase inhibitor, followed by exposure to 20 μM DCVC for 24 h. The concentrations of BkA [31] and AOAA were chosen based on efficacy in previous studies [32].

Mitochondrial Membrane Potential Assay

DCVC-stimulated changes in the MMP were assessed using the fluorescent reagent JC-1 with the JC-1 MMP assay kit (Cayman Chemical) following the manufacturer's protocol. JC-1 is a potential-sensitive lipophilic dye that selectively accumulates in mitochondria and changes fluorescence from red in cells with healthy MMP to green with mitochondrial membrane depolarization. HTR-8/SVneo cells were seeded at a density of 50 000 cells per well and cultured for 24 h in a black, clear-bottom 96-well plate. Cells were treated with 5, 10, or 20 μM DCVC for 5, 10, and 24 h. Following treatment, cells were washed once with 200 μl of HBSS and then incubated with JC-1 dye in HBSS for 30 min at 37°C. Then, JC-1 dye was removed, cells were washed once with HBSS, 200 μl of fresh HBSS was added to each well, and fluorescence was measured using a SpectraMax M2e Multi-Mode microplate reader (Molecular Devices).

In separate experiments, JC-1 fluorescence was visualized by epifluorescence microscopy. Cells were grown at a density of 400 000 cells per well in a 6-well plate. Following incubation with 5, 10, or 20 μM DCVC for 10 or 24 h, JC-1 was added to each well, and plates were incubated at 37°C for 30 min. Cells were then washed, and fresh HBSS was added back to the wells. Cells were viewed using an EVOS digital inverted fluorescence microscope.

Statistical Analysis

All experiments were repeated at least three times and performed in triplicate. The triplicate results were averaged within each experiment, and these values were analyzed either by one-way or two-way ANOVA followed by Tukey post-hoc comparison of means test by using GraphPad Prism software (GraphPad Software Inc.). Data are means ± SEM. N = 3 independent experiments. A P value of <0.05 was considered statistically significant.

RESULTS

Cytotoxicity

To investigate the cytotoxic effect of DCVC on HTR-8/SVneo cells, protease-based viability and cell death assays were performed using the MultiTox-Glo multiplex cytotoxicity assay kit (Promega). Exposure to DCVC with concentrations up to 50 μM for 5 and 10 h did not significantly increase cell death in HTR-8/SVneo cells as measured by increased luminescence due to protease release from cells with compromised membrane integrity (Fig. 1A). However, luminescence due to extracellular protease activity was 1.3-, 2.0-, and 5.8-fold increased in a concentration-dependent manner after 24 h exposure to 10, 20, and 50 μM DCVC, respectively, relative to that of untreated controls ($P < 0.05$) (Fig. 1A). Similarly, decreases in cell viability were not evident with 10, 20, and 50 μM DCVC treatment for 5 and 10 h, as measured by cellular fluorescence, reflecting intracellular protease activity. Consistent with results of the cell death assay, loss of viability at 24 h was indicated by concentration-dependent decreases of fluorescence to 84%, 75%, and 48% of untreated controls with 10, 20, and 50 μM DCVC ($P < 0.05$) (Fig. 1B). Although modest statistically significant increases in fluorescence were observed with 50 μM DCVC treatment for 5 h and with 20 μM DCVC treatment for 10 h compared with untreated control ($P < 0.05$) (Fig. 1B), these increased fluorescence values trend in a direction opposite to that of cytotoxicity and likely reflect variations in the assay. Based on these cytotoxicity results, 50 μM DCVC was not used in subsequent experiments. Furthermore, treatment for 24 h with 10 and 20 μM DCVC, but not 5 μM DCVC, decreased cellular ATP by 19.6% and 26.1%, respectively, compared with untreated controls ($P <$

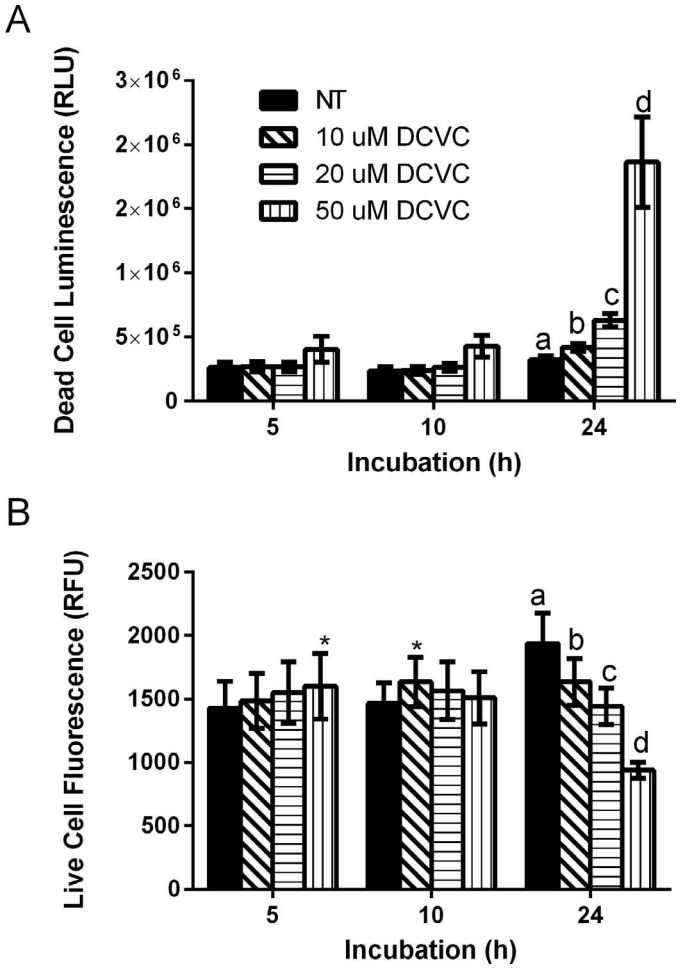

FIG. 1. Time-dependent and concentration-dependent DCVC toxicity in HTR-8/SVneo cells. **A**) Cell death as determined by a luminescence-based assay of extracellular protease activity. **B**) Cell viability as determined by a fluorescence-based assay of intracellular protease activity. Cells were not treated (control) or were exposed to 10, 20, or 50 μM DCVC for 5, 10, or 24 h. Live and dead cells were assessed using a multiplex assay as described in Materials and Methods. Bars represent means ± SEM. Following ANOVA detection of significant treatment and time effects, Tukey post-hoc comparison of means detected significant increases compared with untreated control within the same time point (*$P < 0.05$) and between treatment groups (different letters indicate significantly different groups; $P < 0.05$). N = 3 experiments, and each experiment was performed in triplicate.

0.01) (Supplemental Fig. S1; available online at www.biolreprod.org), suggesting decreased cell number after 24 h of exposure to 10 and 20 μM DCVC.

Cellular Generation of ROS

DCVC stimulated ROS generation, as detected by carboxy-DCF fluorescence. Treatment of HTR-8/SVneo cells with 10 or 20 μM DCVC for 10 h significantly increased carboxy-DCF fluorescence, as quantified by spectrophotometry, by 21.5% and 37%, respectively, compared with that of untreated controls ($P < 0.001$) (Fig. 2A). Representative fluorescence photomicrographs in Figure 2B show increased carboxy-DCF fluorescence in HTR-8/SVneo cells treated with 10 μM DCVC (Fig. 2B, panel b) compared with untreated control (Fig. 2B, panel a), although the fluorescence appeared less intense than that observed with 25 μM *tert*-butyl hydroperoxide, included as a positive control (Fig. 2B, panel c). Figure 2B, panels d, e, and f, show corresponding Hoescht dye nucleus-stained images of cell cultures receiving control, 10 μM DCVC, or 25 μM *tert*-butyl hydroperoxide treatments, respectively.

Cellular Antioxidant Status

Because DCVC increased ROS production, we investigated effects of DCVC on intracellular GSH, a major cellular antioxidant, in HTR-8/SVneo cells. Treatment with 10 or 20 μM DCVC for 24 h decreased GSH concentration in cell lysates by 24.9% and 26.7%, respectively, compared with those in untreated control, as assessed by a luminescence assay ($P < 0.01$) (Fig. 3A). Additionally, 24-h treatment with 20 μM DCVC produced approximately 2.2-fold increased expression of the antioxidant response genes *TXNRD1* and *GLRX2* compared to control ($P < 0.05$) (Fig. 3B), and 10 μM DCVC produced 1.5-fold increased *GLRX2* mRNA expression.

IL-6 Release

Because ROS have been implicated in stimulation of proinflammatory cytokines, we examined the effect of DCVC exposure on IL-6 release. Using ELISA, we detected IL-6 consistently in our technical as well as biological replicates. Treatment of HTR-8/SVneo cells with 10 and 20 μM DCVC for 24 h stimulated concentration-dependent 1.7- and 3.3-fold increases of IL-6, respectively, relative to that of untreated control ($P < 0.05$) (Fig. 4A). In contrast, 10 and 20 μM DCVC had no significant effects at the earlier time point of 10 h. The response observed at 24 h with 20 μM DCVC was similar to the response elicited by LPS (100 ng/ml), included as a positive control ($P < 0.05$), and LPS stimulated 3.3-fold release of IL-6 with 10 h exposure ($P < 0.05$). The β-lyase inhibitor AOAA blocked DCVC-stimulated IL-6 release in HTR-8/Svneo cells ($P < 0.001$) (Fig. 4B), suggesting that DCVC-induced IL-6 release requires β-lyase-dependent metabolic activation of DCVC.

Inhibition of DCVC-Stimulated IL-6 Release by Antioxidant Treatments

We next used antioxidant treatments to test the hypothesis that ROS mediate DCVC stimulation of IL-6 release. HTR-8/SVneo cells were cotreated with the antioxidant (±)-α-tocopherol (50 μM) or pretreated with the iron chelator DFO (1 mM). Treatment with (±)-α-tocopherol (Fig. 5A) or DFO (Fig. 5B) significantly depressed IL-6 release in cells stimulated for 24 h with 10 or 20 μM DCVC compared with cells treated with DCVC without antioxidants ($P < 0.01$).

IL6 *mRNA Expression*

To determine whether the effect of DCVC was mediated at the level of gene transcription, we quantified expression of the *IL6* gene in HTR-8/SVneo cells by using qRT-PCR. Exposure to 10 or 20 μM DCVC for 24 h caused 7.3- or 11.4-fold increased *IL6* mRNA expression, respectively, compared with that in untreated control ($P < 0.01$) (Fig. 6A). No significant increase of *IL6* mRNA was observed with 5 μM DCVC treatment. Similar to its effect on IL-6 release, exposure of HTR-8/SVneo cells to (±)-α-tocopherol significantly inhibited DCVC-stimulated *IL6* mRNA expression by 78.9%, using 10 μM DCVC exposure, and 67.2%, using 20 μM DCVC exposure ($P < 0.05$) (Fig. 6B).

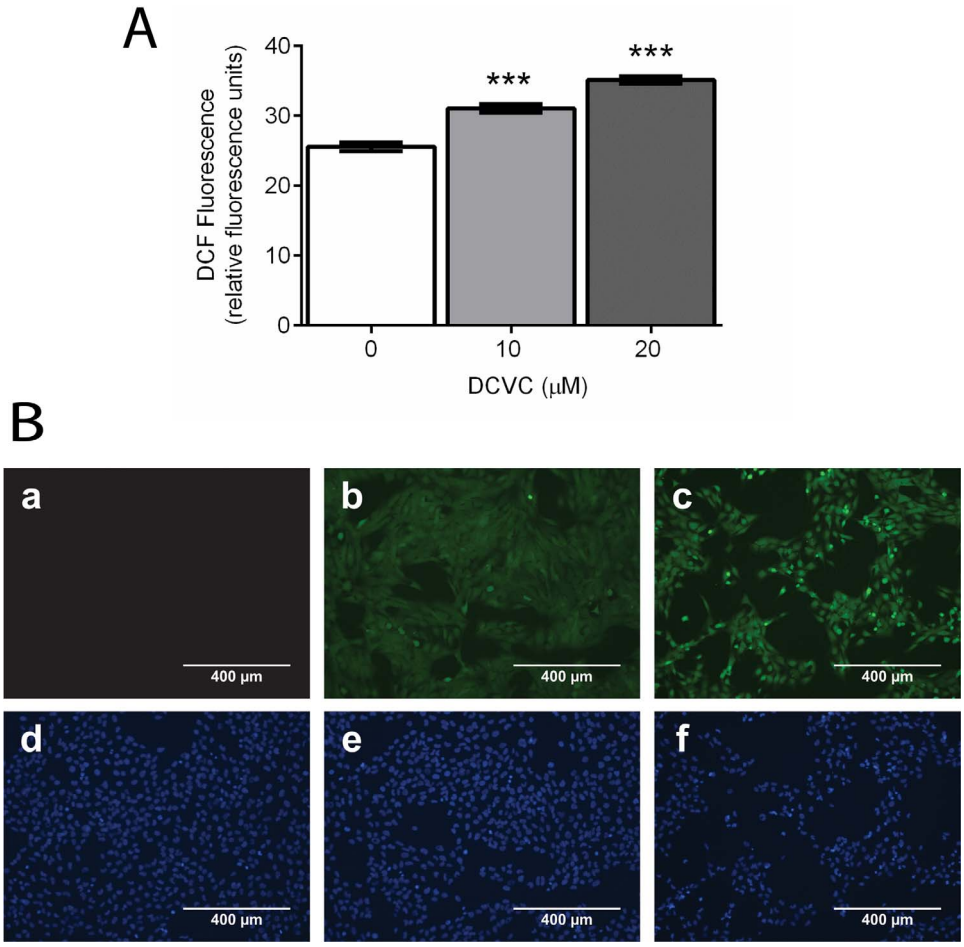

FIG. 2. DCVC-stimulated generation of reactive oxygen species (ROS). **A**) ROS were quantified by spectrophotometric detection of carboxy-DCF fluorescence. Cells were treated with 0 (untreated control), 10, or 20 μM DCVC for 10 h. Data are mean ± SEM. Following ANOVA detection of treatment effect, Tukey post-hoc comparison of means detected indicates statistically significant increases compared with untreated control (***$P < 0.001$). N = 3 experiments, and each experiment was performed in triplicate. **B**) DCVC-stimulated generation of ROS visualized by microscopic detection of carboxy-DCF fluorescence. The HTR-8/SVneo cells were treated with 10 μM DCVC or 25 μM TBHP (positive control) for 10 h, loaded with carboxy-H_2DCF-DA for 1 h, and then counterstained with Hoechst 33342 dye. The top row shows intracellular fluorescence of untreated control (**a**), 10 μM DCVC (**b**), and 25 μM TBHP (**c**). The bottom row shows the corresponding Hoescht nuclear staining images of untreated control (**d**), 10 μM DCVC (**e**), and 25 μM TBHP (**f**). Representative images of 3 experiments are shown.

Mitochondrial Membrane Potential

We used JC-1, a selective MMP dye, to explore the role of mitochondrial dysfunction in DCVC-induced responses. Because JC-1 fluorescence shifts from red to green with membrane depolarization, changes in MMP were quantified by changes in the JC-1 red/green fluorescence intensity ratio by using a spectrofluorometer. Treatment with 5, 10, or 20 μM DCVC significantly decreased JC-1 red/green fluorescence intensity ratio in a concentration-dependent manner by 19.6%, 26.3%, and 36.8% at 5 h; 23.2%, 34.8%, and 44.1% at 10 h; and 24.7%, 31.5%, and 90.5% at 24 h, respectively; compared to untreated control ($P < 0.05$) (Fig. 7A). In separate microscopy experiments, we observed a fluorescence shift from red to green in cells exposed to 5, 10, and 20 μM DCVC for 24 h compared with untreated control, indicative of loss of mitochondrial membrane polarity with DCVC exposure (Fig. 7B). A similar pattern was observed with DCVC treatment for 10 h (data not shown). We then tested the effect of BkA, a mitochondrial membrane transition pore inhibitor [31], on DCVC-stimulated IL-6 release. Pretreatment with 10 μM BkA followed by 20 μM DCVC modestly inhibited IL-6 release by 27% compared with cells treated with DCVC alone ($P < 0.05$) (Fig. 8).

DISCUSSION

Due to improper disposal, TCE has become a widespread air, soil, and drinking water contaminant [1]. Humans rapidly metabolize TCE to bioactive intermediates such as DCVC, a well-characterized kidney toxicant [33]. Although exposure to TCE has been linked to poor birth outcomes [6, 34], DCVC has not previously been considered a potential toxicant to placenta. In the present study, we demonstrated that DCVC stimulated generation of ROS in the human placental cell line HTR-8/SVneo. In addition, we provide new evidence for mechanistic linkages between DCVC-stimulated ROS and increase in the proinflammatory cytokine IL-6.

Our study reports a direct stimulatory effect of DCVC on *IL6* mRNA expression and release of IL-6 protein in HTR-8/SVneo cells, consistent with prior reports that IL-6 is expressed in HTR-8/SVneo cells [35] and in trophoblasts in vivo [36]. The concurrence of DCVC-stimulated increased *IL6* mRNA expression and increased concentration of IL-6 into the medium suggests that DCVC acts at the level of transcription

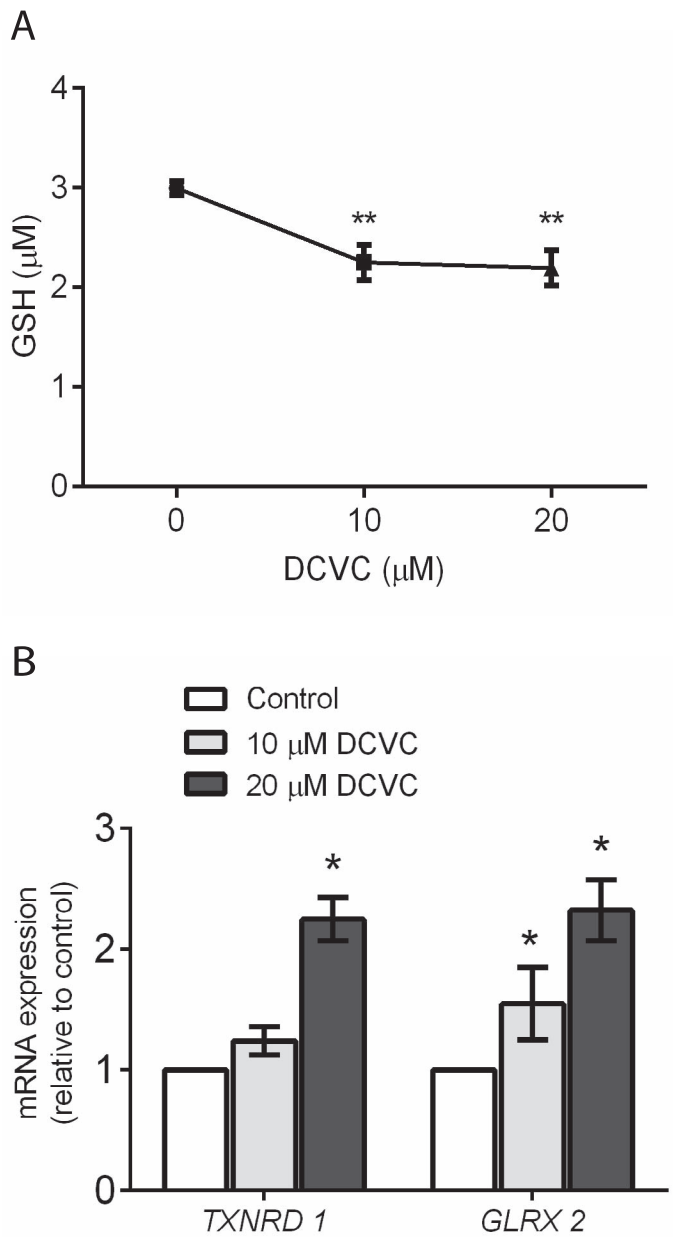

FIG. 3. DCVC effects on antioxidant status of HTR-8/SVneo cells. **A**) DCVC effects on cellular GSH using a luminescence-based assay to quantify GSH concentrations in lysates of cells exposed to 10 or 20 μM DCVC for 24 h. Following ANOVA detection of treatment effect, Tukey post-hoc comparison of means detected statistically significant decreases compared with untreated control (**$P < 0.01$). N = 3 independent experiments, and each experiment was performed in triplicate. **B**) DCVC effects on mRNA expression of the redox-sensitive genes *TXNRD1* and *GLRX2* quantified by qRT-PCR. Cells were exposed to 10 or 20 μM DCVC for 24 h. Bars represent mean ± SEM. Following ANOVA detection of treatment effect, Tukey post-hoc comparison of means detected statistically significant increases compared with untreated control (*$P < 0.05$). N = 3 experiments, and each experiment was performed in triplicate.

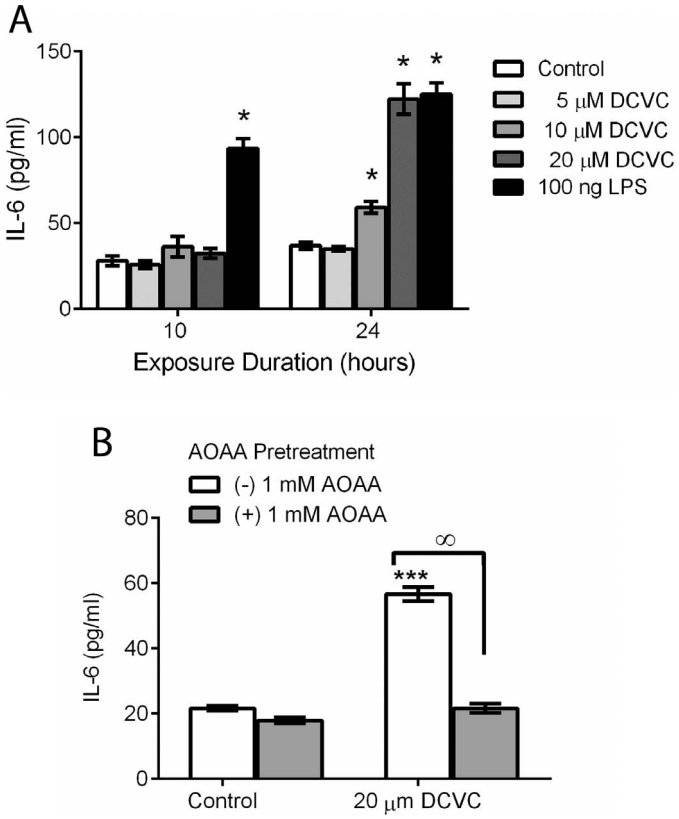

FIG. 4. DCVC effects on IL-6 release from HTR-8/SVneo cells. **A**) Time-dependent and concentration-dependent DCVC-stimulated IL-6 release. IL-6 concentrations were measured in culture medium of cells treated with 0 (untreated control), 5, 10, or 20 μM DCVC or with 100 ng/ml LPS (positive control) for 10 or 24 h. **B**) Effect of treatment with the cysteine-conjugated β-lyase inhibitor aminooxyacetic acid (AOAA) on DCVC-stimulated IL-6 release. Cells were pretreated with 1 mM AOAA for 1 h before a 24-h exposure without or with 20 μM DCVC. IL-6 concentrations were measured in culture medium. The bars represent the means ± SEM. Following ANOVA detection of treatment effect, Tukey post-hoc comparison of means detected statistically significant increases compared with untreated control within the same time point (***$P < 0.001$) and between AOAA treatment groups (∞ $P < 0.05$). N = 3 experiments, and each experiment was performed in triplicate.

to stimulate the IL-6 response. Importantly, DCVC stimulated IL-6 release from HTR-8/SVneo cells to a magnitude comparable to that observed with treatment with the positive control LPS, a strongly immunogenic stimulus. Although there has been little research of DCVC-induced IL-6 release, our findings are consistent with those of a report that DCVC exposure stimulates IL-6 release and increases renal *Il6* mRNA expression in mice [14]. Because growing evidence links improper activation of inflammatory pathways including IL-6 with impaired trophoblast function and poor placental development [19], our results suggest that innate immune response activation may be a mechanism by which DCVC can lead to adverse birth outcomes.

Our finding that exposure to DCVC stimulated increased ROS generation is similar to previously published studies with rat kidney proximal tubular cells [13]. Furthermore, we found that treatment with the antioxidant (±)-α-tocopherol decreased IL-6 release, suggesting that DCVC-stimulated IL-6 release in human placental cells is mediated by ROS. Moreover, treatment with the iron chelator DFO also inhibited IL-6 release, suggesting that Fenton reaction-dependent production of hydroxyl radical from hydrogen peroxide plays an important role in DCVC-stimulated IL-6 release. These findings are in agreement with those of previous reports that DFO decreases DCVC-induced ROS production in rabbit renal cortical slices [13] and inhibits DCVC-induced cytotoxicity in rat kidney epithelial cells [27]. In addition, these results augment our previous reports that the flame retardant chemical brominated diphenyl ether-47 (BDE-47) stimulates release of IL-6 in a ROS-dependent manner from HTR-8/SVneo cells, providing

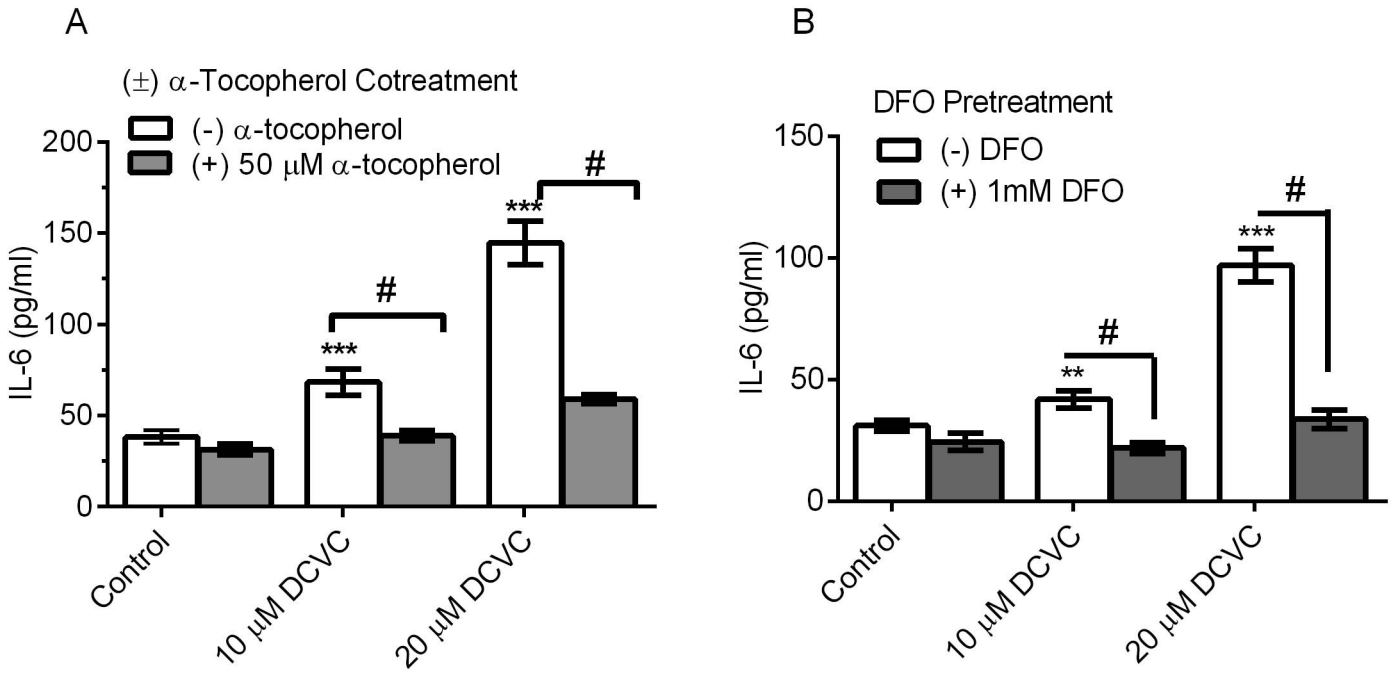

FIG. 5. Effect of antioxidant treatments on DCVC-stimulated IL-6 release. **A**) Cells were cotreated with 50 μM α-tocopherol during a 24-h exposure to 10 or 20 μM DCVC. **B**) Cells were pretreated with 1 mM DFO for 1 h before a 24-h exposure to 10 or 20 μM DCVC. Bars represent means ± SEM. Following ANOVA detection of treatment effect, Tukey post-hoc comparison of means detected statistically significant differences compared with untreated control (no DCVC and no antioxidant; $^{**}P < 0.01$ and $^{***}P < 0.001$), and between cells with and without antioxidant at the same concentration of DCVC ($^{\#}P < 0.05$).

an additional example of an environmental pollutant that modifies cytokine response by an ROS pathway [36, 37].

Consistent with increased ROS, we also found that DCVC decreased GSH in HTR-8/SVneo cells. It should be noted that the assay used in the current study measures GSH concentration relative to cell lysate volume in a 96-well plate. Therefore, further studies using more sensitive approaches such as high-performance liquid chromatography are warranted to determine physiological relevance of DCVC-induced GSH decrease in HTR-8/SVneo cells. Nonetheless, our findings corroborate those from prior studies that show incubation of rat proximal tubular cells with DCVC depletes cellular GSH [38]. GSH, a major intracellular antioxidant, reacts with electrophiles, ROS, and lipid peroxides to protect cells from oxidative stress [39]. Decreased levels of GSH in the maternal blood and in the placenta have been associated with adverse pregnancy outcomes including preeclampsia [40], preterm birth, and IUGR [41]. Concordant with our findings that DCVC increased ROS and decreased GSH levels, we also observed increased expression of the antioxidant genes *GLRX2* and *TXNRD1*. These redox-sensitive genes are disulfide reductases that defend the cells against oxidative stress, DNA damage, and apoptosis [42]. We suggest that HTR-8/SVneo cells increased expression of the redox-sensitive genes *GLRX2* and *TXNRD1*

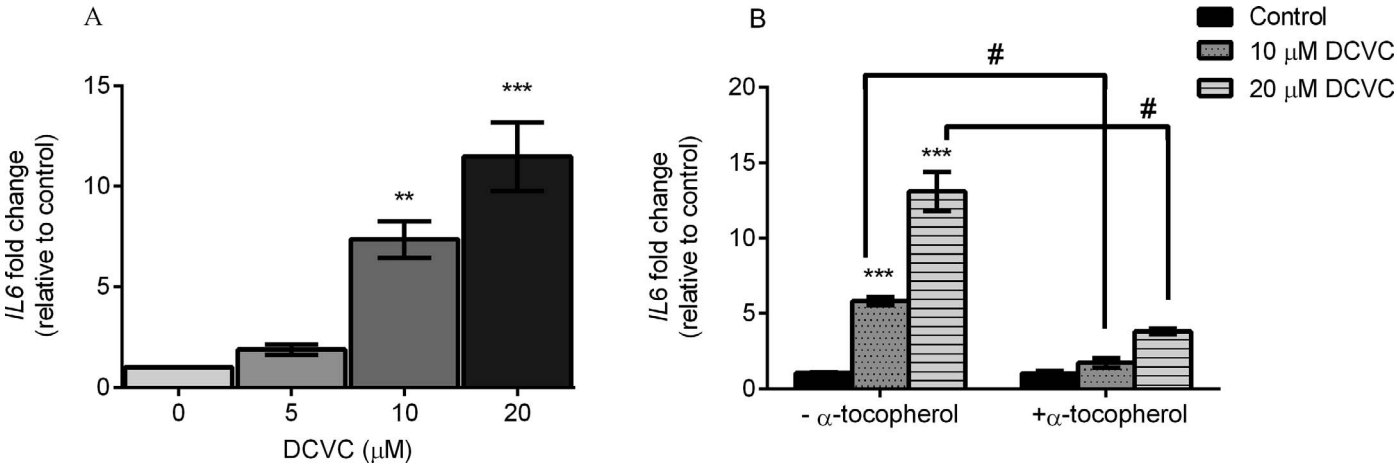

FIG. 6. DCVC effects on *IL6* mRNA expression in HTR-8/SVneo cells. **A**) Concentration-dependent response of *IL6* mRNA expression to treatment with 0 (control), 5, 10, or 20 μM DCVC for 24 h. **B**) Effect of cotreatment with 50 μM α-tocopherol on DCVC-stimulated *IL6* mRNA expression. Data are means ± SEM. Following ANOVA detection of treatment effect, Tukey post-hoc comparison of means detected statistically significant increases compared with untreated control ($^{**}P < 0.01$, $^{***}P < 0.001$) and statistically significant differences comparing samples treated with and without α-tocopherol at the same concentration of DCVC ($^{\#}P < 0.05$). N = 3–4 experiments, and each experiment was performed in triplicate.

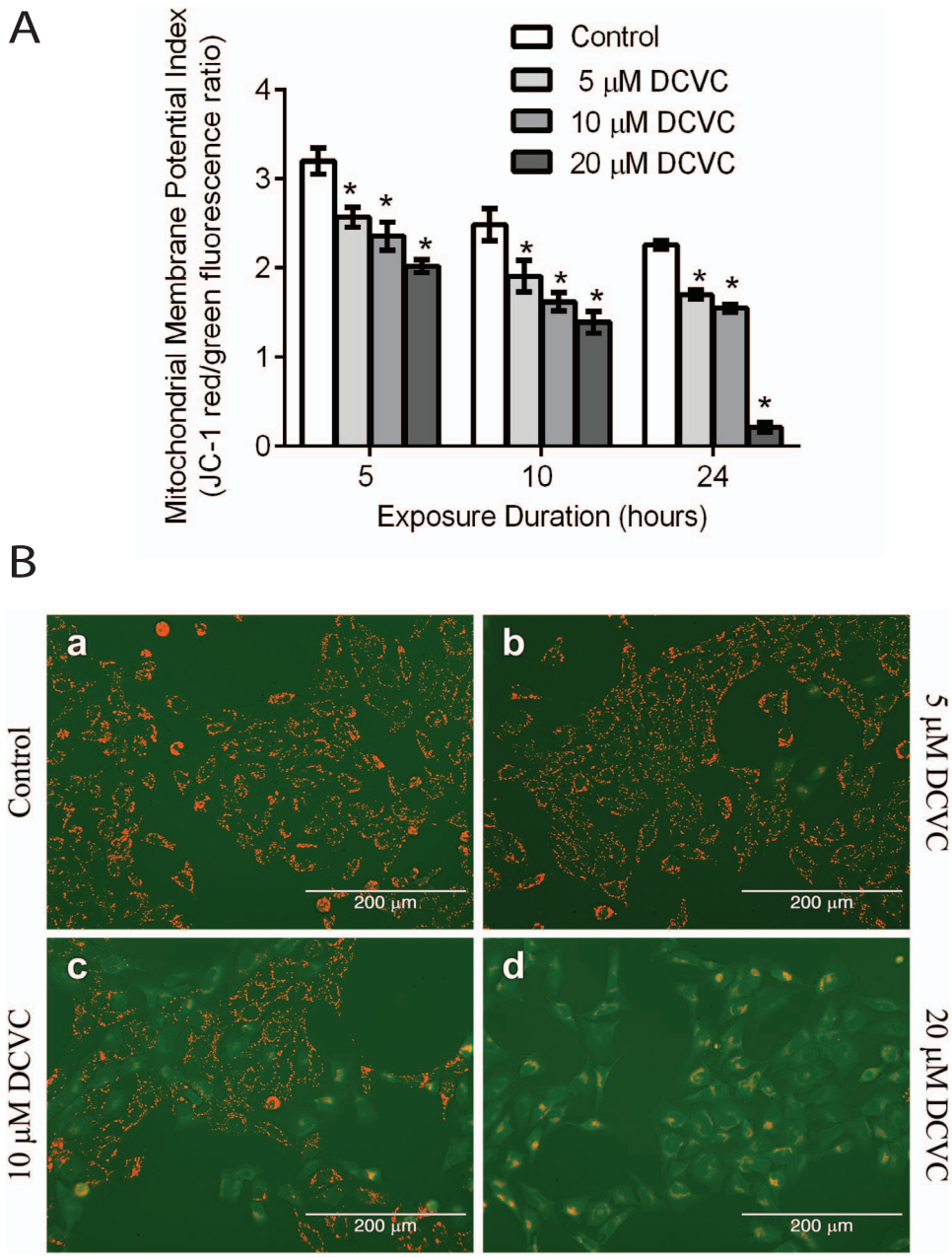

FIG. 7. Effect of DCVC on MMP. JC-1 dye was used to measure changes in MMP in HTR-8/SVneo cells. **A**) Cells were treated for 5, 10, or 24 h with 0 (control), 5, 10, or 20 μM DCVC. Bars are means ± SEM of the red or green fluorescence intensity ratio of JC-1 aggregate-to-monomer, an index of polarization of the mitochondrial membrane. Following ANOVA detection of treatment and time effects, Tukey post-hoc comparison of means detected statistically significant differences compared with untreated control within the same exposure duration (*$P < 0.05$). N = 3 experiments, and each experiment was performed in triplicate. **B**) DCVC-stimulated change of MMP visualized by fluorescence microscopy. Red fluorescence represents the mitochondrial aggregate form of JC-1, indicating normal MMP. Green fluorescence represents the monomeric form of JC-1, indicating depolarized MMP. Cells were exposed for 24 h to: untreated controls (**a**); 5 μM DCVC (**b**); 10 μM DCVC (**c**); or 20 μM DCVC (**d**). Representative images from 3 experiments are shown.

as a response to DCVC-induced increase in cellular ROS. In humans, increased levels of the protein products of *GLRX2* and *TXNRD1* in the placenta have been associated with pre-eclampsia [43]. Furthermore, differential expression of antioxidant response genes has been observed with pregnancy complications such as IUGR and preeclampsia [44–46].

Under normal physiological conditions, small amounts of ROS generated as oxidative phosphorylation byproducts can leak from the mitochondria and form hydrogen peroxide or hydroxyl radical [47]. Because DCVC decreased MMP and mitochondrial dysfunction can lead to enhanced mitochondrial production of ROS [48], we suggest that the DCVC-stimulated ROS in HTR-8/SVneo cells might have originated from mitochondria, at least in part. However, because the mitochondrial membrane transition pore inhibitor BkA significantly attenuated but did not completely inhibit IL-6 release, mitochondrial dysfunction is likely only partly responsible for DCVC-induced IL-6 release in HTR-8/SVneo cells. We obtained similar results in limited experimentation with another mitochondrial membrane transition pore inhibitor, cyclosporine A (data not shown). These findings corroborate previous reports that DCVC induces mitochondrial toxicity in other cells

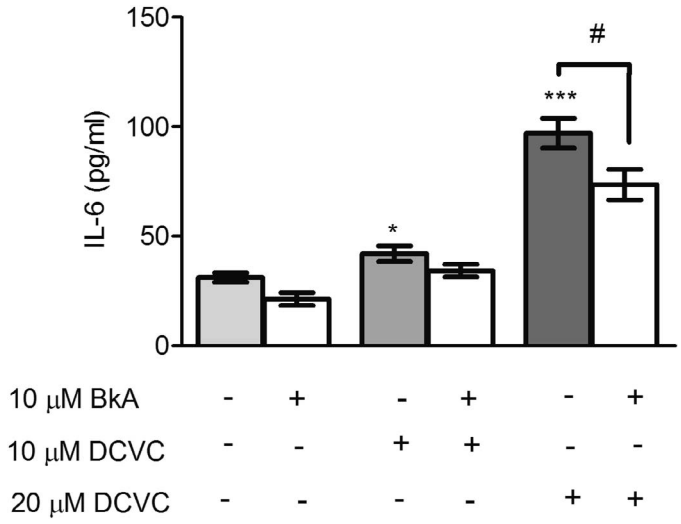

FIG. 8. Effect of BkA, a mitochondrial membrane transition pore inhibitor, on DCVC-stimulated IL-6 release. IL-6 concentrations were measured in culture medium of HTR-8/SVneo cells pretreated with 10 μM BkA for 1 h, followed by culture without or with 10 or 20 μM DCVC for 24 h. Bars are means ± SEM. Following ANOVA detection of treatment effect, Tukey post-hoc comparison of means detected statistically significant increases in DCVC-treated cultures compared with untreated control (no DCVC or BkA; $^{*}P < 0.05$, $^{***}P < 0.001$) and between cultures exposed to 20 μM DCVC without and with BkA ($^{\#}P < 0.05$). N = 3 experiments, and each experiment was performed in triplicate.

and tissues. Specifically, studies in rat proximal tubular cells found that DCVC dissipated MMP [13] and decreased ATP production [49]. In human proximal tubular cells, mitochondrial dysfunction is a necessary step in DCVC-induced toxicity [12]. Moreover, DCVC-stimulated decreases in concentrations of ATP and GSH were the most sensitive endpoints in a cataractogenesis study in rat [32].

The present study supports the role of metabolism in DCVC stimulation of IL-6 release. We observed that treatment with AOAA, a renal β-lyase inhibitor, almost completely inhibited DCVC-induced IL-6 release in HTR-8/SVneo cells. Our findings are consistent with other studies that report DCVC toxicity is partly due to bioactivation by the β-lyase in other cells and tissues [32, 50]. DCVC is bioactivated by the β-lyase to generate the highly reactive intermediate *S*-(1,2-dichlorovinyl)thiol (DCVT) [51]. DCVT is chemically unstable and rearranges to form chlorothioketene (CTK) and chlorothionoacetyl chloride (CTAC), two highly reactive species that are thought to be responsible for DCVC-induced formation of covalent adducts that bind to DNA, protein, and phospholipids [51]. Findings from the current study suggest that β-lyase activity is present in human placental cells, although this was not directly demonstrated. Moreover, the present study suggests that DCVC-stimulated increase in IL-6 is due to a reactive metabolite of DCVC generated via the β-lyase which leads to mitochondrial dysfunction and increased ROS production.

Although it contributes new information on DCVC activity in human placental cells, our study has some limitations. First, the concentrations used in the present study are a magnitude higher than the U.S. Environmental Protection Agency maximum contaminant level standard for drinking water of 5 ppb for the parent compound TCE [1]. Nonetheless, in a study by Lash et al., workers who were exposed to 100 ppm of TCE by inhalation had 50 μM concentration of DCVG, a precursor to DCVC, in plasma [11, 12]. Moreover, the concentrations used in the present study (i.e., 5–50 μM) are in the lower range of concentrations used to determine DCVC-induced toxicity in kidney cells [11, 12].

Second, the current study lacks experiments for functional consequences of the changes observed. Therefore, further investigation is warranted to determine the potential relevance of DCVC on placental development, for example, by assessing the effect of DCVC on trophoblast migration and invasion. Third, although use of a cell line allowed us to study mechanistic linkages between DCVC-stimulated ROS and IL-6, we recognize limitations related to the absence of cell-cell interactions or tissue-tissue interactions when using cell culture models. Moreover, different culture conditions (e.g., cell densities) were used for some assays, which may influence the responses in ways not accounted for here. Therefore, further investigation of placental tissues, including in vivo studies, will be needed to validate the potential relevance of our results to pregnancy. Finally, we used HTR-8/SVneo, an immortalized cell line transfected with SV40. As such, the HTR-8/SVneo cells may respond to stimuli such as LPS and toxicants in a manner different from normal trophoblasts in vivo. Although HTR-8/SVneo cells have a phenotype similar to primary human trophoblasts [25, 52], and retain the ability to migrate and invade, and express the essential trophoblast markers HRA-G, cytokeratin 7 and α5β1 integrin up to passage number 105 [53, 54], HTR-8/SVneo cells have a different epigenetic profile than primary extravillous trophoblasts [55]. Nevertheless, HTR-8/SVneo cells have been a useful model in other studies to examine placentation and physiologically invasive extravillous trophoblast [56–59].

In summary, we demonstrated that DCVC, a bioactive metabolite of TCE, stimulated reactive oxygen species (ROS) generation in human placental cells. In addition, we report novel findings linking DCVC-stimulated ROS to increased expression and release of the proinflammatory cytokine IL-6. To our knowledge, this is the first study to report DCVC-induced ROS-mediated stimulation of IL-6 in human placental cells. We observed significant effects with DCVC concentrations ranging from 5 to 20 μm, which is a lower concentration range than has been shown to be toxic in kidney cells, suggesting that risk assessors should include the placenta as a potential target organ for TCE-induced toxicity in humans. Furthermore, results from our study suggest that exposure to TCE and other environmental toxicants that increase generation of ROS in placental cells may contribute to increased risk for adverse pregnancy outcomes by dysregulating proinflammatory cytokine production.

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