THE EFFECT OF TRICHLOROETYLENE ON ADVERSE BIRTH OUTCOMES

by

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DEDICATION

To my family and friends.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Trichloroethylene (TCE) is a chlorinated solvent and a widespread environmental pollutant implicated in adverse reproductive outcomes in humans. TCE toxicity primarily occurs through its biotransformation to toxic metabolites, including S-(1,2-dichlorovinyl)-L-cysteine (DCVC), which induce oxidative stress and inflammation in the liver and kidney. This thesis used both in vivo and in vitro approaches to test the hypothesis that TCE induces oxidative stress-mediated inflammation in gestational tissues, which contributes to adverse pregnancy outcomes.

To investigate TCE-induced oxidative stress and inflammation in gestational tissues, pregnant Wistar rats were exposed daily to 480 mg/kg of TCE from gestational day 6 – 16. Placenta, maternal serum, and amniotic fluid were collected prior to onset of parturition. Exposure to TCE significantly decreased average fetal weights, increased placental 8-hydroxyguanosine, a biomarker of oxidative DNA damage, and increased placental global 5-hydroxymethylcytosine, a marker of DNA methylation changes. A significant increase in interleukin (IL)-6 levels in maternal serum was observed, and immunohistochemistry analysis showed presence of neutrophils in the decidua. These results suggest that TCE exposure in vivo induces systemic inflammation and oxidative stress in the placenta, which can lead to adverse pregnancy outcome. Moreover, DCVC, the bioactive metabolite of TCE, was detected in the amniotic fluid by HPLC/MS/MS, suggesting that the placenta may be capable of metabolizing TCE to DCVC.

The effect of DCVC on proinflammatory cytokine release and stimulation of reactive oxygen species (ROS) was tested in human placental cells (HTR-8/SVneo). Results show a direct stimulatory effect of DCVC on release of IL-6. DCVC induced mitochondrial dysfunction and stimulated ROS. DCVC-induced IL-6 release was inhibited by the antioxidants (±)α-tocopherol and deferoxamine, implicating the involvement of ROS in stimulation of IL-6 release.

In conclusion, results from the present study show that exposure to TCE stimulates oxidative stress and inflammation in gestational tissues and cells. Because oxidative stress and inflammation have been associated with adverse birth outcomes, these data provide support for a plausible biological explanation for TCE exposure associations with poor pregnancy outcomes. As such, this thesis provides new knowledge about the potential mechanisms by which TCE and other environmental contaminants may contribute to adverse pregnancy outcomes.

CHAPTER 1. INTRODUCTION.

Adverse birth outcomes: a significant public health problem

Adverse birth outcomes including spontaneous abortion, stillbirth, congenital malformation, intrauterine growth restriction (IUGR), and small-for-gestational-age (SGA) or low birth weight are significant determinants of infant health and survival. Normal term pregnancy is between 37 and 41 completed weeks (Martin *et al.*, 2015). Preterm birth is defined as birth before 37 weeks of completed gestation (Martin *et al.*, 2015) . Infants that are born premature have low birth weight but also some infants can be born SGA. The term SGA is used to refer to newborns whose birth weights are below the 10th percentile for their gestational age at birth whether due to pathologic or nonpathologic factors. By comparison, IUGR refers to fetuses with in utero weights below the 10th percentile for gestational age due to some factor(s) that inhibit their growth potential (Stillerman *et al.*, 2008). Conditions related to SGA and preterm birth are the second leading cause of infant death in the United States (Mathews and MacDorman, 2013). The infant mortality rate for low birth weight babies is approximately 24 times that of the infant rate for normal weight babies (Mathews and MacDorman, 2013). Likewise, infants born before 32 weeks of completed gestation are 74 times more likely to die than those born at term (Mathews and MacDorman, 2013). Infants that are

born prematurely and those that are born SGA are likely to have underdeveloped organs, suffer from respiratory distress syndrome and have general health problems that may persist throughout their lifetime (Stillerman *et al.*, 2008).

Although preterm births rates have declined in recent years in the US (from 11.55% in 2012 to 11.39% in 2013, they remain high among Hispanic and non-Hispanic Black women who have rates of 11.31% and 16.27% of births, respectively (Martin *et al.*, 2015). In comparison, for non-Hispanic White women the rate is 10.17% (Martin *et al.*, 2015) . In 2013 the overall rate for low birth weight remained unchanged at 8.02% (Martin *et al.*, 2015) . Low birth weight remained unchanged between 2012 and 2013 among non-Hispanic White infants at 6.98% and for non-Hispanic Black infants the rate was 13.08%. The rate for Hispanic infants was slightly increased from 6.97% in 2012 to 7.09% in 2013 (Martin *et al.*, 2015) . Factors that contribute to low birth weight and preterm birth include medical interventions such as labor induction, as well as in vitro fertility treatments that have increased the rates of multiple births. Other factors related to preterm birth, SGA, and IUGR include impaired placenta growth and function, maternal alcohol and tobacco use, and maternal and fetal infections (Stillerman *et al.*, 2008).

Adverse birth outcomes and environmental pollution

Exposure to environmental contaminants is increasingly thought to play a role in low birth weight and preterm birth. Several epidemiological studies have found significant association between maternal exposure to environmental contaminants and adverse birth outcomes (Stillerman *et al.*, 2008; Ferguson *et al.*, 2013). For example, exposure to airborne particulate matter is correlated with low birth weight, IUGR, and perterm delivery (Kannan *et al.*, 2006; Stillerman *et al.*, 2008). Furthermore, cigarette smoke exposure during pregnancy has been associated with preterm delivery (Windham *et al.*, 2000). Additionally, a meta-analysis conducted by Leonardi-Bee et al. found that exposure to environmental tobacco smoke was associated with increased risk of low birth weight and SGA (Leonardi-Bee *et al.*, 2011). In addition, women exposed during pregnancy to phthalates, chemicals found in plastics, personal

care products, and other consumer products, have significantly increased odds of delivering preterm (Meeker *et al.*, 2009; Ferguson *et al.*, 2011). Similarly, a prospective study found in utero exposure to dichlorodiphenyltrichloroethane (DDT) was associated with preterm delivery (Longnecker *et al.*, 2001). Moreover, some studies have found that polychlorinated biphenyl (PCB) exposure is associated with increased risk of low birth weight and restricted fetal growth, including a large prospective cohort study of the Danish National Birth Cohort (Halldorsson *et al.*, 2008).

Trichloroethylene – a common environmental pollutant linked to adverse pregnancy outcomes

TCE is a chlorinated organic solvent that has been used to degrease metal parts, to strip paint, and as a dry cleaning solvent. Although the amount of TCE released in the environment has decreased from >57 million pounds in 1988 to approximately 2.4 million pounds in 2010 (U.S. EPA, 2011), TCE is still an abundant pollutant (Chiu *et al.*, 2013). Due to improper disposal, TCE is commonly found as a contaminant in drinking water, soil and air (U.S. EPA, 2011). Additionally, it is estimated that about 3.5 million workers in the United States are exposed to TCE (Bakke *et al.*, 2007). TCE is a liver and kidney carcinogen (Rhomberg, 2000; Forkert *et al.*, 2006) and has been recently classified as a known human carcinogen by the International Agency for Review on Cancer (IARC) (Guha *et al.*, 2012; IARC, 2014). TCE is also a developmental and reproductive toxicant (Green *et al.*, 1997; Lash *et al.*, 2000c; Forkert *et al.*, 2002; Forkert *et al.*, 2003).

Epidemiologic and animals studies indicate that TCE is a reproductive and developmental toxicant (Chiu *et al.*, 2006). TCE is capable of crossing the placenta (Beppu, 1968; Laham, 1970). In humans, maternal inhalation exposure to TCE levels ranging from 0.18

to 140 µg/m^3 through in-home vapor intrusion has been significantly associated with term low birth weight (LBW) (risk ratio (RR) = 1.68; 95% CI: 1.20, 2.34), and SGA (RR= 1.23 ; 95% CI: 1.03, 1.48) (Forand *et al.*, 2012). Another epidemiological study reported an association between maternal exposure to TCE in drinking water with SGA (odds ratio (OR) = 1.55), fetal death (OR= 2.57), eye malformation (OR=4.41), cleft lip (OR=2.21), and neural tube defect (OR=2.21) (Bove *et al.*, 2002). Furthermore, inhalation exposure of pregnant Wistar rats to 100 ppm (535 mg/m^3) TCE produced increased fetal loss, decreased average fetal weight, and increased incidence of abnormal ossification (Healy *et al.*, 1982). In addition, Narotsky and Kavlock found increased incidences of full-litter resorptions, decreased litter sizes, and decreased mean pup birth weights at all treatment levels in pregnant rats orally exposed to TCE at 0, 1,125, and 1,500 mg/kg-day (Narotsky and Kavlock, 1995). Likewise, oral exposure of TCE at 475 mg/kg-d in rats increased incidences of fetal loss and percentage of pups with eye abnormalities (Narotsky *et al.*, 1995). Despite these observations, the mechanism by which TCE induces adverse pregnancy outcomes is unknown.

TCE metabolism

TCE toxicity is primarily dependent on its metabolism, which is tissue-, species- and sex-dependent (Lash *et al.*, 2000b). The major routes of human exposure to TCE are inhalation and ingestion. With oral exposure, TCE is absorbed from the gastrointestinal tract and distributed to the liver and kidney where it is metabolized (Lash *et al.*, 2000b). TCE is metabolized via two separate pathways (Figure 1). In humans and rodents, the major pathway for TCE is by cytochrome P450-mediated oxidation in the liver to an epoxide, then to chloral hydrate, then to trichloroethanol and its glucuronide conjugate, and eventually to trichloroacetate and dichrloroacetate, which are excreted in the urine (Lash *et al.*, 2000b; Lash *et al.*, 2000a). The

second, but lower kinetically favored pathway, is through conjugation with glutathione (GSH) (Lash *et al.*, 2000a; Lash *et al.*, 2000b). In rats and humans, TCE can be conjugated in the liver and kidney by glutathione S-transferase (GST) to S-(1,2-dichlorovinyl)glutathione (DCVG) (Lash *et al.*, 2000b; Lash *et al.*, 2000a). Subsequently, DCVG is biotransformed in the kidney by γ-glutamyl transpeptidase (GGT) to S-(1, 2-dichlorovinyl) cysteine (DCVC), which is further metabolized by cysteine-conjugated β-lyase to an unstable intermediate capable of forming covalent bonds with cellular nucleophiles, leading to cytotoxicity. DCVC can also be metabolized by flavin monooxygenase (FMO) to form a reactive sulfoxide (Lash *et al.*, 2000b; Lash *et al.*, 2000a). Alternatively, DCVC undergoes N-acetylation and is excreted in the urine as NAcDCVC or the sulfur conjugated form NAcDCVCS (Lash *et al.*, 2000a; Lash *et al.*, 2000b). TCE metabolism is species dependent, and studies show that human metabolism of TCE is more similar to metabolism in rats compared with mice (Lash *et al.*, 2000b). For example, chloral hydrate formation in mouse lung due to TCE, which causes lung injury, is significantly higher than in rats or humans (Lash *et al.*, 2000a; Lash *et al.*, 2000b). Moreover, mice metabolize and eliminate TCE more rapidly than rats and humans (Lash *et al.*, 2000a; Lash *et al.*, 2000b). Although the placenta expresses GST and cytochrome P450 enzymes (Juchau, 1980), and TCE has been shown to cross the placenta (Beppu, 1968), little is known about TCE metabolism and toxicity in the placenta.

Placenta as a toxicant target

The placenta is a critical organ for life-long maternal and fetal health. Growing rapidly in the first few weeks of pregnancy, the placenta is a multifaceted organ. It functions as immune, hepatic, renal, and respiratory systems for the fetus (Furukawa *et al.*, 2011; Guttmacher *et al.*, 2014). In addition, the placenta performs nutritional transport, drug metabolizing activity, and

endocrine action (Prouillac and Lecoeur, 2010). Therefore, the placenta is a highly susceptible target organ for drug- or chemical-induced adverse effects, and many placenta-toxic agents have been reported (Juchau, 1973; Leazer and Klaassen, 2003).

In women during the first trimester, the maternal-fetal placenta unit develops a unique immunologic tolerance coupled with profound remodeling of the uterine vasculature and invasive growth of the placenta into the decidua (Cross *et al.*, 1994; Cross, 2006). The extravillous trophoblast cells migrate and invade the decidua resulting in remodeling of the spiral arteries (Pijnenborg *et al.*, 1983). This vascular remodeling is important to ensure adequate blood supply in the placenta (Pijnenborg *et al.*, 1981). Trophoblast migration and invasion is a highly regulated process during placentation. Although incompletely understood, key factors responsible for regulating trophoblast migration and invasion include cell adhesion molecules, extracellular matrix proteins, and matrix degrading enzymes (Cross *et al.*, 1994; Burrows *et al.*, 1996). Furthermore, maternal environment also plays a role in regulating trophoblast invasion by regulating cytokines, prostaglandins, production of maternal leukocytes and decidua natural killer cells (Martinez-Varea *et al.*, 2014). Thus, impaired placenta structure and function is associated with several pathologies of pregnancy, including preeclampsia, preterm birth, and fetal growth restriction (Pijnenborg *et al.*, 1991; Norwitz, 2007; Cotechini *et al.*, 2014). Moreover, insufficient nutrient supplement to the fetus resulting from poor placentation has been associated with later onset of adult disease, including cardio-vascular disease and type-2 diabetes (Barker and Thornburg, 2013; Hennington and Alexander, 2013).

Comparison between rat and human placentas

Rat models have been useful in evaluating environmental toxicants and drug-induced placental injury and pregnancy-related problems, including preeclampsia, IUGR, and gestational

diabetes (Rossant and Cross, 2001; Acar *et al.*, 2014). The overall structure and development of human and rat placenta are similar (Acar *et al.*, 2014), and both have hemochorial placentas (Furukawa *et al.*, 2011). However, there are some differences between rat and human placentas, including embryo/fetal period ratio, implantation type, function of the yolk sac, and hormone synthesis (Furukawa *et al.*, 2011). Furthermore, rat placenta has a single cell layer but the human placenta has three cell layers between the maternal and fetal blood supply (Furukawa *et al.*, 2011). Consequently, the rat may be a poor animal model choice for studies that aim to investigate placental endocrine disrupting chemicals (Nakanishi, 2007). In addition, trophoblasts in humans secrete much larger amounts of estrogen and progesterone than trophoblasts in rats (Freemark *et al.*, 1993). However, in the present study, rat is an adequate model because TCE metabolism in rat is similar to humans, and we are not investigating endocrine-related endpoints. Nevertheless, the differences and similarities between human and rat placenta must be taken into account when extrapolating toxicant-induced adverse effects in the placenta leading to poor pregnancy outcome. However, rat is an adequate model to use in the present study because TCE metabolism in rat is similar to humans, and we are not investigating endocrine related endpoints.

Proinflammatory cytokines relevant to pregnancy outcomes

The innate immune response plays critical roles in placentation and parturition (Lappas *et al.*, 2003). Dysregulated release of cytokines in the gestational tissues is associated with various pathologies of pregnancy, including preterm labor, preeclampsia, and intrauterine growth restriction (Challis *et al.*, 2009). Increased levels of cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α, as well as C-reactive protein, were found in plasma of women with preeclampsia (Ouyang *et al.*, 2009). Furthermore, elevated levels of IL-6 are frequently

found in pregnancy disorders including recurrent miscarriage, preeclampsia and preterm delivery (Prins *et al.*, 2012).

IL-6 is a multifunctional cytokine with an important role in innate immune response during pregnancy (Jovanovic and Vicovac, 2009). Studies show that during the first trimester, IL-6 is released in human gestational membranes and placenta (Kameda et al., 1990). Furthermore, it is highly expressed in syncytiotrophoblasts and extravillous trophoblasts, and plays a crucial role in placentation (Jauniaux et al., 1996; Kauma et al., 1993). In humans, elevated levels of IL-6 in the second trimester are associated with preterm delivery (Wenstrom *et al.*, 1998). Notably, IL-6 and TNF-α can initiate the production of matrix metalloproteases and prostaglandins, which are important factors in term and preterm birth (Romero *et al.*, 2007; Challis *et al.*, 2009). In humans, TNF-α stimulates the production of MMP1, MMP3, and prostaglandin E2 (PGE2) in cultured amnion fibroblasts and epithelial cells (Uchide *et al.*, 2012), and MMP9 in extraplacental membranes (Kumar *et al.*, 2006). Similarly, IL-6 stimulates the production of PGE2 in human decidual and amnion cells (Uchide *et al.*, 2012). These findings suggest that increased levels of pro-inflammatory cytokine release in gestational tissues can lead to untoward pregnancy outcomes.

Environmental toxicants have been shown to induce cytokines and prostaglandin release in gestational tissues and cells. For example, studies from our laboratory showed that *tert*-butyl hydroperoxide, a prototypical pro-oxidant, stimulated IL-6 and prostaglandin release in a human placental cell line (Korte, 2013). Other studies demonstrated that polybrominated diphenyl ether 47 induced IL-6 and IL-8 release (Park *et al.*, 2014), and monoethylhexyl phthalate stimulated prostaglandin release (Tetz *et al.*, 2013) in human placental cells in culture. Furthermore, polychlorinated biphenyls (PCB) stimulated release of arachandonic acid and prostaglandins in

cultured rat amnion cells (Brant and Caruso, 2006). Because proinflammatory cytokines and prostaglandins are important mediators for parturition, their modulation by environmental contaminants suggests a mechanism for toxicant-induced disruption of pregnancy.

Oxidative stress relevant in parturition

Oxidative stress is defined as the imbalance between cellular pro-oxidants and antioxidants resulting in increase of reactive oxygen species (ROS) (Jones, 2006). Although increased intracellular ROS production can be physiologically harmful, ROS function as important regulators of intracellular signaling pathways (Finkel, 2001). ROS, including hydrogen peroxide, hydroxyl radical, superoxide anion, and singlet oxygen, are primarily produced through phagocytosis and through leakage from the mitochondria during cellular respiration (Fisher, 2009). ROS can function in signal transduction by modifying proteins and alter gene transcription leading to activation of inflammatory and anti-inflammatory mediators (Reuter et al., 2010). In addition, decreased glutathione, a major intracellular antioxidant, is associated with increased ROS leading to augmentation of pro-inflammatory signals (Haddad, 2002).

In human gestational tissues, increased oxidative stress is associated with pathophysiology of pregnancy, including preterm labor, preeclampsia, intrauterine growth restriction, and SGA (Al-Gubory *et al.*, 2010). For example, SGA is associated with increased markers of oxidative stress, including malondialdehyde (MDA) in the maternal serum and cord blood, increased expression of 4-hydroxynonenal in the placenta (Gveric-Ahmetasevic *et al.*, 2009), and higher urinary 8-hydroxy-deoxyguanosine (8-OHdG) (Hsieh *et al.*, 2012). In humans, preeclampsia is also associated with increased levels of oxidative stress markers, including F_2-isoprostanes in the amniotic fluid, nitrotyrosine in maternal plasma, and 4-hydroxynonenal staining in the placenta (Raijmakers *et al.*, 2004; Wang *et al.*, 2011). In addition, placenta

antioxidant capacity as indicated by vitamin E concentrations or expression and activity of antioxidant enzymes is decreased in preeclampsia (Raijmakers *et al.*, 2004).

A growing body of literature reports a link between increased ROS and activation of innate immune response within the gestational compartment leading to adverse birth outcomes. ROS can act as signaling molecules by stimulating prostaglandin and cytokine release in human placenta and extraplacental membranes (Lappas *et al.*, 2003). For example, treatment of extraplacental membranes with the antioxidant, N-acetylcysteine (NAC), suppressed the expression of proinflammatory cytokines and MMPs by inhibiting the nuclear factor-κB (NF-κB), an ROS activated transcription factor (Lappas *et al.*, 2003). Treatment with NAC also prevented lipopolysaccharide (LPS)-stimulated parturition and fetal death in mice (Buhimschi *et al.*, 2003) and LPS-induced pro-inflammatory cytokine and prostaglandin release from human extraplacental membranes (Lappas *et al.*, 2003). In mice, pretreatment with NAC has been shown to significantly attenuate LPS-induced elevation in TNF-α concentration in maternal serum and amniotic fluid and lipid peroxidation in maternal and fetal livers, as well as fetal mortality, growth, and skeletal development retardation (Xu *et al.*, 2005). Together, these findings implicate interplay between oxidative stress and inflammation in the etiology of adverse pregnancy outcomes.

TCE-induced oxidative stress and inflammation

Due to pervasive presence of TCE in the environment, people are mostly exposed to the compound through indoor air, ambient outdoor air, and contaminated drinking water; limited exposure can occur through ingestion of contaminated food (Chiu *et al.*, 2006). Environmental and occupational exposures to TCE have been associated with inflammatory diseases in humans, including dermatitis, arthritis and autoimmune disease (Weinhold, 2009; Chiu *et al.*, 2013).

Additionally, oral exposure to TCE is associated with oxidative stress including increased lipid peroxidation, oxidative DNA damage, and decreased antioxidant defense, in the liver and kidneys of rodents (Cojocel *et al.*, 1989; Ogino *et al.*, 1991). Furthermore, the TCE metabolites trichloroacetate (TCA) and dichloroactetate (DCA) increase reactive oxygen species and inflammation in liver and kidney of mice (U.S. EPA, 2011). The TCE metabolite DCVC, a nephrotoxicant, covalently binds to protein and lipids leading to mitochondrial dysfunction, formation of reactive oxygen species, and activation of stress response genes in kidney cells (van de Water *et al.*, 1993; van de Water *et al.*, 1994; Xu *et al.*, 2008). DCVC also induces lipid peroxidation and covalently binds to protein, leading to thiol depletion, effects which were reversed by antioxidants diphenyl-p-phenylenediamine (DPPD) and deferoxamine (an iron chelator) in rat proximal tubular cells (Chen *et al.*, 1990). Perturbation of mitochondrial calcium homeostasis is an early effect of DCVC exposure and a key step in DCVC toxicity in the kidney (Xu *et al.*, 2008). DCVC stimulates hydroperoxide formation in rat kidney proximal tubular cells, which is blocked by treatment with antioxidants (van de Water *et al.*, 1994). Additionally, DCVC activates NF-κB, a protein complex involved in cellular response to stimuli including reactive oxygen species, free radicals and cytokines (van de Water *et al.*, 1994). Furthermore, a recent study found that exposure to DCVC in mice induced IL-6, TNF-α and cyclooxygenase 2 (cox2) mRNA expression in the kidney (Shirai *et al.*, 2012). Although exposure to TCE has been shown to induce oxidative stress and inflammation in other tissues, to our knowledge, no studies have explored TCE-induced stimulation of these pathways in gestational tissues leading to adverse birth outcomes.

Overall Hypothesis

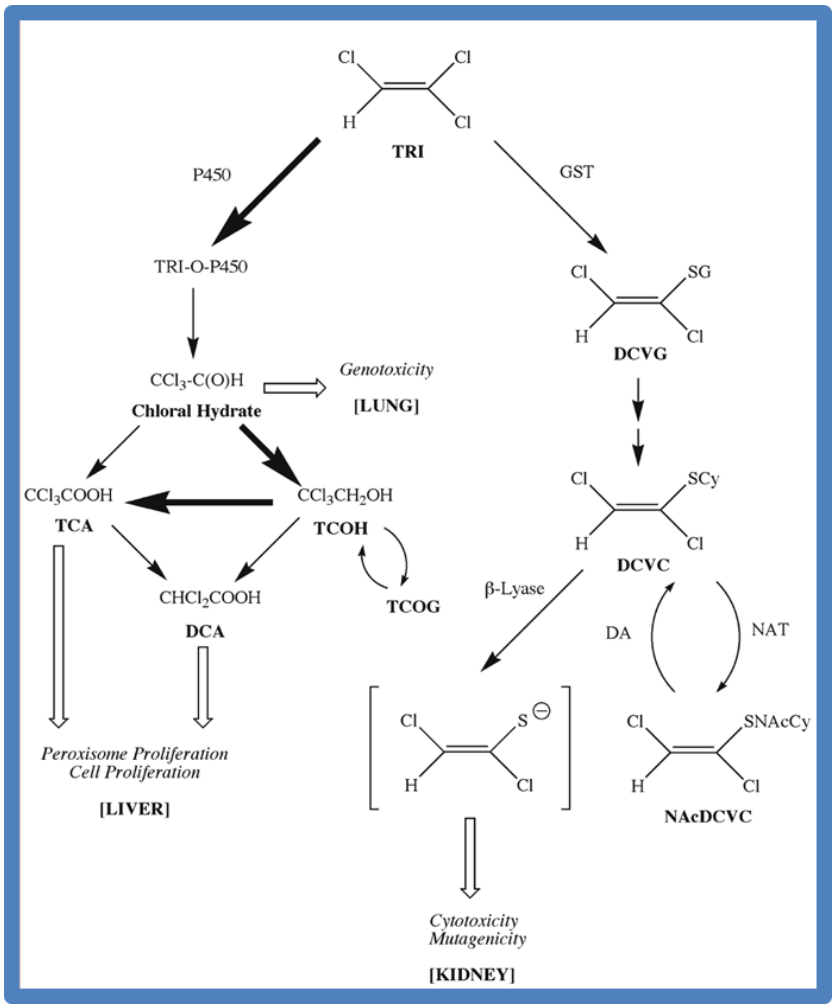
TCE and its bioactive metabolite DCVC have been shown to induce oxidative stress and inflammation in the liver and kidney (Toraason *et al.*, 1999; Lock and Reed, 2006; Wang *et al.*, 2013), the primary known sites for TCE metabolism and toxicity (Lash *et al.*, 2000b). Although the placenta is a highly metabolic organ (Gude *et al.*, 2004), and oxidative stress and inflammation in the placenta have been associated with adverse pregnancy outcomes (Challis *et al.*, 2009; Al-Gubory *et al.*, 2010), little is known about TCE metabolism and toxicity in placental tissue. Because the placenta is a critical tissue for pregnancy, this dissertation addresses data gaps in our understanding of the mechanism by which TCE exposure may contribute to poor obstetrical outcomes. In this thesis, we used the Wistar rat model to evaluate whether exposure to TCE activates inflammatory pathways and stimulates oxidative stress in vivo, in combination with vitro human cell culture methods to test the hypothesis that oxidative stress initiates activation of inflammatory pathways. Because in vivo and in vitro approaches each have advantages and disadvantages, utilizing both approaches in this thesis allowed unique opportunities for exploring mechanistic relationships with potential physiologic relevance regarding TCE exposure and poor pregnancy outcomes.

We hypothesize that exposure to TCE induces oxidative stress-mediated activation of inflammatory pathways in gestational tissues and cells leading to adverse pregnancy outcomes. The specific aims of this dissertation are:

Specific Aim 1: Test the subhypothesis that exposure to TCE increases production of reactive oxygen species and activates inflammatory pathways in the placenta of rats.

Specific Aim 2: Test the hypothesis that DCVC is a bioactive TCE metabolite for toxicity in human placental cells.

Specific Aim 3: Determine the metabolic profile of TCE in the rat placenta and amniotic fluid.



(Lash *et al.*, 2000a)

Figure 1.1. Metabolism of TCE by cytochrome P450-dependent oxidation and GSH conjugation pathway. CYP, cytochrome P-450; DCA, dichloroacetic acid; DCVC, S-(1,2-dichlorovinyl)-L-cysteine; DCVG, S-(1,2-dichlorovinyl)glutathione; DCVT, S-(1,2-dichlorovinyl)thiol; GST, glutathione S-transferase; NAcDCVC, N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine; TCA, trichloroacetic acid; TCE, trichloroethylene; TCE-O-CYP, trichloroethylene-oxide-cytochrome P-450 complex; TCOH, trichloroethanol; TCOG, trichloroethanol glucuronide; NAT, Nactyltransferase.

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CHAPTER 2. TRICHLOROETHYLENE-INDUCED OXIDATIVE STRESS AND INFLAMMATION IN PREGNANT WISTAR RAT MODEL OF FETAL GROWTH RESTRICTION

Abstract

Trichloroethylene (TCE) is a widespread environmental contaminant that has recently been classified as a known human carcinogen by IARC, as well as by the National Toxicology Program (NTP). A recent epidemiologic study reported significant associations between maternal TCE exposure and decreased birth weight; however, limited studies have explored mechanisms of TCE toxicity in the placenta and its impact on pregnancy outcomes. The objective of this study is to investigate TCE-induced oxidative stress and inflammation in rat placenta tissues leading to adverse birth outcomes. Placental tissues were collected before onset of parturition from pregnant Wistar rats administered daily oral doses of 480 mg TCE /kg body weight or vehicle (controls) from gestational day (gd) 6 – 16. The tissues were analyzed for markers of oxidative stress, inflammation, and epigenetic changes. Additionally, maternal serum was analyzed for levels of the proinflammatory cytokine interleukin (IL)-6. We found that exposure to TCE significantly decreased average fetal weights. We also found that TCE significantly increased 8-hydroxy-deoxyguanosine, a biomarker of oxidative DNA damage, as well as global 5-hydroxymethylcytosine, a marker of DNA methylation changes, in the placenta: however, levels of 5-methylcytosine remained unchanged. We observed a significant increase in proinflammatory cytokine interleukin (IL) 6 levels in maternal serum. Immunohistochemistry analysis of the placenta showed the presence of neutrophils in the decidua basalis in TCE-treated

rats. Moreover, microarray gene expression analysis detected increased mRNA levels of genes that are involved in immune system regulation, including cd74, Rt1-Ba, and Rt1-Da. Pathway analysis showed an up-regulation of inflammation-related pathways and down-regulation of metabolic hemostasis pathways. Our results demonstrate that exposure to TCE in time-pregnant Wistar rats induces oxidative DNA damage in the placenta, activates inflammatory pathways in the maternal system and in the placenta, and may lead to decreased average fetal weight. Our findings warrant further investigation into TCE-induced placental toxicity and its adverse effects on pregnancy outcomes.

Introduction

Trichloroethylene (TCE) is a chlorinated organic solvent that has been used to degrease metal parts, to strip paint, and as a dry cleaning solvent. In the United States, 2.5 million pounds per year of TCE was utilized in 2011, while the global consumption was 945 million pounds per year (Glauser *et al.*, 2012). TCE is a widespread environmental pollutant due to improper disposal (U.S. EPA, 2011). TCE exposure is common in occupational settings (Chiu *et al.*, 2013a), with an estimated 3.5 million workers in the United States being exposed to TCE with short-term exposure levels in air ranging from 1.3 mg/m^3 to 1,084 mg/m^3 (the highest mean concentration reported for a degreasing operation) (NTP, 1990).

TCE metabolism is tissue, species, and sex dependent, and is important in determining organ-specific toxicity (Lash *et al.*, 2000a; Forkert *et al.*, 2002). With oral exposure, TCE is readily absorbed from the gastrointestinal tract and distributed to the liver and kidney where it is metabolized (Lash *et al.*, 2000a). TCE is metabolized in animals and humans via two separate pathways (Lash *et al.*, 2000a). The major metabolism pathway is by oxidation in the liver and glutathione (GSH) conjugation is the secondary pathway. In the oxidative pathway, TCE is

metabolized in the liver by cytochrome (CYP) P450 enzymes, particularly CYP2E1, to an epoxide, 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 (Lash *et al.*, 2000a). The second pathway is through conjugation with glutathione (GSH) (Lash *et al.*, 2000a). In rats and humans, TCE can be conjugated in the liver and kidney by glutathione S transferase (GST) to S-(1,2-dichlorovinyl) glutathione (DCVG) (Lash *et al.*, 2000a), which is subsequently biotransformed in the kidney by γ-glutamyl transpeptidase (GGT) to DCVC and then by cysteine conjugated β-lyase to an unstable intermediate capable of forming covalent bonds with cellular nucleophiles, leading to cytotoxicity and renal injury (Lash *et al.*, 2014). Alternatively, the metabolite can undergo N-acetylation to a mercapturate, which is excreted in the urine either as NAcDCVC or the sulfur conjugated form NAcDCVCS (Lash *et al.*, 2014). Because the liver and the kidney bioactivate TCE, they are key target organs for TCE-induced intoxication (Rhomberg, 2000; Forkert *et al.*, 2006). In addition, TCE exposure is associated with immunotoxicity, neurotoxicity, and male reproductive toxicity (Green *et al.*, 1997; Lash *et al.*, 2000c; Forkert *et al.*, 2002; Forkert *et al.*, 2003). Limited studies have explored the impact of TCE on pregnancy outcomes.

A recent epidemiologic study of women in New York State reports a significant association between exposure to TCE and decreased birth weight (Forand *et al.*, 2012). In addition, exposure to TCE has been associated with inflammatory diseases in humans, including dermatitis, arthritis, and autoimmune disease (Watanabe, 2011; Chiu *et al.*, 2013a). TCE has also been shown to increase markers of oxidative stress, including malondialdehyde and 8-hydroxy-deoxyguanosine, in humans (Abusoglu *et al.*, 2014) and rodents (Toraason *et al.*, 1999; Khan *et al.*, 2001). In humans, oxidative stress (Al-Gubory *et al.*, 2010) and inflammation (Challis *et al.*,

2009) play critical roles in placentation and parturition. Increased release of the proinflammatory cytokines interleukin (IL)-6, IL-1β, IL-8 and tumor necrosis factor-α (TNF-α) is associated with adverse birth outcomes in humans (Challis *et al.*, 2009). Throughout *in utero* development, the placenta plays an important role in regulating growth and development, including nutrient transfer from the maternal to the fetal compartment (Koukoura *et al.*, 2012). In rodents and humans, TCE has been shown to induce oxidative stress and inflammation in the liver and kidney, as well as epigenetic changes in the liver, which maybe be linked to adverse birth outcomes (Tao *et al.*, 2000; Chiu *et al.*, 2013a).

Gene expression is regulated by epigenetic mechanisms that include changes in DNA methylation, histone modification, and expression of miRNA (Maccani *et al.*, 2009). Gene expression regulation by DNA methylation plays an important role in placenta function including regulating the intrauterine environment (Maccani *et al.*, 2009). Furthermore, epigenetic alterations related to environmental exposures have been associated with adverse pregnancy outcomes (Koukoura *et al.*, 2012). Although TCE has been shown to induce DNA hypomethylation and oxidative stress in the mouse liver (Tao *et al.*, 2000) and mouse brain (Blossom *et al.*, 2013), there is a paucity of data regarding TCE-induced epigenetic changes in the placenta.

Reactive oxygen species (ROS) can interact directly with DNA and thereby induce both genetic and epigenetic changes (Thompson *et al.*, 2012). Furthermore, oxidative DNA damage affect the ability of DNA methyltransferases to interact with DNA and thus resulting in altered methylation of cytosine residues (Turk *et al.*, 1995; Valinluck *et al.*, 2004). Therefore, oxidative stress can induce epigenetic changes in the placenta and also DNA methylation can affect gene

expression of oxidative stress and inflammation related genes which may be associated with poor pregnancy outcomes.

Although oxidative stress (Jauniaux *et al.*, 2006) inflammation (Redline, 2004), and epigenetic changes (Maccani *et al.*, 2009) in the placenta have been associated with adverse pregnancy outcomes, limited studies have investigated TCE effects on the placenta. Therefore, the goal of the current study was to examine whether effects of TCE exposure on reproductive outcomes were associated with activation of inflammatory pathways and oxidative stress in the placenta of pregnant Wistar rats.

Materials and Methods

Materials

We purchased trichloroethylene (99% pure) from Sigma Chemical Company (St. Louis, MO). RNAlater, RNeasy Plus Mini Kit and QIAamp DNA Mini Kit were purchased from SABiosciences (Valencia, CA). The Colorimetric MethylFlash Hydroxymethylated DNA Quantification Kit and EpiQuik 8-OHdG DNA Damage Quantification Direct Kit were purchased from Epigentek. Anti-MMP-8 antibody was purchased from Abcam (Cambridge, MA)

Animals

Timed-pregnant Wistar rats between 60-90 days of age weighing 200-250 grams were obtained from Charles River (Portage, MI). The day after copulation was designated as day 0 of pregnancy. Rats were shipped at gestational day (gd) 2 and individually housed in a controlled environment with a 12-hour light/dark cycle. Dams were fed standard rat chow (Purina 5001) and water *ad libitum*.

Exposure method

Animals were exposed to TCE orally using a vanilla wafer, a method that was adapted from work conducted by Seegal et al. for polychlorinated biphenyls (PCB) exposure (Seegal *et al.*, 1997). Initially, N=8 rats were assigned to either control or TCE treatment group, however one rat in the control group was excluded from all subsequent analysis because the rat was not pregnant. The sample size was increased with a second exposure set of rats, with three rats assigned to the control group and two rats assigned to the TCE treatment group, for a total of 10 rats per treatment group. Rats received vanilla wafer only (vehicle controls) or were exposed to 480 mg TCE/kg body weight once a day, between 8:00 – 9:00 am, from gd 6-16. First, rats were trained to eat the wafer by placing them in individual exposure cages and placing one vanilla miniwafer without TCE in the cage for three consecutive days. After the wafer-training period, TCE was pipetted onto a miniwafer and immediately offered to the rats, which they readily ate. Oral exposure was used because it is a relevant environmental TCE exposure route for humans. The 480 mg TCE/kg/d dose was selected because 400 – 500 mg TCE/kg/d was previously shown to stimulate oxidative stress (Toraason *et al.*, 1999) and inflammatory response (Griffin *et al.*, 2000) in rat liver.

Dissections

Rats were euthanized at gd 16 with carbon dioxide followed by cardiac puncture. Dams were euthanized in a random fashion alternating between controls and treated. The uterine horn was removed and examined for resorbed or dead fetuses. Dissections were conducted within a 4-h period in the morning. Fetal weights and litter size were recorded as well as maternal kidney and liver weights. The collected blood was centrifuged at 2000 x g at 4°C for 30 min, and then

stored at -80°C until assayed. Gestational tissues were snap frozen in liquid nitrogen then stored in the -80°C freezer or in RNAlater reagent for future analysis.

RNA extraction, sample preparation and gene expression profiling

Because we observed decreased fetal weight with exposure to TCE, we evaluated changes in gene expression using a Rat ST 1.1 microarray, (>27,000 protein coding transcripts), followed by biological pathway analysis. First, RNA was extracted from placental tissue of TCE-treated rats and from vehicle-treated controls. Three placentas per dam from 4 treated dams and 4 control rats were selected at random, weighed, and then homogenized using a FastPrep-24 tissue and cell lyser (MP Biomedicals, Solon, OH). The RNA was extracted using a RNeasy kit (SA Biosicence) according to the manufacturer's protocol. RNA concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA).

The RNA from each of the three placentas was pooled for each rat and used for downstream microarray gene chip analysis using the Rat Gene ST 1.1 Array (Affymetrix). Biotinylated cDNA was prepared according to the Ambion WT Kit protocol from 250 ng total RNA (GeneAtlas™ WT Expression Kit User Manual P/N 702935 Rev. 3). Following fragmentation, 3.7 ug of cDNA were hybridized for 20 h at 48 °C on the Rat Gene ST 1.1 Array Strips using the Affymetrix GeneAtlas System (software version 1.0.4.267). Arrays were scanned using the Affymetrix GeneAtlas System. Data were analyzed using the limma and oligo Bioconductor packages were implemented in the R-statistical environment version 2.15.1. Robust Multi-array Average (RMA) was used to normalize the data and fit log2 transformed

expression values. Differentially expressed genes were defined as fold change ≥1.5 and $p<0.05$ by unpaired t-test.

Further analysis of the gene expression data was conducted according to pathway analysis using Gene Ontology (GO) (Falcon *et al.*, 2007). Over-represented GO terms and enriched pathways associated with differentially expressed genes were generated. Fisher's exact test was first conducted on each term in the database and p-values were used to sort the terms when pathway analysis was conducted. Conditional hypergeometric tests were conducted using the GOstats package of Bioconductor probesets and variance across all samples less than 0.02 was removed as a non-specific filter to reduce the gene universe.

Out of 100 differentially expressed genes identified by the Rat Gene ST 1.1 Array, six genes were chosen for validation analysis using quantitative real-time polymerase chain reaction (qRT-PCR). These six genes were selected based on the degree of expression, p-value, involvement in immune system regulation, and expression during fetal growth and development. First, 1µg of RNA was reverse transcribed to cDNA with iScript Reverse Transcriptase (BIO-RAD, Hercules, CA). Then, using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) qRT-PCR was conducted according to the manufacturer's protocol. Primer sequences were designed using National Center for Biotechnology Information gene databases. The ΔΔ Ct method was used to calculate the relative expression levels of transcription: the geometric mean of the three housekeeping genes was calculated and then used to normalize expression values of the genes of interest (Vandesompele *et al.*, 2002). The following primers were used: β2-microglobulin 5' ACTGAATTCACACCCACCGAG 3' and 5'ACATGTCTCGGTCCCAGGT 3'; β-actin 5'AAGCCGGCCTTGCACAT 3' and 5' CGCCACCAGTTCGCCA 3'; and TATA binding protein (TBP) 5' AATATAATCCCAAGCGGTTTGCTG 3' and 5'

TGCTGCTAGTCTGGATTGTTCTT 3'. Results were reported as fold change in mRNA expression of TCE-treated rat placenta compared with controls.

Proinflammatory cytokine analysis in maternal serum

TCE-induced release of IL-6 in maternal serum was determined using enzyme linked immunosorbant assay (ELISA). Blood was collected from cardiac puncture and then left to coagulate at room temperature for 30 min. The coagulated blood was then centrifuged at 2000 x g at 4°C for 30 min. Serum was collected and stored at -80°C until assayed for IL-6 by ELISA (Peprotech, Rocky hill, NJ) following the manufacturer's protocol.

Immunohistochemistry detection of neutrophils in rat placenta tissue

Placentas were harvested at necropsy, bisected transversely on midline and fixed in 10% formalin (Fisher, Waltham, MA) for a minimum of 48 hours. Immunohistochemistry was performed by the University of Michigan In Vivo Animal Core (IVAC) within the Unit for Laboratory Animal Medicine. Specifically, neutrophil detection was performed using a commercially available rabbit monoclonal antibody against matrix metalloproteinase 8 (anti-MMP8, clone EP1252Y, ab81286, Abcam). Unstained 5-µm sections were cut from paraffin-embedded, formalin-fixed tissue using a rotary microtome and mounted on glass slides. Heat-induced antigen retrieval was performed by incubation in a commercially available retrieval buffer (Reveal Antigen Retrieval buffer, RV1000M, Biocare Medical, Concord, CA) within a pressure chamber (Decloaking Chamber, Biocare Medical) at a temperature of 95°C for 40 min, followed by cooling to room temperature for 20 min. Immunohistochemistry was performed with an automated immunohistochemical stainer (Intellipath, Biocare Medical) using a polymer-

based, biotin-free detection system (Promark Rabbit-on-Rodent HRP polymer, Biocare Medical) following manufacturer instructions. The primary antibody step consisted of incubation of the primary antibody at a dilution of 1:200 for 30 min at room temperature. The detection polymer was applied for 30 min and diaminobenzidine was used as the chromogen. Negative controls consisted of non-immune rabbit serum (NC499, Biocare Medical) used in place of the primary antibody.

Patterns of immunohistochemical labelling were evaluated by a board-certified veterinary pathologist (ILB). Representative images were taken using an Olympus DP72 12.5 megapixel digital camera mounted to an Olympus BX45 light microscope. Images were acquired using the manufacturer's software (CellSensStandard 1.7.1, Olympus Corporation), and image processing and composite construction were performed in Adobe Photoshop CS2, version 9.0. Image processing was confined to global adjustments of brightness, contrast, exposure, sharpness, image size, and color balance. Correction of peripheral lens distortion was performed if needed.

Sample preparation for DNA extraction

Genomic DNA was extracted from placental tissue of TCE-treated and control rats. Three placentas per dam were selected. Each tissue was weighed, and homogenized using a FastPrep-24 tissue and cell lyser (MP Biomedicals, Solon OH). DNA was extracted from the homogenized tissue using the QIAmp DNA Mini Kit (SABiosciences, Qiagen) according to manufacturer's protocol. Concentration and purity of the extracted DNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). The samples were stored at -20ºC until assayed for levels of 8-hydroxy-deoxyguanosine and 5-hydroxymethylcytosine.

Quantification of 8-hydroxy-deoxyguanosine (8-OHdG) levels in placenta

We measured levels of oxidative DNA damage in the extracted placental DNA with an EpiQuik 8-OHdG DNA Damage Quantification Direct Colorimetric Kit, an absorbance based assay method (Epigentek, Farmingdale, NY) according to the manufacturer's instructions. Briefly, 300 ng of DNA was added to strip wells that were pretreated to have high affinity for DNA. Then capture and detect antibodies were used to detect 8-OHdG. The detected signal was quantified by reading absorbance at 450 nm on Molecular Devices SpectraMax Gemini M2e spectrophotometer. The amount of 8-OHdG was proportional to the OD intensity measured. Experiments were performed in triplicate. Quantity of 8-OHdG was calculated based on the manufacturer's recommended formula.

Global DNA methylation quantification assay

Because we found increased level of 8-OHdG, which can induce DNA hypomethylation by inhibiting methylation at a nearby cytosine base (Wu *et al.*, 2015), DNA methylation changes in the placenta were investigated. In addition, because TCE has been shown to induce DNA hypomethylation in the mouse liver (Tao *et al.*, 2000), 5-hydroxymethylcytosine (5-hmC), a cytosine residue oxidation intermediate that is associated with active DNA demethylation, was also investigated (Wu *et al.*, 2015). Levels of 5-methylcytosine (5-mC) and 5-hmC in the TCE-treated and control rat placentas were quantified. Levels of 5-mC and 5-hmC in rat placental tissues were analyzed using the MethylFlash Methylated DNA Quantification Kit (Colorimetric) and MethylFlash Hydroxymethylated DNA Quantification Kit (Epigentek, Farmingdale, NY), respectively, following the manufacture's protocol. Briefly, 200 ng of the genomic DNA was used, in separate experiments, to measure levels of 5-mC and 5-hmC. DNA was added to strip wells that were pretreated to have a high affinity for DNA. Then, capture and detection

antibodies were used to determine the methylated and hydroxymethylated fractions of the DNA. The absorbance was read at 450 nm on Molecular Devices SpectraMax Gemini M2e spectrophotometer. The amount of methylated or hydroxymethylated DNA was proportional to the OD intensity measured. The experiment was carried out in triplicate. Relative quantities of 5-mC and 5-hmC were calculated based on the manufacturer's guidelines.

Quantitative RT-PCR for mRNA levels of ten-eleven translocation (Tet) enzyme

TET enzymes oxidize 5-mC to 5-hmC, leading to active DNA demethylation, and are thought to be activated as a response to oxidative stress (Chia *et al.*, 2011; Branco *et al.*, 2012). Thus, qRT-PCR was used to quantify gene expression levels of Tet2 and Tet3 in the TCE-exposed rat placental tissues compared with control. Placenta tissues (25 mg) were homogenized using the MP Tissue Lyser. The DNA samples used in 5-mC and 5-hmC experiments and the mRNA samples used to determine the levels of Tet enzymes were extracted from the same placentas. The mRNA was then extracted using RNeasy mini plus kit (SABiosciences, Qiagen,CA) following manufacture's protocol. Aliquots of 1 µg of mRNA were used for cDNA synthesis using the RT2 First Strand Kit (SA Biosciences, CA) following the manufacturer's protocol. Quantitative real-time polymerase chain reactions (qRT-PCR) were performed using 12.5 µL of RT^2SYBR Green qPCR Master Mix, 1 µL of gene-specific primer target (Tet) (SABiosciences, CA), 4 µL of cDNA template and 7.5 µL of nuclease-free H_2O, for a total volume of 25 µL. Samples were analysed using a CFX96 Real-Time PCR Detection System (Bio-Rad Labs, Hercules, CA). The qRT-PCR with an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 5 s at 60 °C, and ΔΔCt method (Yuan *et al.*, 2006) was used to quantify gene expression. The target gene was normalized to the geometric mean of

three housekeeping genes, β2-microglobulin 5’ CGTGCTTGCCATTCAGAAAACT and 5’ GGTGGGTGGAACTGAGACAC 3’ ; β-actin 5’AAGCCGGCCTTGCACAT 3’ and 5’ CGCCACCAGTTCGCCA 3’; Tata binding protein (Tbp) 5’GAATAAGAGAGCCACGAACAACTG 3’ and 5’ATTGTTCTTCACTCTTGGCTCCT 3’ using Bio-Rad CFX manager software (Bio-Rad Labs, Hercules, CA). All samples were analysed in triplicate.

Statistical analysis

For average fetal weight and cytokine data, t-tests were performed using GraphPad Prism 5.0 (LaJolla, CA). All data were expressed as mean ± standard error mean (SEM). Statistical analyses of 5-hmC and 8-OHdG data were conducted using mixed model regression analysis in SPSS software (SPSS Inc., Chicago, IL), with treatment as the fixed effect and litter as the random effect.

Results

Reproductive effects of TCE

To characterize TCE effects on pregnancy outcome, pregnant rats were exposed daily to 480 mg TCE/kg-day on vanilla wafers on gd 6-16. Fetal weights were averaged for each litter and a grand mean calculated for each treatment group. As shown in Figure 2.1, TCE decreased average fetal body weight by 10% (Fig. 2.1; $p<0.05$; N=10 rats per group). The maternal body weight, litter size, maternal kidney weights, and maternal liver weights of TCE-treated rats were not significantly different from controls (Fig. 2.2).

TCE Effects on gene expression in rat placenta

To determine TCE exposure effects on gene expression in placenta, we conducted a Rat ST 1.1 microarray analysis. Three placentas from each of four rats in each treatment group were selected and pooled for microarray analysis. We identified nineteen unique genes whose mRNA expression was significantly changed one-half-fold or more with TCE treatment compared with control (Table 2.1; unadjusted $p < 0.05$; N=4 rats per group). We found that TCE treatment significantly increased expression of hla class II histocompatibility antigen gamma chain also known as (cluster of differentiation 74) Cd74, heat shock protein family member 7 (Hspb7), gamma enolase 2 (Eno2), nadph dehydrogenase quinone 2 (Nqo2), RT1 class II, locus Ba (Rt1-Ba), lecithin-retinol acyltransferase (Lrat), RT1 class II, locus Da (Rt1-DA), ras-related GTP binding B (RragB), fas apoptotic inhibitory molecule 3 (Faim3), Fc receptor-like A (Fcrla), and Cd79b molecule, immunoglobulin associated beta (Cd79b); whereas expression significantly decreased for hepatic lipase (Lipc), secreted frizzled related protein 2 (Sfrp2), keratin 72 (Krt72), guanine nucleotide binding protein (G protein) gamma 11 (Gng11), purinergic receptor P2X, ligand-gated ion channel 7 (P2rx7), versican (Vcan), chemokine (c-x-c motif) ligand 14 (Cxcl14), and src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites (Srms). We selected the top six differentially expressed genes ($p<0.05$) for qRT-PCR analysis. With qRT-PCR, we confirmed TCE-induced changes in gene expression of Cd74, Nqo2, Eno2, Rt1-Ba, and Rt1-DA and decreased expression of Sfrp2, Krt72, Cxcl14 and P2rx7. Statistically significant differences in gene expression were not observed with qRT-PCR analysis for Hspb7, Lipc or Gng11. Furthermore, results from the GO pathway analysis showed an up-regulation of inflammatory pathways and down-regulation of metabolic homeostasis pathways (Table 2.2).

Detection of biomarkers of inflammation in the maternal serum and placenta

Because we identified an up-regulation of inflammatory gene expression pathways in the placenta, we examined whether TCE stimulates inflammatory responses. TCE exposure during pregnancy significantly increased concentrations of the pro-inflammatory cytokine IL-6 in maternal serum of rats compared with vehicle controls by 4-fold (Fig. 2.3; p=0.03; N=10 rats per group), which may indicate that exposure to TCE induced maternal systemic inflammation: however, neutrophil blood count would be needed to confirm this conclusion. Additionally, we used immunohistochemical staining for matrix metalloproteinase-8 (MMP-8) to identify neutrophils in formalin-fixed placental tissue from a subset of three rats per group (2 placentas analyzed per litter with 7 to 8 images analyzed for each placenta). Neutrophils were detected in the blood vessels of the decidua basalis (without tissue infiltration) of the rat placentas (N= with (representative images are shown in Figure 2.4).

Levels of 8-OHdG in placenta of TCE-exposed rats

Because oxidative stress is linked to inflammation and TCE increases 8-OHdG, a marker for oxidative DNA damage, in rodent liver (Channel *et al.*, 1998; Toraason *et al.*, 1999), we investigated the effect of TCE exposure on levels of 8-OHdG in the placenta of TCE-treated rats compared with controls. We observed that 8-OHdG was significantly increased by 42.2% (Fig. 2.5; p=0.02; vehicle control N= 7 rats with 4 placentas per litter and N=8 for TCE treated rats with 6 placentas per litter). This experiment was conducted with placentas from the first set of exposed rats: there were initially 8 rats per treatment group, but one rat in the control group was not pregnant

Global DNA 5-methyl cytosine (5-mC) and 5-hydroxymethyl cytosine (5-hmC) levels and Tet gene expression in placentas of TCE-exposed rats

Because we observed decreased fetal weights in TCE-exposed litters and changes in DNA methylation in human placentas have been significantly associated with SGA and fetal growth restriction in humans (Banister *et al.*, 2011), we examined the effect of TCE exposure on global 5-hmC and 5-mC levels in rat placentas DNA. We found that global DNA levels of 5-hmC were significantly increased 53.9% in placentas of TCE-exposed rats compared with controls (Fig. 2.6A; $p=0.005$; N= 8 litters for the TCE-treated group and N = 7 litters for the vehicle control group, with 3 placentas per litter). However, global 5-mC was not significantly increased (Fig. 2.6B; $p=0.84$, not significant). In addition, we assessed mRNA expression of the Tet2 and Tet3 genes because Tet enzymes oxidize 5-mC to 5-hmC. We observed a statistically significant, albeit modest, increase in mRNA expression for Tet3 ($p<0.02$) but not Tet2 (Fig. 2.8). These assays were conducted with placentas from the first set of exposed rats: there were initially 8 rats per treatment group, but one rat in the control group was not pregnant.

Discussion

Despite reductions of release into the environment over recent decades, TCE exposure remains significant due to exposures in the workplace and contamination of water, soil, and air (U.S. EPA, 2011). In 2014, TCE was reclassified by IARC as well as by NTP as a "human carcinogen" (Guha *et al.*, 2012; IARC, 2014; NTP, 2014).

Although less studied than cancer risk, TCE is also linked to adverse pregnancy outcomes in humans (Chiu *et al.*, 2013a). Our finding that TCE exposure during pregnancy decreased fetal weight in Wistar rats is consistent with previous epidemiological and animal studies that have associated TCE exposure with low birth weight. Specifically, a retrospective cohort study of

TCE-contaminated drinking water in Woburn, Massachusetts found an association between exposures to TCE-contaminated well water and SGA, as well as fetal deaths, eye defects, choanal atresia, neural tube defects (NTDs), cleft lip, and hypospadias (ATSDR, 1997). A study of TCE-contaminated drinking water at Camp LeJeune also found increased risk of SGA associated with TCE (Bove *et al.*, 2002a). More recently, Forand et al. found an association between maternal exposure to TCE through in home vapor intrusion in the home in New York State and low birth weight (Forand *et al.*, 2012). In a rat study, Fisher et al. found that TCE induced cardiac malformation and decreased fetal weights (Fisher *et al.*, 2001). Of added interest is our finding that TCE decreased average litter fetal weight without significantly changing maternal body and organ (liver and kidney) weights.

Consistent with decreased fetal weight, our microarray results demonstrated that expression of placental genes involved in metabolic homeostasis and cell growth was decreased. In addition, we conducted pathway analysis that showed down-regulation of metabolic homeostasis pathways. The down-regulated genes include Lipc, Sfrp2, P2rx7; Gng11, Hspb7, and Vcan. Lipc catalyzes hydrolysis of phospholipids and triglycerides and is an important enzyme in HDL metabolism and in receptor-mediated lipoprotein uptake (Baroni *et al.*, 2003), and a Lipc polymorphism, LIPC-514TT genotype, has been associated with increased risk of preeclampsia in women (Enquobahrie *et al.*, 2005). Sfrp2 plays an important role in embryonic development, stem cell maintenance and differentiation, and tissue homeostasis (Sonderegger *et al.*, 2010) and has been shown to inhibit Wnt signaling during development. P2rx7 has been shown to mediate ATP-stimulated rise in calcium levels, an important signaling molecule that ensures normal cell function and is a significant factor in regulation of events during early pregnancy (Roberts *et al.*, 2007). Gng11, a member of the gamma-subunit of G-protein family,

encodes a lipid-anchored cell membrane protein. Chemical oxidants such as hydrogen peroxide induce expression of Gng11 (Hossain *et al.,* 2006), and TCE induced this response in our study. Hspb7 is expressed during embryonic development and functions as a chaperone to ensure protein homeostasis; its suppression disrupts normal cardiac development (Rosenfeld *et al.*, 2013). Finally, Vcan, an extracellular matrix protein, is highly expressed during development and is involved in the control of cell adhesion, migration and extracellular matrix assembly; its suppression affects embryo implantation and decidualization (Naso *et al.,* 1994). Because placental nutrient exchange between fetus and mother is dependent on the health of the placenta, our results suggest that TCE modulates genes that sustain fetal growth and development leading to decreased fetal weights.

Microarray analysis also showed TCE-induced up-regulation of MHC class II antigen immunity-related genes important for maternal-fetal tolerance including, Cd74, Rt1-Ba, Rt1-DA, and Cd79b. In normal pregnancies in humans and rodents, MCH class II antigens are suppressed in the placenta to facilitate maternal-fetal tolerance whereas enhanced expression of MHC class II antigen correlates with adverse pregnancy outcomes (Sun *et al.*, 2005).

Our finding that exposure to TCE during pregnancy increased IL-6 concentrations in rat maternal serum is consistent with previously published reports that link TCE with increased proinflammatory cytokine levels in humans. Specifically, increased serum IL-6, IL-1β, IL-8, and TNF-α concentrations were associated with occupational TCE-induced hypersensitivity dermatitis (Jia *et al.*, 2012). Furthermore, increased IL-6 concentrations in maternal serum of TCE-exposed rats are consistent with prior studies that associated release of proinflammatory cytokines including IL-6 in amniotic fluid with preterm labor (Romero *et al.*, 1990) and in umbilical cord blood of SGA infants (Lindner *et al.*, 2013). In addition, increased levels in

amniotic fluid of the proinflammatory cytokines IL-6, IL-1β, IL-8, and TNF-α, have been associated with SGA (Amarilyo *et al.*, 2011) preterm birth (Christiaens *et al.*, 2008). In addition, we found that exposure to TCE increased the presence of neutrophils in the blood vessels of the decidua basalis of the TCE-treated rat placenta compared with controls, suggesting that exposure to TCE led to placental inflammation and that TCE-induced inflammation may have contributed to fetal growth restriction we observed.

Oxidative stress in gestational tissues has been associated with several pathologies of pregnancy, including preterm labor, preeclampsia, and intrauterine growth restriction (IUGR) in humans (Biri *et al.*, 2007; Al-Gubory *et al.*, 2010). In humans, increased levels of the urinary oxidative stress markers 8-isoprostane and 8-OHdG in early or mid-gestation have been associated with increased risk for preeclampsia and decreased gestational length (Peter Stein *et al.*, 2008). In our gene expression microarray results we found that exposure to TCE induced expression of Nadph dehydrogenase quinone 2 (Nqo2), a flavoprotein that protects cells against chemical-induced oxidative stress (Iskander *et al.*, 2006; Xu *et al.*, 2013). As such, induction of Nqo2 in the placentas of TCE-exposed rats may be in response to oxidative stress.

We also found that exposure to TCE increased levels of 8-OHdG in the rat placenta. This finding is in agreement with other studies in animals and humans that implicate TCE exposure with oxidative stress. Specifically, in an occupational study levels of 8-OHdG were significantly higher in the urine of TCE exposed workers compared with controls ((Hu *et al.*, 2008; Abusoglu *et al.*, 2014). Moreover, TCE exposure in mice resulted in a two-fold increase in thiobarbituric acid-reactive substance and 8-OHdG concentrations in the liver (Channel *et al.*, 1998). In addition, Toraason et al. showed that TCE exposure elevated levels of 8-OHdG in rat liver (Toraason *et al.*, 1999). Although previous studies in animals and humans have linked TCE

exposure with oxidative stress, the present study is the first, to our knowledge, that reports evidence of TCE-induced oxidative stress in placenta.

Findings from recent human and rodent studies suggest that placental epigenetic and genetic profiles may serve as markers of intrauterine environment (Koukoura *et al.*, 2012). In humans, decreased placental DNA methylation was associated with adverse birth outcomes including preterm birth and IUGR (Nelissen, van Montfoort et al. 2011). TCE has been shown to alter epigenetic mechanisms. In mice, hypomethylation of DNA was observed in liver following exposure to TCE (Tao *et al.*, 1998). DNA hypomethylation has been linked to changes in expression of proinflammatory cytokine (e.g., IL-6) and oxidative stress-related genes. Our results also show that exposure to TCE induced an increase gene expression of Tet3, an enzyme involved in DNA demethylation through oxidation of 5-mC to 5-hmC (Branco *et al.*, 2012). It has been hypothesized, by Chia and co-workers, that TET enzyme activation and its role in oxidation of 5-mC to 5-hmC could be due to oxidative stress (Chia et al, 2011). The oxidative DNA damage marker, 8-OHdG, has also been shown to induce DNA hypomethyaltion by decreasing methylation on adjacent cytosine (Weitzman *et al.*, 1994; Turk *et al.*, 1995). Alternatively, oxidative stress could induce global demethylation by decreasing the efficiency of one-carbon metabolism (Fleming *et al.*, 2012). Our findings of increased levels of Tet3 enzyme and 5-hmC suggest that exposure to TCE could lead to global hypomethylation of DNA in TCE-treated rat placenta through an oxidative stress mechanism, with subsequent alteration of gene expression of inflammatory and oxidative stress genes and potential to contribute to increased risk for poor obstetrical outcome.

By using time pregnant Wistar rats, we have established an experimental model to study potential mechanisms by which exposure to environmental pollutants including TCE contributes

to adverse pregnancy outcomes. We chose to work with rat because of its similarity to human metabolism of TCE: e.g., mice metabolize and eliminate TCE more rapidly than do rats and humans (Lash *et al.*, 2000b). An additional strength of our study is the use of oral exposure, which is a more relevant route of exposure to TCE than oral gavage or intraperitoneal injection. However, we evaluated only one concentration of TCE (480 mg/kg-d), which provides a lowest observable effect level for TCE toxicity in the placenta. Therefore, future toxicological studies are needed to characterize the full dose-response relationship for TCE toxicity in pregnant rats. The US EPA maximum contaminant level for drinking water is 5 µg/L, but levels as high as 1400 µg/L has been measured in municipal drinking water supply (ATSDR, 2003; U.S. EPA, 2011). Furthermore, the US EPA drinking water standard fails to account for other exposure routes including inhalation and dermal exposure during showers.

In summary, TCE decreased fetal weight and increased biomarkers of inflammation and oxidative stress that have been associated with adverse pregnancy outcomes in humans, including IUGR. To our knowledge, this is the first study that explores TCE-induced oxidative stress and inflammation in the placenta and their impacts on pregnancy in rats. Results of the current study suggest that oxidative stress and inflammation in the placenta are possible mechanisms that may underlie epidemiological associations of TCE exposure and adverse birth outcomes (Bove *et al.*, 2002b; Forand *et al.*, 2012; Chiu *et al.*, 2013b). Based on our findings, further studies are warranted to investigate the mechanisms by which TCE exposure may lead to fetal growth restriction and SGA in humans. In addition to contributing to the weight for evidence for TCE-induced SGA outcome, our results should raise public awareness of the potential adverse effects of environmental pollution on pregnancy outcomes.

Table 2.1**.** Rat Gene ST 1.1 Microarray Expression of Significantly Modified Genes in the Placentas of Wistar Rats*****

Gene	Protein Product	Fold change	p-value
Eno2	Gamma Enolase 2	2.69	0.001
Hspb7	Heat shock protein family member 7 (cardiovascular)	2.04	0.025
Cd74	Cd74 molecule major histocompatibility complex, class II invariant chain	1.96	0.009
Lipc	Hepatic lipase	0.53	0.01
Nqo2	NADPH dehydrogenase quinone 2	1.88	0.03
Sfrp2	Secreted frizzled related protein 2	0.55	0.04
RT1-Ba	RT1 class II, locus Ba	1.76	0.009
Lrat	lecithin-retinol acyltransferase (phosphatidylcholine-retinol-O-acyltransferase)	1.75	0.02
RT1-Da	RT1 class II, locus Da	1.7	0.008
Krt72	Keratin 72	0.58	0.002
Gng11	Guanine nucleotide binding protein (G protein), gamma 11	0.59	0.001
P2rx7	Purinergic receptor P2X, ligand-gated ion channel, 7	0.6	0.01
RragB	Ras-related GTP binding B	1.66	0.01
Faim3	Fas apoptotic inhibitory molecule 3	1.66	0.0004
Vcan	Versican	0.60	0.04
Fcrla	Fc receptor-like A	1.64	0.008
Cd79b	Cd79b molecule, immunoglobulin associated beta	1.63	0.002
Srms	src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites	0.61	0.0004

*N=4 rats per treatment group, 3 placentas/litter. The placentas were pooled within each litter.

Table 2.2**.** Rat Placenta Gene ST 1.1 Microarray Gene Ontology (GO) Pathway Analysis

GOBPID	Up-regulated biological pathways
GO:0006954	inflammatory response
GO:0030593	neutrophil chemotaxis
GO:0002438	acute inflammatory response to antigenic stimulus
GO:0006950	response to stress
GO:0071219	cellular response to molecule of bacterial origin
GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class II
GO:0048771	tissue remodeling

GOBPID	Down-regulated Pathways
GO:0034368	protein-lipid complex remodeling
GO:0071827	plasma lipoprotein particle organization
GO:0034374	low-density lipoprotein particle remodeling
GO:0033344	cholesterol efflux
GO:0034380	high-density lipoprotein particle assembly
GO:0034370	triglyceride-rich lipoprotein particle remodeling
GO:0034375	high-density lipoprotein particle remodeling
GO:0043933	macromolecular complex subunit organization

Pathway analysis conducted on microarray results from placentas of time pregnant Wistar rats exposed daily to 480 mgTCE/kg-day or vehicle (control) from gd 6-16. N=4 rats, 3 placentas/litter. The placentas were pooled within litter.

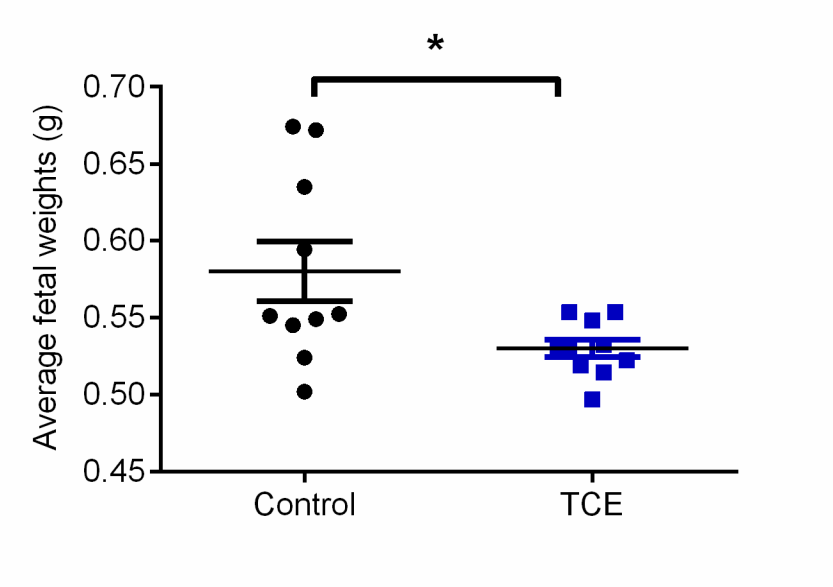


Figure 2.1. Average fetal weight per litter of control and TCE-treated time-pregnant rats. Rats were exposed to 0 (control) or 480 mg TCE/kg-day on vanilla wafers from gestational day 6 – 16. Fetal weights were averaged per litter and a grand mean was calculated for statistical comparison (N=10 rats per group; *$p<0.05$). The bars indicate means ± SE.

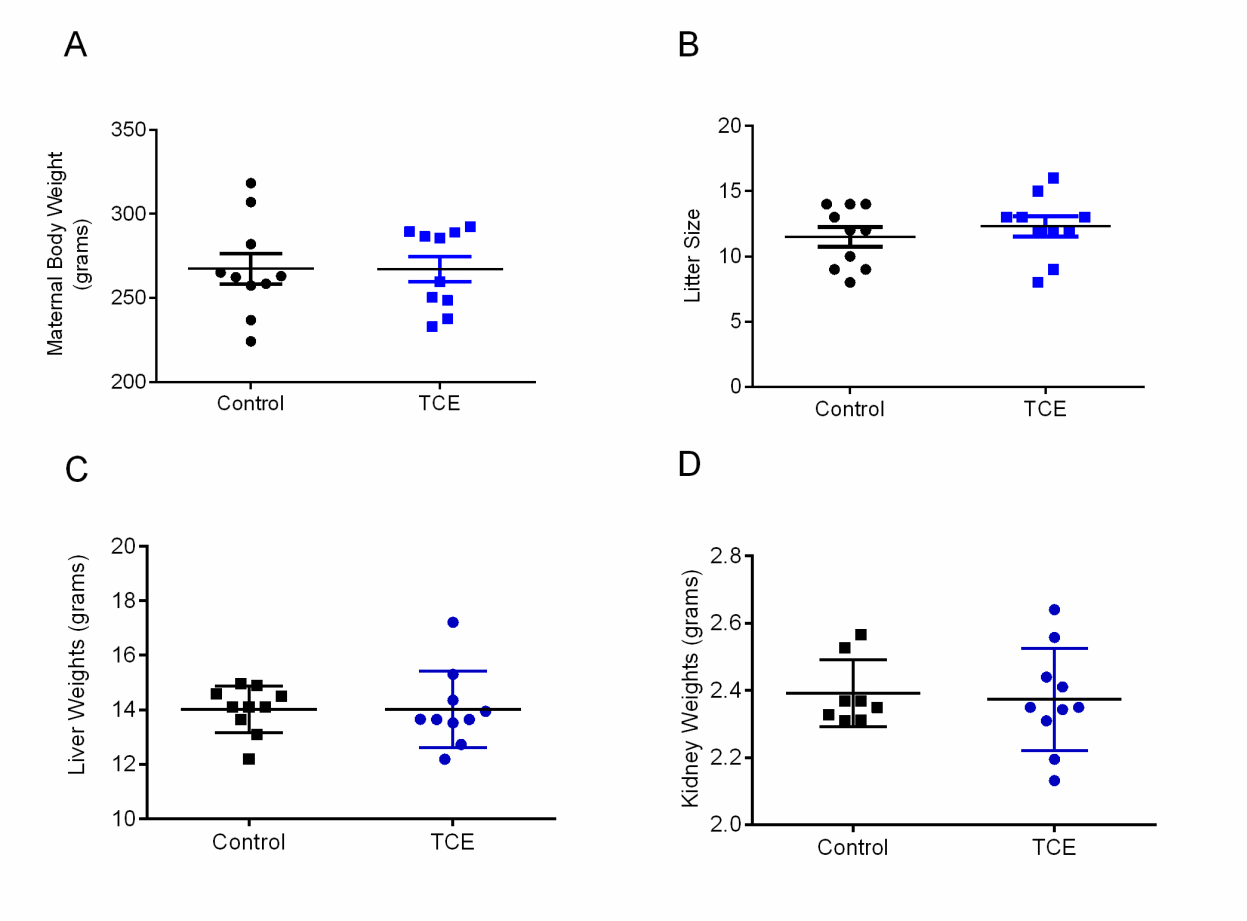


Figure 2.2. Effect of TCE exposure on weights**:** A) maternal body weights. B) litter size, C) maternal liver weights, and D) maternal kidney weights. Time-pregnant rats were exposed daily to 0 (control) or 480 mg TCE/kg-d (N = 10 per group) from gestational day 6 – 16 as described in the Materials and Methods section. Daily maternal weights were averaged and a mean was calculated for statistical comparison. The bars indicate means ± SE.

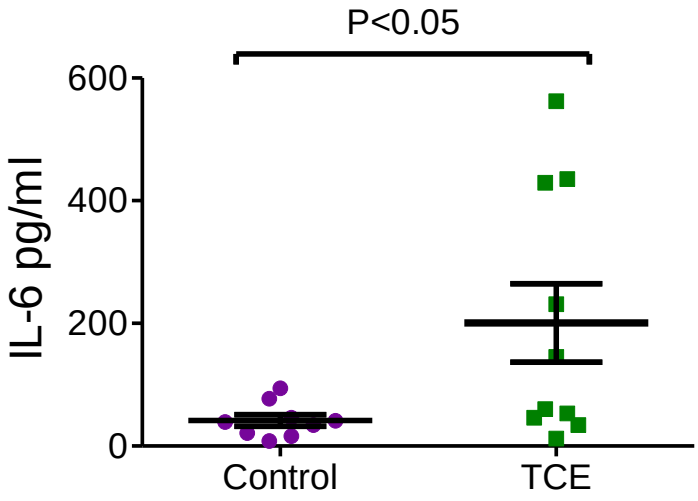


Figure 2.3. Effect of TCE treatment on interleukin (IL)-6 levels in maternal serum of time-pregnant rats exposed daily to 480 mg TCE/kg-d or 0 (vehicle control) (N= 10 per group; *p=0.03). The bars indicate means ± SE.

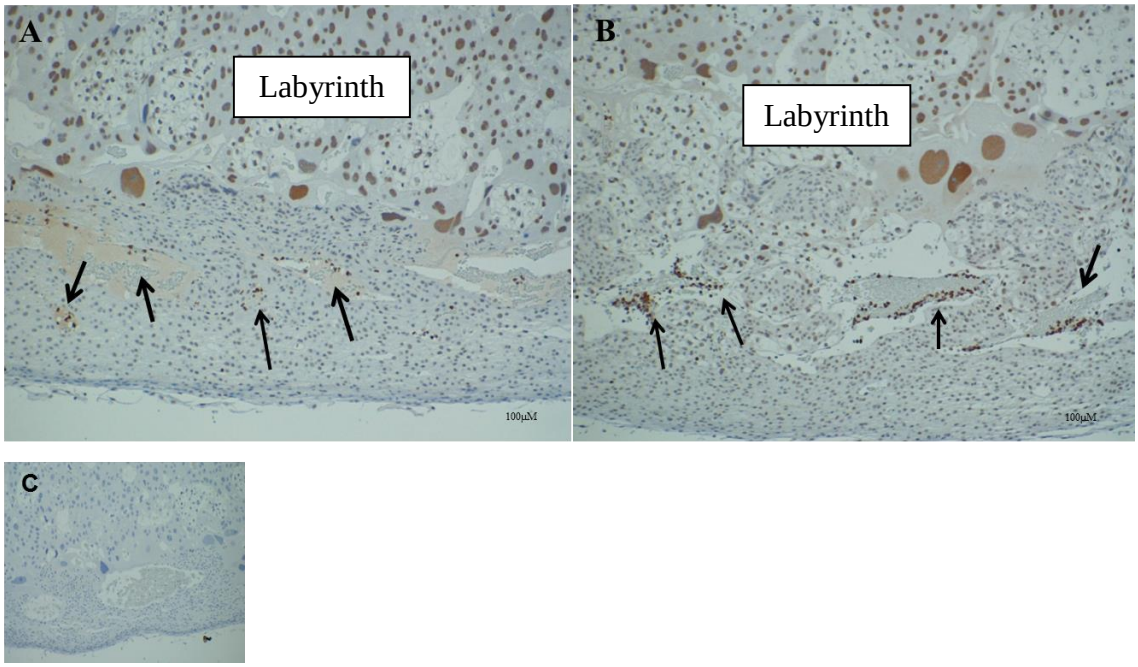


Figure 2.4. Immunohistochemical staining for matrix metalloproteinase-8 (MMP-8) as a marker of neutrophils in rat placenta. Time-pregnant rats were exposed daily to vehicle (control) or 480 mg TCE/kg-d from gestational day 6 -16. Representative images show low to moderate numbers of MMP-8-labeled neutrophils within the maternal blood vessels in the decidua basalis (indicated by arrows) in placenta from (A) TCE-treated rat, and (B) vehicle control rat. (C) Tissue section from control rat with secondary antibody alone (negative control). (The brown staining throughout the tissue sections is because MMP-8 marker also stains trophoblast of the labyrinth and trophoblastic giant cells of the basal zone.)

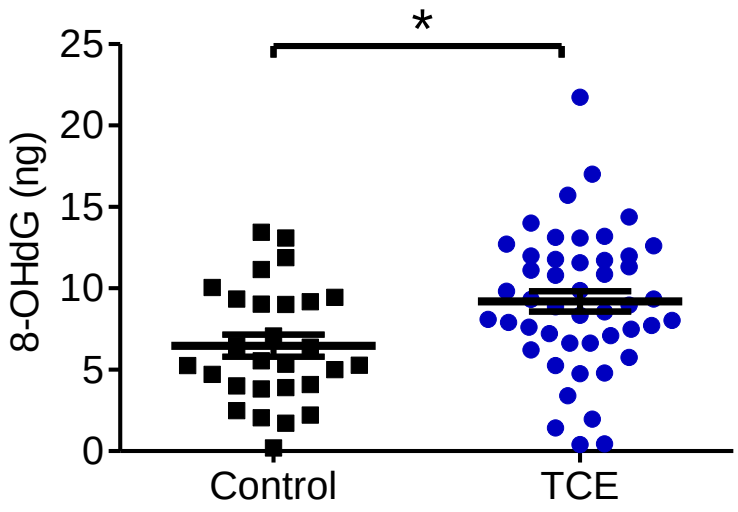
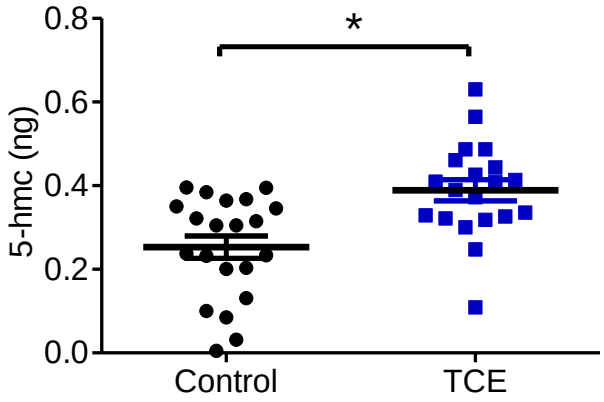


Figure 2.5. Levels of 8-OHdG in placentas of rats with or without exposure to TCE. Rats were exposed to vehicle control (N= 7 rats with 4 placentas per litter) to 480 mg TCE/kg-d (N=8 rats with 6 placentas per litter) from gd 6-16. Although values of individual placentas are shown, the data were analyzed on a per litter basis. The bars indicate litter means ± SE. *p=0.02

A



B

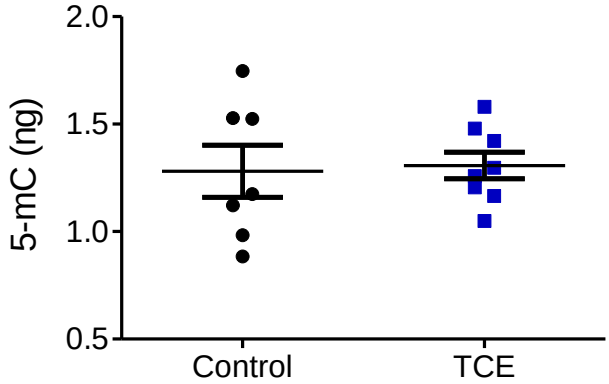
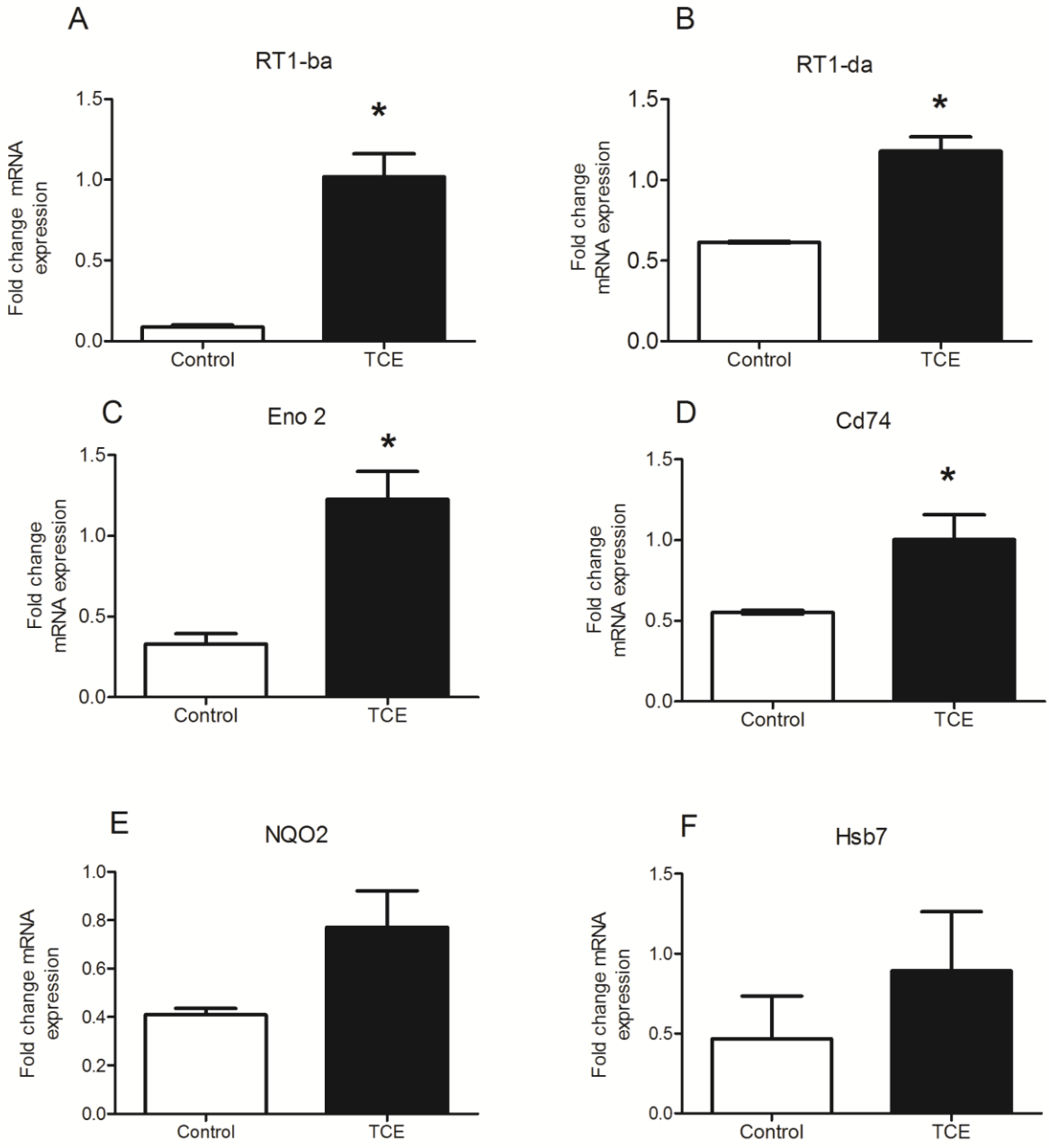


Figure 2.6. TCE-induced DNA methylation changes levels in placentas of time-pregnant rats exposed daily to 480 mg TCE/kg-day. A) 5-hydroxymethylcytosine (5-hmC); N= 8 rats for the TCE-treated group and N = 7 rats for the vehicle control group, with 3 placentas per litter; *significantly different (P=0.005). B) 5-methylcytosine (5-mC); N=8 rats for the TCE-treated group and N=7 rats for the control group, with 3 placentas pooled within each litter; P= 0.84, not statistically significantly different. The bars indicate means ± SE.


A
RT1-ba
Fold change mRNA expression
1.5
1.0
0.5
0.0
*
Control
TCE
B
RT1-da
Fold change mRNA expression
1.5
1.0
0.5
0.0
*
Control
TCE
C
Eno 2
Fold change mRNA expression
1.5
1.0
0.5
0.0
*
Control
TCE
D
Cd74
Fold change mRNA expression
1.5
1.0
0.5
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*
Control
TCE
E
NQO2
Fold change mRNA expression
1.0
0.8
0.6
0.4
0.2
0.0
Control
TCE
F
Hsb7
Fold change mRNA expression
1.5
1.0
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0.0
Control
TCE

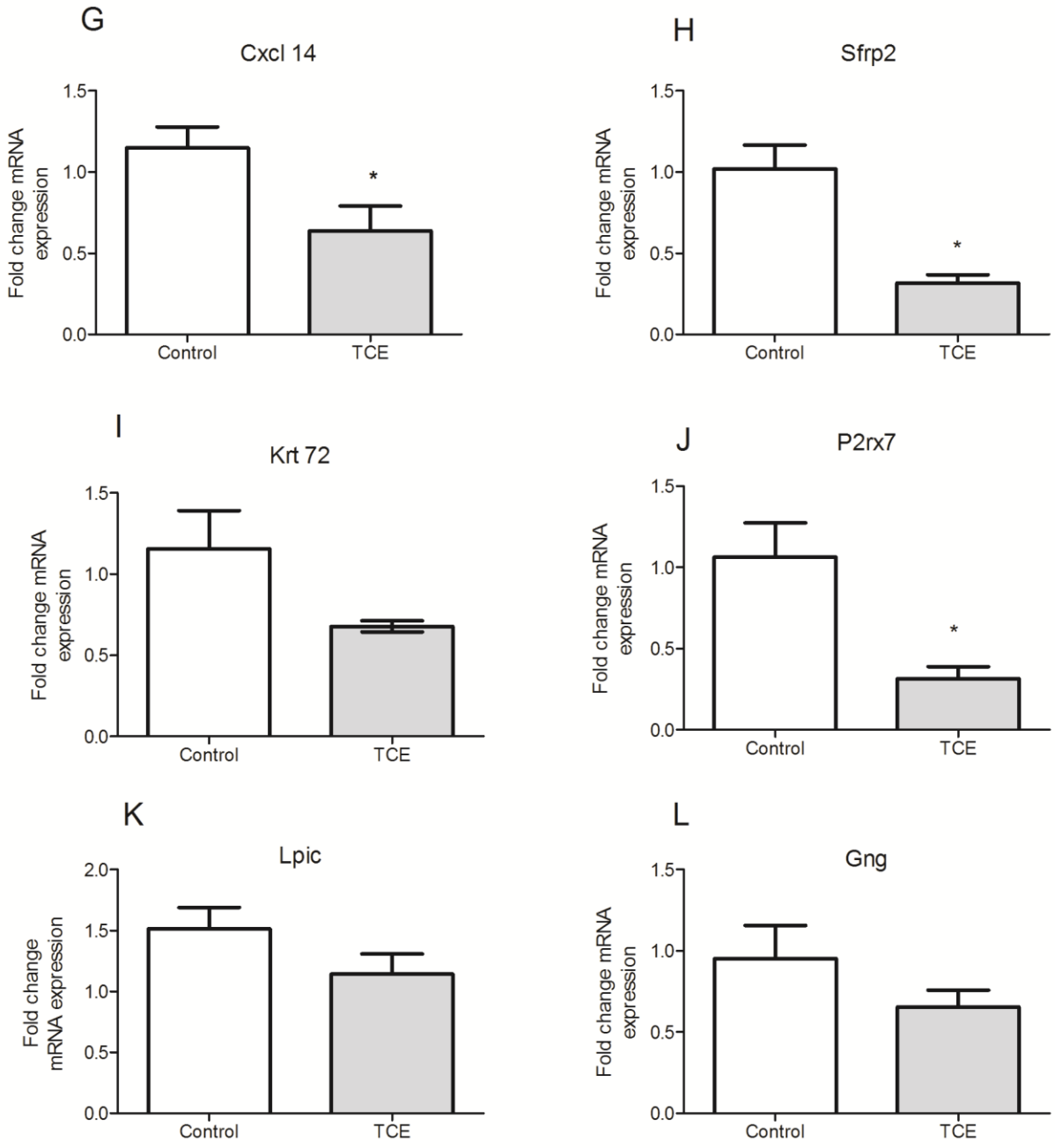

Figure 2.7. Validation of Rat Gene ST 1.1 Microarray Gene Expression changes using qRT-PCR in the placentas of time pregnant Wistar rats exposed daily to 480 mg TCE/kg-day or vehicle control from gestational day 6 – 16. Panels A-F show up-regulated genes. Panels G-L show down-regulated genes. The bars indicate means ± SE. N=4 rats per TCE-treated group and N=3 rats per vehicle control group, with 3 placentas/litter. The placentas were pooled within litter. *Significantly different ($p \leq 0.05$).

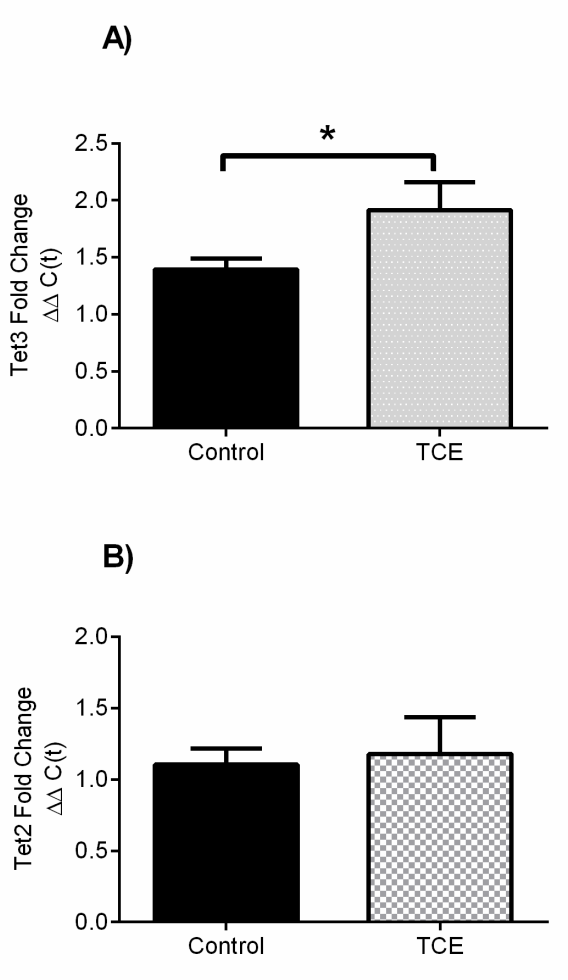

Figure 2.8. TCE-induced ten eleven translocation (Tet) enzyme mRNA expression levels in placentas of time-pregnant rats exposed daily to 480 mg TCE/kg-d (N=8 rats per group) or vehicle control (N=7 rats per group) with 3 placentas per litter. The placentas were pooled within litter**.** The bars indicate means ± SE. *Significantly different $p<0.05$.

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CHAPTER 3. REACTIVE OXYGEN STIMULATION OF INTERLEUKIN-6 RELEASE IN HUMAN PLACENTAL CELLS BY TRICHLORETHYLENE METABOLITE S-(1, 2-DICHLORO)-L-CYSTEINE

Abstract

Trichloroethylene (TCE) is a common environmental pollutant that has been associated with adverse reproductive and developmental outcomes in humans. TCE toxicity is primarily through its biotransformation to toxic metabolites, including S-(1,2-dichlorovinyl)-L-cysteine (DCVC). TCE its metabolites have been shown to induce oxidative stress and inflammation in other tissues including the liver and kidney. Although the placenta is a highly perfused organ capable of xenobiotic metabolism and oxidative stress and inflammation in placenta have been associated with adverse pregnancy outcomes, TCE metabolism and toxicity in the placenta remains poorly understood. Therefore, we determined the effects of DCVC on human placental cells using the human extravillous trophoblast cell line HTR-8/SVneo. We tested the hypothesis that DCVC increases generation of reactive oxygen species that mediate proinflammatory cytokine release. HTR-8/SVneo cells were treated with concentrations of DCVC ranging from 5-50 µM. We assessed mitochondrial dysfunction by quantifying ATP levels and measuring mitochondrial membrane potential. The HTR-8/SVneo cells exhibited decreased cellular concentrations of ATP at 10 and 20 µM DCVC and decreased mitochondrial membrane potential at 5, 10 and 20 µM DCVC, showing that DCVC induces mitochondrial dysfunction in HTR-8/Svneo cells. Exposure to 10 and 20 µM DCVC increased reactive oxygen species (ROS)

production as measured by carboxydichlorofluorescein fluorescence ($p<0.05$). Consistent with increased ROS generation, 10 and 20 µM DCVC decreased cellular glutathione and increased mRNA expression of the redox-sensitive genes glutaredoxin 2 (*GLRX2*) and thioredoxin reductase 1 (*TXNRD1*) ($p<0.05$). Moreover, 10 and 20 µM DCVC increased mRNA expression and release of IL-6, and these responses were inhibited by co-treatment with the antioxidant (±)α-tocopherol (50 µM). Additionally, pre-treatment with deferoxamine (1 mM), which can act as an antioxidant by chelating intracellular iron, significantly decreased IL-6 release. The results with antioxidant treatments suggest that DCVC-stimulated IL-6 release in HTR-8/SVneo cells is dependent on ROS. Additionally, pre-treatment with 1 mM aminooxyacetic acid (AOAA), a cysteine conjugated beta lyase inhibitor, significantly decreased DCVC-stimulated IL-6 release ($p<0.05$), suggesting that DCVC toxicity is dependent on its bioactivation by cysteine conjugated beta lyase. These data show that human placental cells respond to the TCE metabolite DCVC with activation of ROS-mediated IL-6 release. Because abnormal activation of proinflammatory cytokines can disrupt trophoblast functions necessary for placental development and successful pregnancy, these findings suggest that exposure to TCE during pregnancy may contribute to increased risk for adverse pregnancy outcomes.

Introduction

Trichloroethylene (TCE) is a chlorinated industrial solvent used as a metal degreaser and as an additive in paint removers and adhesives (U.S. EPA, 2011). Although, the amount of TCE released in the environment has decreased from >57 million pounds in 1988 to approximately 2.4 million pound in 2010, approximately 3 million people are exposed annually to TCE through environmental and occupational exposures in the US (U.S. EPA, 2011) . TCE has been recently classified by IARC as “carcinogenic to humans” (Guha *et al.*, 2012; IARC, 2014).

TCE is also a reproductive and developmental toxicant (Chiu *et al.*, 2006). 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 (Chiu *et al.*, 2013). Additionally, Forand et al. in a recent retrospective cohort study of 1,440 live births among New York residents found significant associations between exposure to TCE and decreased birth weight (Forand *et al.*, 2012). Animal studies have also shown that exposure to TCE during pregnancy increases fetal loss, alters glucose metabolism in the brain, and delays organ and bone development (Noland-Gerbec *et al.*, 1986; Das and Scott, 1994; Narotsky *et al.*, 1995; Johnson *et al.*, 1998) .

TCE toxicity is primarily dependent on its metabolism, which is tissue, species and sex dependent (Lash *et al.*, 2000a; Chiu *et al.*, 2013). TCE is metabolized via two separate pathways (Lash *et al.*, 2000a). The major pathway is oxidative metabolism in the liver by cytochrome P450 (CYP) enzymes to an epoxide, 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 (Lash *et al.*, 2000a). The second pathway is through conjugation with glutathione (GSH) in the liver and kidney by glutathione S transferase (GST) to S-(2,2-dichlorovinyl)glutathione (DCVG), which is subsequently biotransformed in the kidney by γ-glutamyl transpeptidase (GGT) to S-(1, 2-dichlorovinyl) cysteine (DCVC), then by cysteine conjugated β-lyase to a transient metabolite that rearranges to generate more toxic species (Lash *et al.*, 2000a). In addition, DCVC undergoes N-acetylation and is excreted in the urine as NAcDCVC or the sulfur-conjugated form NAcDCVCS (Lash *et al.*, 2000a).

DCVC induces cytotoxicity, mitochondrial dysfunction, and oxidative stress, and stimulates increased mRNA expression of proinflammatory mediators such as tumor necrosis

factor-α (TNF-α), interleukin (IL)-6, and cyclooxygenase-2 in animal and human kidney cells and tissue (Lash *et al.*, 1995; Cummings and Lash, 2000; Cummings *et al.*, 2000; Lash *et al.*, 2001; Shirai *et al.*, 2012). Perturbation of mitochondrial calcium homeostasis is an early effect of DCVC exposure and a key step in DCVC toxicity in the kidney (Xu *et al.*, 2008). DCVC stimulates hydroperoxide formation in human kidney proximal tubular cells, which is blocked by treatment with antioxidants (van de Water *et al.*, 1994). Additionally, in mice, exposure to DCVC has been shown to induce increased levels of IL-6 in the plasma and in the kidney (Shirai *et al.*, 2012).

Increased release of proinflammatory cytokines plays a critical roles in placentation and parturition (Lappas *et al.*, 2003). 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 (Vrachnis *et al.*, 2010). 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 (Goncalves *et al.*, 2002; Hendson *et al.*, 2011). Similarly, bacterial infection elicits a proinflammatory cascade in extraplacental membranes with enhanced release of cytokines and matrix metalloproteases (MMPs) (Goncalves *et al.*, 2002; Vrachnis *et al.*, 2010). Studies with 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. Although widely used as a proinflammatory stimulus, exposure of human fetal membranes to LPS leads to oxidative stress with increased production of 8-isoprostanes (Lappas *et al.*, 2003). Moreover, the antioxidant N-acetylcysteine (NAC)

prevents LPS-stimulated parturition in mice as well as LPS-induced proinflammatory cytokine release from extraembryonic membranes in vitro (Lappas *et al.*, 2003).

Dysregulation of cytokine release (Challis *et al.*, 2009) and oxidative stress (Al-Gubory *et al.*, 2010) in gestational tissues are associated with pathophysiology of pregnancy, including preterm labor, preeclampsia, and IUGR. 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 (Ouyang *et al.*, 2009). Together, these findings suggest that oxidative stress mediates at least some proinflammatory responses in gestational tissues with relevance to untoward 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 investigate the effects of DCVC on ROS-mediated stimulation of IL-6 in the human extravillous trophoblast cell line HTR-8/SVneo.

Materials and Methods

Materials

S-(1, 2-dichlorovinyl)-L-cysteine (DCVC) was synthesized by the University of Michigan Medicinal Chemistry Core according to procedures described by McKinney et al. (McKinney *et al.*, 1959). Purity (98.7%) was determined by HPLC analysis and identity was confirmed by proton nuclear magnetic resonance spectroscopy. Phosphate buffered saline (PBS), Hank's balanced salt solution (HBSS), 6-carboxy-2',7'-dichlorodihydrofluoresceine diacetate (H2DCF-DA), Hoechst 33342, and 0.25% trypsin were purchased from Invitrogen Life Technologies (Carlsbad, CA). Amniooxyacetic acid (AOAA), *tert*-butyl hydroperoxide, and (±)-

α-tocopherol and deferoxamine mesylate (DFO) were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 culture medium with $_{L}$-glutamine and without phenol red, 10,000 U/mL penicillin/10,000 µg/mL streptomycin (P/S) and fetal bovine serum (FBS) were from Gibco (Grand Island, NY). Lipopolysaccharide (LPS) from *Salmonella typhimurium*, was purchased from List Biological Laboratories, Inc. (Campbell, CA). The JC-1 Mitochondrial Membrane Potential Assay was purchased as a kit from Cayman Chemical (Ann Arbor, MI). Bongkrekic acid (BkA) was purchased from Calbiochem (Billerica, MA).

Cell culture and treatment

HTR-8/SVneo cells were kindly provided by Dr. Charles H. Graham (Queen's University, Kingston, Ontario, Canada). The HTR-8/SVneo cell line was derived from first trimester human cytotrophoblast cells and immortalized with simian virus 40 large T antigen (Graham et al, 1993). HTR-8/SVneo cells were cultured as previously described (Tetz et al. 2013). Briefly, cells were cultured between passages 73-85 in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin (P/S) at 37°C in a 5% CO_2 humidified atmosphere. Cells were grown to a 70-80% confluence 24 h after subculture and before starting an experiment. A stock solution of 1 mM DCVC was prepared in PBS and stored at -20 °C. Prior to each experiment, DCVC stock solution was 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 on DCVC exposures of human placental cells are lacking, exposure concentrations for the current study were selected based on the lower range of concentrations used in studies of kidney cells (Chen *et al.*, 1990a; Xu *et al.*, 2008).

Cytotoxicity and viability

Cytotoxicity and viability were assayed using a MultiTox-Glo Multiplex kit following the manufacturer's directions (Promega). 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 an additional 24 h. The MultiTox-Glo Multiplex assay measures intracellular protease activity to determine cell viability and extracellular protease activity as a measure of membrane integrity. Cell viability was measured by using the flourogenic substrate glycyl-phenylananyl-aminoflourocoumarin (GF-AFC), which is cleaved by live cell proteases to yield the fluorescent product aminoflourocoumarin (AFC). Then, fluorescence was measured using a SpectraMax M2e Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA), with fluorescence inversely correlated to number of viable cells. After fluorescence was measured, the luminescent cell-impermeable protease substrate alanyl-alanyl-phenylalanyl-aminoluciferin (AAF-Glo) was added to the cultured cells to determine cytotoxicity. Luminescence was quantified using the Glomax Multi Plus Detection System (Promega, Madison, WI). Luminescence was proportional to protease leakage from cells with compromised cell membranes.

Measurement of reactive oxygen species (ROS)

A modification of the dichlorofluorescein (DCF) assay was used to assess DCVC-stimulated generation of oxidant species in HTR-8/SVneo (Tetz *et al.*, 2013). Cells were seeded at a density of 30,000 cells per well in a 96-well black clear-bottom plate. After 24 h incubation, cells were treated with 10 or 20 μM DCVC for 10 h. Then, treatment medium was removed, cells were rinsed twice with HBSS, and cells were incubated with 100 μM 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) in HBSS for 1 h at 37 °C. The H_2DCF-DA concentration of 100 μM H_2DCF-DA was chosen based on prior studies with HTR-8/SVneo

cells (Tetz *et al.*, 2013). After loading cells with the probe, cells were washed twice with HBSS. Fresh HBSS was added back to the cells. Fluorescence was measured immediately by using Molecular Device SpectraMax Gemini M2e at 492 nm excitation and 522 nm emission wavelengths.

Stimulation of intracellular ROS generation was also visualized by microscopic detection of 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 DCF fluorescence as measured by spectrofluorimetry. *Tert*-butyl hydroperoxide (25 µM), a prototypical chemical oxidant used to generate intracellular ROS (Korte, 2013; Tetz *et al.*, 2013), 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 H_2DCF-DA in HBSS for 1 h. The HBSS containing 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, intracellular DCF fluorescence was visualized at 470 nm excitation and 525 nm emission, and Hoechst 33342 was visualized at 360 nm excitation and 447 nm emission. Five images were taken, one in each of the four quadrants and one in the middle of the well. Equivalent adjustment for brightness and contrast was applied to each image using ImageJ software (National Institutes of Health).

Measurement of intracellular glutathione levels

The effect of DCVC on intracellular glutathione levels (GSH) was measured in HTR-8/SVneo cells using the commercial GSH-Glo Glutathione Assay (Promega) following the manufacturer's protocol. 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 then the cells were washed with PBS. 100 µl of prepared GSH-Glo reagent was 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 was 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 on a Glomax Multi Plus Luminescence Detection System (Promega).

RNA extraction and quantitative real-time PCR

HTR-8/SVneo cells were seeded at a density of 50,000 cells per well in 24-well plates and cultured for 24 h. Cells were exposed to 5, 10 or 20 µM DCVC or untreated for 24 h then cell culture medium was removed and cells were lysed using RNA lysis buffer (Qiagen). Cell lysates were collected and homogenized using QIA shredder, and homogenates were pooled. The mRNA was then extracted using RNeasy mini plus kit (Qiagen) following the manufacturer's protocol. Aliquots of 1 µg of mRNA were used for cDNA synthesis using the RT2 First Strand kit (Qiagen) following the manufacturer's protocol. Quantitative real-time polymerase chain reactions (qRT-PCR) was performed using 12.5 µL of RT^2SYBR Green qPCR Master Mix, 1 µL of gene-specific primer target (*TXNRD*1, *GLRX*2 and *IL*6), 4 µL of cDNA template, and 7.5 µL of nuclease-free H_2O, for a total volume of 25 µL. Samples were analysed using a CFX96 Real-Time PCR Detection System (Bio-Rad Labs). 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, 5 s at 60 °C. The ΔΔCt method (Yuan *et al.*, 2006) was used to quantify and normalize the signal intensities for

the target gene to the housekeeping gene signal using Bio-Rad CFX manager software (Bio-Rad Labs). The mRNA levels were represented as fold change relative to nontreatment controls. All samples were analysed in triplicate.

Measurement of cellular ATP content

Relative cellular ATP content was measured by the Cell Titer-Glo Luminescent 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. Cell Titer Glo reagent was added to cells and incubated in the dark at room temperature for 30 min and luminescence was measured using Glomax Multi Plus Luminescence Detection System (Promega). The acquired luminescence signal is proportional to the amount of ATP in the cells.

Mitochondrial membrane potential assay (MMP) measurement

Mitochondrial membrane potential was assayed using the fluorescent reagent 5, 5′, 6, 6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) with the Mitochondrial Membrane Potential Assay (Cayman Chemical) following the manufacturer's protocol. 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 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 per well of fresh HBSS was added, and fluorescence was measured using a Molecular Device SpectraMax Gemini M2e spectrofluorometer.

In separate experiments, JC-1 fluorescence was measured 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.

DCVC-stimulated IL-6 release

DCVC-stimulated release of the proinflammatory cytokine IL-6 was 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) for 10 or 24 h. LPS (100 ng/mL) was included as a positive control. The concentration of IL-6 in cell culture medium was quantified using ELISA (R&D Systems).

Modulation of DCVC-stimulated IL-6 release

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. Because DCVC stimulated IL-6 release in cell culture medium with 24 h but not 10 h of exposure, we chose the 24-h exposure duration to determine the effect of antioxidants on DCVC-stimulated IL-6 release. First, HTR-8/SVneo cells were co-treated for 24 h with DCVC and 50 μM (±)-α-tocopherol, an antioxidant and peroxyl radical scavenger, or were pretreated with 1 mM deferoxamine (DFO), an iron chelator, for 1 h prior to DCVC exposure. We used 1 mM DFO because this concentration blocks DCVC-induced oxidative stress in rabbit renal proximal tubular cells (Groves *et al.*, 1991) and is not cytotoxic to HTR-8/SVneo cells (Tetz *et al.*, 2013). Second, to investigate mitochondrial

dysfunction in DCVC stimulation of IL-6, HTR-8/SVneo cells were pretreated for 1 h with 10 μM bongkrekic acid (BkA), an inhibitor of the mitochondrial permeability transition pore (Lieven *et al.*, 2003). Finally, to test cysteine conjugate β-lyase-catalyzed metabolism in DCVC-induced toxicity, 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 (Lieven, Vrabec et al. 2003) and AOAA (Lash, Sausen et al. 1994) was chosen based on efficacy in previous studies (Walsh Clang and Aleo, 1997).

Statistical analysis

All experiments were repeated at least three times and performed in triplicate. The triplicates were averaged within each experiment, and these values were analysed either by one-way or two-way analysis of variance (ANOVA), followed by Tukey post-hoc comparison of means using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Data are expressed as means ± SEM. N=3 independent experiments. $P < 0.05$ was considered statistically significant.

Results

Effect of DCVC on HTR-8/SVneo cytotoxicity and viability

Treatment with 10, 20 or 50 μM DCVC for 24 h did not induce statistically significant cytotoxicity at 24 h as assessed by intracellular protease activity (Fig. 3.1A). Treatment with 50 μM DCVC, but not lower DCVC concentrations, significantly decreased cell viability by 34.5% after 24 h, as assessed by a membrane integrity assay of extracellular protease activity (Fig. 3.1B; $p<0.05$). On the other hand, treatment with 30 ng/mL digitonin, the positive control,

significantly increased cytotoxicity 4-fold (Fig. 3.1A, $p<0.001$) and decreased cell viability by 87.30% (Fig. 3.1B, $p<0.001$).

DCVC-stimulated generation of reactive oxygen species (ROS)

DCVC stimulation of reactive oxygen species generation was assessed with a modification of the DCF fluorescence assay. Treatment of HTR-8/SVneo cells with 10 or 20 μM DCVC for 10 h significantly increased carboxy-DCF fluorescence by 21.5% and 37%, respectively, compared with untreated control as quantified by spectrophotometry (Fig. 3.2; $p<0.001$). Representative fluorescence microscopy images in Figure 3.3 show increased carboxy-DCF fluorescence in HTR-8/SVneo cells treated with 10 μM DCVC (Fig. 3.3 B) compared with nontreated control (Fig. 3.3A), although the fluorescence appeared less intense than that observed with 25 μM *tert*-butyl hydroperoxide, the positive control (Fig. 3.3 C). Figures 3.3D, E, and F show the corresponding Hoescht nuclear staining images of cell cultures receiving control, 10 μM DCVC, or 25 μM *tert*-butyl hydroperoxide treatments, respectively.

Treatment effects of DCVC on intracellular glutathione (GSH) levels

Because DCVC increased ROS production, we investigated DCVC effects on the levels of 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 nontreated control, as assessed by a luminescence assay (Fig. 3.4; $p<0.01$).

DCVC effects on mRNA expression of redox-sensitive genes

Considering that redox-sensitive genes *TXNRD1* and *GLRX2* were previously shown in our lab to be induced in HTR-8/SVneo cells by treatments that stimulate ROS (Korte, 2013; Tetz *et al.*, 2013), we evaluated the effect of DCVC on the expression of these genes. Treatment of HTR-8/SVneo cells with 10 or 20 μM DCVC for 24 h increased *GLRX2* mRNA expression 1.5 and 2.3-fold, respectively, compared to control (Fig. 3.5; $p<0.05$). As shown also in figure 3.5, exposure to 20 μM DCVC resulted in a 2.2-fold increase in *TXNRD1* mRNA expression compared with nontreated controls ($p<0.05$), but there was not a statistically significant effect on *TXNRD1* mRNA with 10 μM DCVC treatment.

DCVC effects on cellular ATP content

Because toxicants that target mitochondria can lead to increased ROS generation (Xu *et al.*, 2008), we investigated DCVC-induced changes in cellular ATP levels in HTR-8/SVneo cells. Treatment for 24 h with 10 and 20 μM DCVC, but not 5 μM DCVC, significantly decreased ATP content by 19.6% and 26.1%, respectively, compared with nontreated controls (Fig. 3.6; $p<0.01$).

Effect of DCVC treatment on mitochondrial membrane potential (MMP)

To further elucidate the role of mitochondrial dysfunction in DCVC-induced increase in ROS and decrease of ATP levels in HTR-8/SVneo cells, we used JC-1, a selective mitochondrial membrane potential dye. Because JC-1 fluorescence shifts from green to red with JC-1 membrane potential-dependent accumulation and aggregation in mitochondria, mitochondrial depolarization was quantified by a decrease in JC-1 red/green fluorescence intensity ratio. 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 nontreated control (Fig. 3.7; $p<0.05$), as quantified using a spectrofluorometer. In separate experiments, JC-1 dye was used to visualize a decrease in MMP with fluorescence microscopy. We observed a fluorescence shift from red to green in cells exposed to 5, 10, or 20 μM DCVC for 24 h compared with nontreated control, indicative of decreased MMP with DCVC exposure (Fig. 3.8; representative images). A similar pattern was observed with DCVC treatment for 10 h (data not shown).

DCVC-stimulated IL-6 release

Because we found DCVC-induced increased levels of ROS in the HTR-8/SVneo cells and reactive oxygen species have been implicated in stimulation of proinflammatory cytokines, we elucidated the effect of DCVC exposure on IL-6 production. Treatment of HTR-8/SVneo cells with 10 or 20 μM DCVC for 24 h stimulated concentration-dependent increases of 1.7 and 3.3-fold in IL-6, respectively, relative to nontreated control (Fig. 3.9; $p<0.05$). 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 ($p<0.05$). After 10 h of treatment, no statistically significant changes in IL-6 release were observed with DCVC, although LPS stimulated IL-6 release by 3.3-fold at this time-point ($p<0.05$).

Effects of treatment with antioxidants and inhibitors on DCVC-stimulated IL-6 release

To test the hypothesis that DCVC-induced ROS mediates stimulation of IL-6 release, HTR-8/SVneo cells were cotreated with the antioxidant (±)-α-tocopherol or pretreated with the iron chelator deferoxamine mesylate (DFO). DFO was used because it has been shown to prevent DCVC-stimulated hydroperoxide formation in rat proximal tubular cells (van de Water

et al., 1993). Cotreatment with 50 µM (±)-α-tocopherol (Fig. 3.11A) and pretreatment with 1 mM DFO (Fig. 3.11B) significantly depressed IL-6 release in cells stimulated for 24 h with 10 or 20 µM DCVC compared with samples treated with DCVC without antioxidants ($p<0.01$). Because we found evidence of DCVC-induced mitochondrial dysfunction, we tested the effect of BkA, a mitochondrial membrane transition pore inhibitor (Lieven *et al.*, 2003), on DCVC-stimulated IL-6 release. Pretreatment with 10 µM BkA followed by 20 µM DCVC modestly decreased IL-6 release by 27%, compared with cells treated with DCVC alone (Fig. 3.12; $p<0.05$). We used the cysteine conjugated β-lyase inhibitor AOAA to explore the dependence of DCVC-induced IL-6 release on metabolic activation of DCVC. As shown in figure 3.13, exposure to AOAA blocked DCVC-stimulated IL-6 release in HTR-8/Svneo cells (Fig. 3.13; $p<0.001$).

Effects of DCVC and antioxidant treatments on IL6 mRNA expression

Due to our observation that DCVC-stimulated IL-6 release, we quantified expression of the IL6 gene in HTR-8/SVneo cells using qRT-PCR. Exposure to 10 or 20 µM DCVC for 24 h significantly increased IL6 mRNA expression by 7.3 and 11.4-fold, respectively, compared with nontreated control (Fig. 10A; $p<0.01$). No significant increase 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% with 10 µM DCVC and 67.2%, with 20 µM DCVC Fig. 3.10B; $p<0.05$).

Discussion

Due to improper disposal, TCE has become a widespread air, soil and drinking water contaminant (U.S. EPA, 2011). It is rapidly metabolized *in vivo* to highly reactive intermediates

such as S-(1,2-dichlorovinyl)-L-cysteine (DCVC), which is a well-characterized kidney toxicant (Lash *et al.*, 2000b). Although exposure to TCE has been linked to poor birth outcomes (Bove *et al.*, 2002; Forand *et al.*, 2012), DCVC has not previously been considered as a potential toxicant to placenta. In the present study, we demonstrate that the trichloroethylene metabolite DCVC stimulates reactive oxygen species (ROS) generation in human placental cells. In addition, we provide evidence of linkages between DCVC-stimulated ROS and increase in 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.

Our study reports a direct stimulatory effect of DCVC on release of IL-6 in HTR-8/SVneo cells. 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. Additionally, DCVC increased mRNA expression of the IL6 gene in a parallel manner, suggesting that DCVC acts at the level of transcription to stimulate the IL-6 response. Although there has been a paucity of research on DCVC-induced IL-6 release, our findings are consistent with a report that DCVC exposure stimulated IL-6 release and increased renal IL-6 mRNA expression in mice (Shirai *et al.*, 2012). Because growing evidence links improper activation of inflammatory pathways including IL-6 with impaired trophoblast function and poor placental development (Challis *et al.*, 2009), 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 levels is similar to previously published studies in rat kidney proximal tubular cells (van de Water *et al.*, 1994). 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 with DCVC treatment plays an important role in IL-6 release. These findings are in agreement with previous reports that DFO decreases DCVC-induced ROS production in rabbit renal cortical slices (van de Water *et al.*, 1994) and inhibits DCVC-induced cytotoxicity in rat kidney epithelial cells (Chen *et al.*, 1990).

Consistent with increased ROS, we also found that DCVC decreased glutathione 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 (HPLC) are warranted in order to determine physiological relevance of DCVC-induced GSH decrease in HTR-8/SVneo cells. Our findings corroborate prior studies that show incubation of rat proximal tubular cells with DCVC depletes cellular GSH (Lash and Anders, 1986). GSH, a major intracellular antioxidant, reacts with electrophiles, reactive oxygen species, and lipid peroxides to protect cells from oxidative stress (Lushchak, 2012). Decreased levels of GSH in the maternal blood and in the placenta have been associated with adverse pregnancy outcomes including preeclampsia (Knapen *et al.*, 1998), preterm birth, and IUGR (Zadrozna *et al.*, 2009). Therefore, our results suggest that exposure to DCVC decreases GSH levels and increases oxidative stress in placental cells which could lead to poor placentation with possible increased risk for poor obstetrical outcomes.

Concordant with our findings that DCVC increased ROS and decreased glutathione levels, we also observed increased expression of antioxidant genes *GLRX2* and *TXNRD1*. These redox-sensitive genes are disulphide reductases that defend the cells against oxidative stress,

DNA damage, and apoptosis (Arner and Holmgren, 2000). In humans, increased levels of the protein products of *GLRX2* and *TXNRD1* in the placenta have been associated with preeclampsia (Shibata *et al.*, 2001). Furthermore, differential expression of antioxidant response genes have been observed in pregnancies that are complicated by pathologies such as intrauterine growth restriction and preeclampsia (Biri *et al.*, 2006; Biri *et al.*, 2007; Hoegh *et al.*, 2010). Therefore, we suggest that HTR-8/SVneo cells increased expression of the redox-sensitive genes *GLRX2* and *TXNRD1* as a response to DCVC-induced increase in cellular ROS.

In mitochondria, ROS are generated as oxidative phosphorylation by-products, and under normal physiologic conditions superoxide anions can leak from the mitochondria to form hydrogen peroxide or hydroxyl radical (Fariss *et al.*, 2005). We found that exposure to DCVC decreased mitochondrial membrane potential and ATP levels, suggesting that DCVC-induced mitochondrial dysfunction may be contributing to DCVC-stimulated generation of ROS in HTR-8/SVneo cells. Furthermore, treatment with the mitochondrial membrane transition pore inhibitor bongkrekic acid (BkA) significantly attenuated IL-6 release. However, treatment with BkA did not completely inhibit IL-6 release, suggesting that mitochondrial dysfunction is 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). Because mitochondrial dysfunction can lead to enhanced mitochondrial production of ROS (Sohal *et al.*, 1995), we suggest that the DCVC-stimulated ROS in HTR-8/SVneo cells may have originated from mitochondria, at least in part. These findings corroborate previous reports that DCVC induces mitochondrial toxicity in other cells and tissues. Specifically, studies in rat proximal tubular cells found that DCVC decreased mitochondrial membrane potential (van de Water *et al.*, 1994) and ATP production (Lash *et al.*,

1986). In human proximal tubular cells, mitochondrial dysfunction is a necessary step in DCVC-induced toxicity (Xu *et al.*, 2008). Moreover, DCVC-stimulated ATP and glutathione decreases were the most sensitive endpoints in a cataractogenesis study in rat, (Walsh Clang *et al.*, 1997).

The present study supports the role of DCVC metabolism in stimulation of IL-6 release. We observed that treatment with AOAA, a renal cysteine conjugated 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 cysteine conjugated β-lyase in other cells and tissues (Walsh Clang *et al.*, 1997; McGoldrick *et al.*, 2003). DCVC is bioactivated by cysteine conjugated β-lyase to generate the highly reactive intermediate S-(1,2-dichlorovinyl)thiol (DCVT) (Lash *et al.*, 2014,). DCVT is chemically unstable and rearranges to form chlorothioketene (CTK) and chlorothionoacyl 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 (Lash *et al.*, 2014). Findings from the current study suggest that β-lyase 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 cysteine conjugated β-lyase.

While contributing new information on DCVC activity in human placental cells, our study has some limitations. Firstly, the concentrations used in the present study are a magnitude higher than the US EPA maximum contaminant level standard for drinking water of 5 ppb for the parent compound TCE (U.S. EPA, 2011). 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 (Lash *et al.*, 2000a; Xu *et al.*, 2008). 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.

Secondly, the current study lacks experiments of functional consequences of the changes observed. Therefore, further investigation is warranted to determine the potential relevance of DCVC on placenta development, for example, by assessing the effect of DCVC on trophoblast migration and invasion. Thirdly, although utilizing a cell line allows us to study ROS-mediated activation of proinflammatory cytokine by using cell permeable ROS sensitive probes and other methods used to study cell signaling, we recognize limitations related to the absence of cell-cell interactions or tissue-tissue interactions when using cell culture models. Therefore, further investigation using placental tissues 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 similar phenotype to primary human trophoblasts (Graham *et al.*, 1993; Biondi *et al.*, 2006), 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 (Nicola *et al.*, 2005; Khan *et al.*, 2011), HTR-8/SVneo cells have a different epigenetic profile compared with primary extravillous trophoblast (Novakovic *et al.*, 2011). Nevertheless, HTR-8/SVneo cells have been a useful model in other studies to examine placentation and physiologically invasive extravillous trophoblast (Liu *et al.*, 2012; Wang *et al.*, 2012; Weber *et al.*, 2013; Xu *et al.*, 2014).

In summary, we demonstrate that DCVC, a bioactive metabolite of TCE, stimulates reactive oxygen species (ROS) generation in human placental cells. In addition, we report the 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 10 to 50 μm, which is a lower concentration range than what 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. 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.

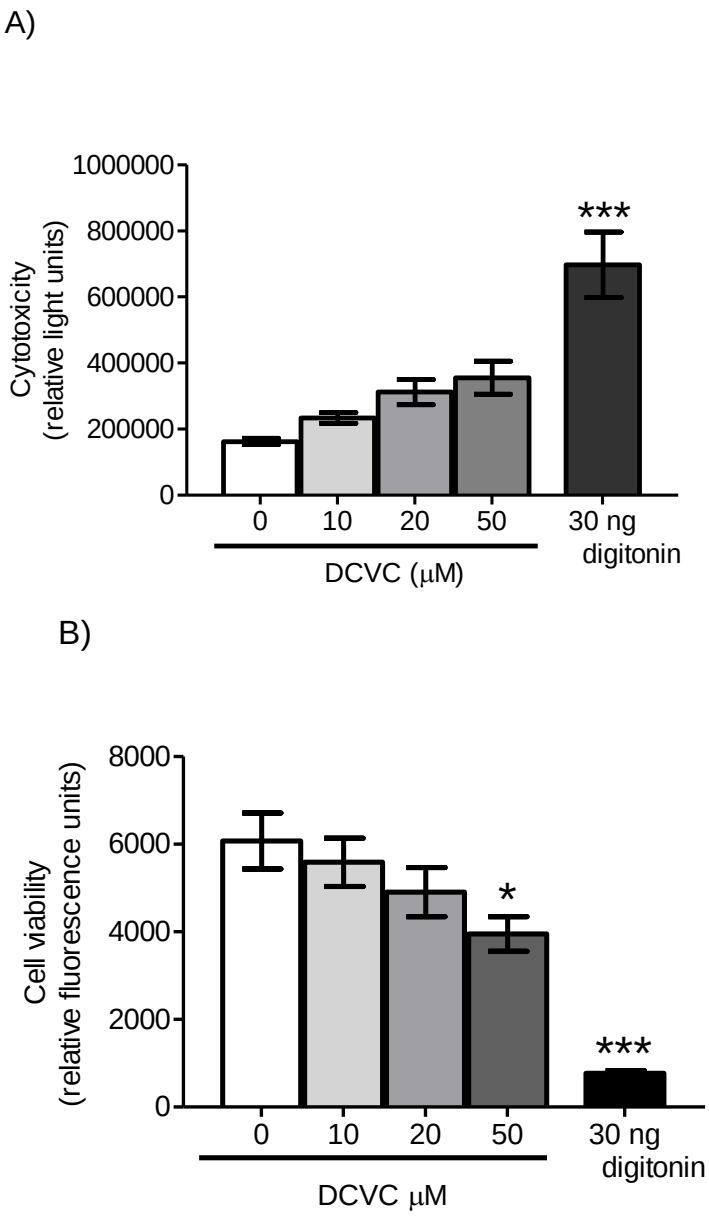

Figure 3.1. DCVC toxicity in HTR-8/SVneo cells. (A) Cytotoxicity as determined by a luminescence-based assay of intracellular protease activity. (B) Cell viability as determined by a membrane integrity assay of extracellular protease activity. Cells were treated for 24 h with 0 (untreated control), 10, 20 or 50 μM DCVC, or with 30 ng/mL digitonin (positive control). Cytotoxicity and cell viability were assessed using a multiplex assay as described in Materials and Methods section. Bars represent means ± SE. *P<0.05 and ***p<0.001 are statistically significantly decreased compared with untreated control. N=3 experiments and each experiment was performed in triplicate.

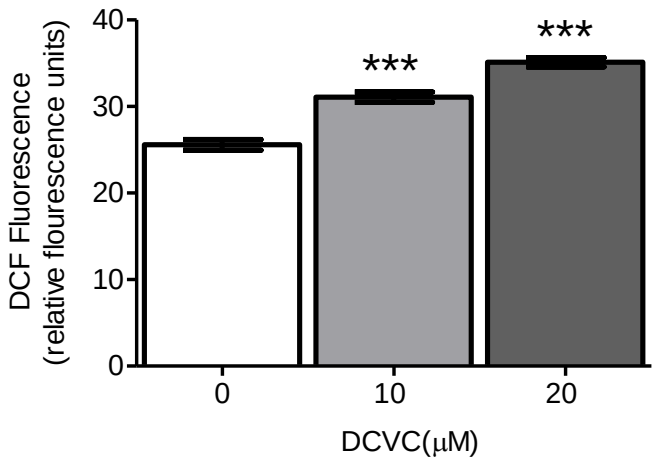

Figure 3.2. DCVC-stimulated generation of reactive oxygen species (ROS) quantified by carboxy-DCF fluorescence. Cells were treated with 0 (untreated control), 10 or 20 μM DCVC for 10 h, then carboxy-DCF fluorescence was used to measure ROS as described in the Material and Methods section. Data are represented as mean ± SE. ***P<0.001 is statistically significantly increased compared with untreated control. N=3 experiments and each experiment was performed in triplicate.

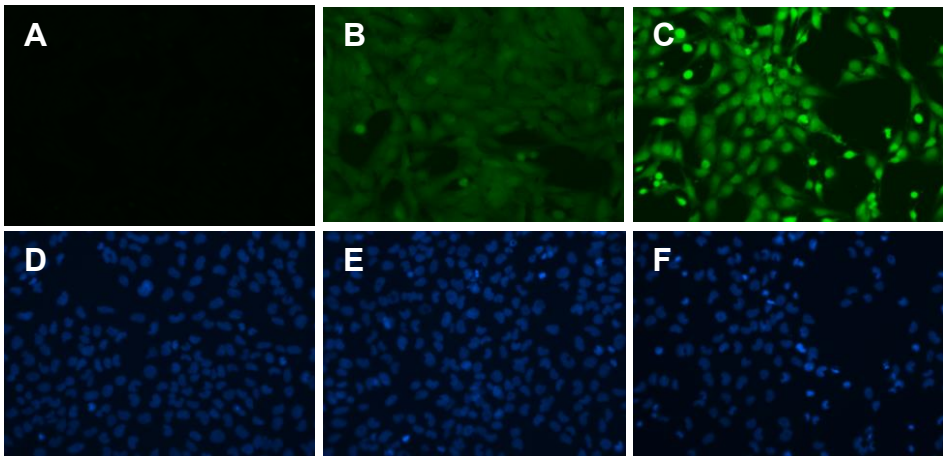

Figure 3.3. DCVC-stimulated generation of reactive oxygen species (ROS) visualized by microscopic detection of carboxy-DCF fluorescence. The HTR-8/SVneo cells were treated with 10 μM DCVC or 25 μM TBHP (positive control) for 10 h, loaded with H_2DCF-DA for 1 h, and then counterstained with Hoechst 33342. The top row shows intracellular fluorescence of A) nontreated control, B) 10 μM DCVC, and C) 25 μM TBHP. The bottom row shows the corresponding Hoescht nuclear staining images of D) nontreated control, E) 10 μM DCVC, and F) 25 μM TBHP. Representative images of 3 experiments are shown.

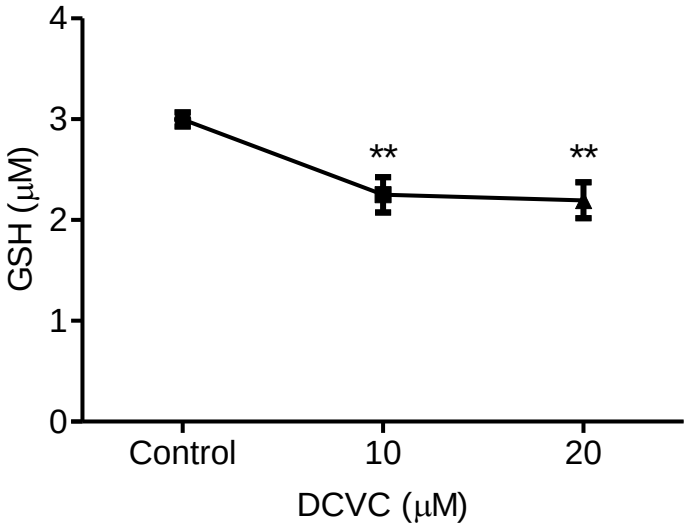

Figure 3.4. Treatment effects of DCVC on intracellular glutathione (GSH) levels. A luminescence-based assay was used to quantify GSH concentrations in cellular lysates of HTR-8/SVneo cells exposed to 10 or 20 μM DCVC for 24 h. **P<0.01 is statistically significantly decreased compared with nontreated control. N=3 independent experiments and each experiment was performed in triplicate.

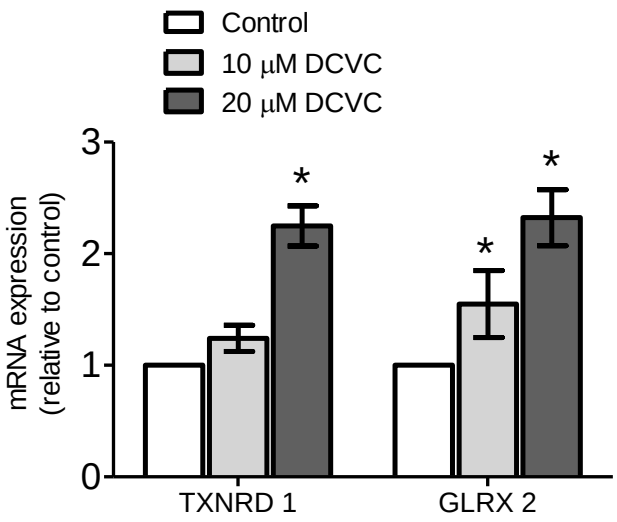

Figure 3.5. DCVC effect on mRNA expression of redox-sensitive genes. Increased mRNA expression of TXNRD1 and GLRX2 was quantified in HTR-8/SVneo cells by qRT-PCR in cells exposed to 10 or 20 μM DCVC for 24 h. The bars represent mean ± SE. *P<0.05 is statistically significantly increased compared with untreated control. N=3 experiments and each experiment was performed in triplicate.

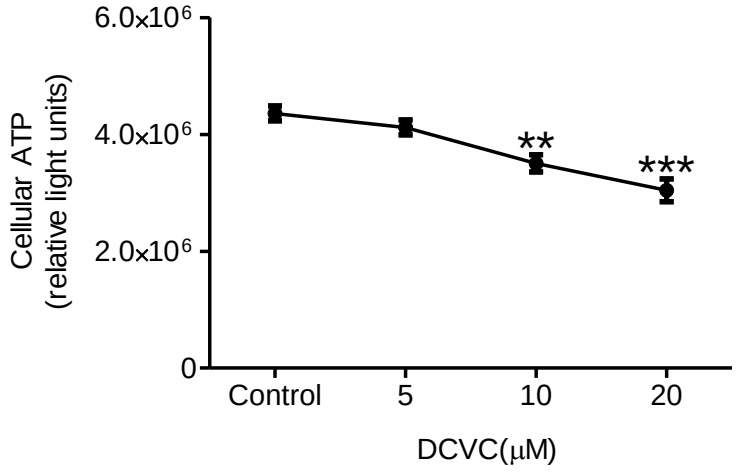

Figure 3.6. DCVC effects on cellular ATP content in HTR-8/SVneo cells. Cellular ATP content was measured after 24 h treatment with 5, 10 or 20 μM DCVC using the CellTiter-Glo™ luminescence assay (described in Materials and Methods). Data are represented as means ±SE. **p<0.01 and ***p<0.001 are statistically significantly decreased compared with nontreated control. N=3 experiments and each experiment was performed in triplicate.

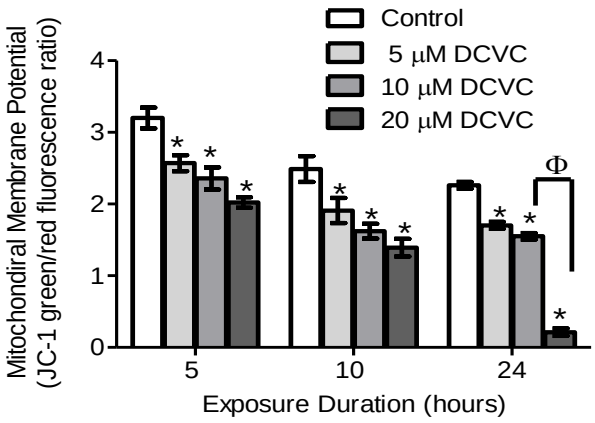

Figure 3.7. Effect of DCVC on mitochondrial membrane potential (MMP). JC-1 dye was used to measure MMP in HTR-8/SVneo cells treated for 5, 10, or 24 h with 0 (control), 5, 10 or 20 μM DCVC. Bars represent the means ±SE of the red /green fluorescence intensity ratio of JC-1 aggregate/monomer, an index of MMP. *P<0.05 is statistically significantly different compared with nontreated control within the same exposure duration. $^{\Phi}$P<0.05 indicates the difference is statistically significant. N=3 experiments and each experiment was performed in triplicate.

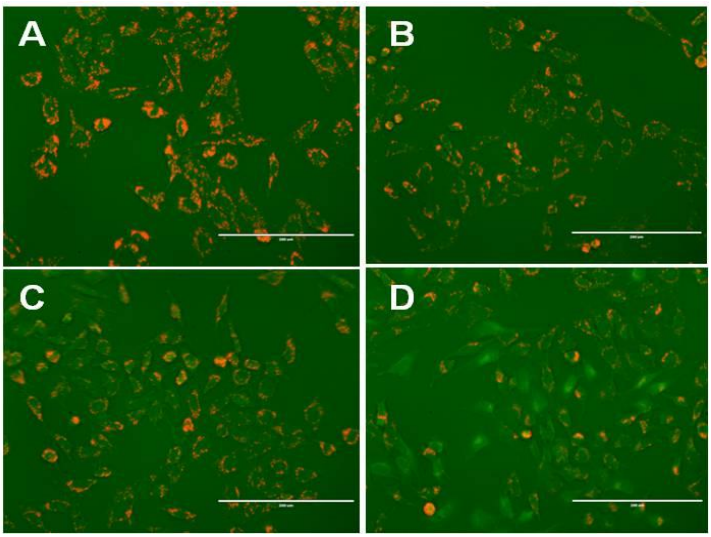

Figure 3.8. DCVC-stimulated change of mitochondrial membrane potential (MMP) analysis in HTR-8/SVneo cells visualized by fluorescence microscopy. Red fluorescence represents the mitochondrial aggregate form of JC-1 indicating high MMP. Green fluorescence represents the monomeric form of JC-1 indicating diminished MMP. Change in mitochondrial membrane potential was detected by JC-1 dye in cells exposed to: (A) untreated controls; (B) 5 μM DCVC; (C) 10 μM DCVC; or (D) 20 μM DCVC. Representative images from 3 experiments are shown.

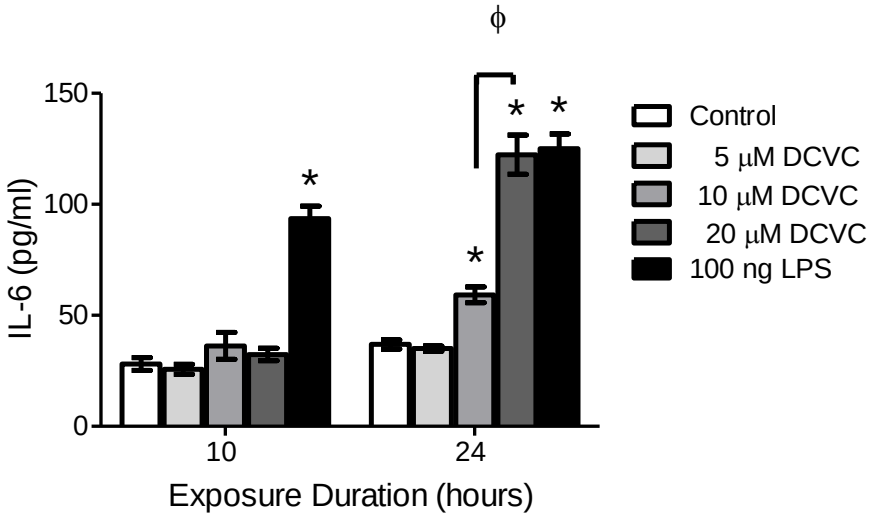

Figure 3.9. DCVC-stimulated IL-6 release. IL-6 concentrations were measured in culture medium of HTR-8/SVneo cells treated with 0 (nontreated control), 5, 10 or 20 μM DCVC or 100 ng/mL LPS (positive control) for 10 or 24 h. The bars represent the means ± SE. *P<0.05 is statistically significantly increased compared with untreated control at the same time point. $^{\Phi}$P<0.05 is significantly different from next lowest concentration within time point.

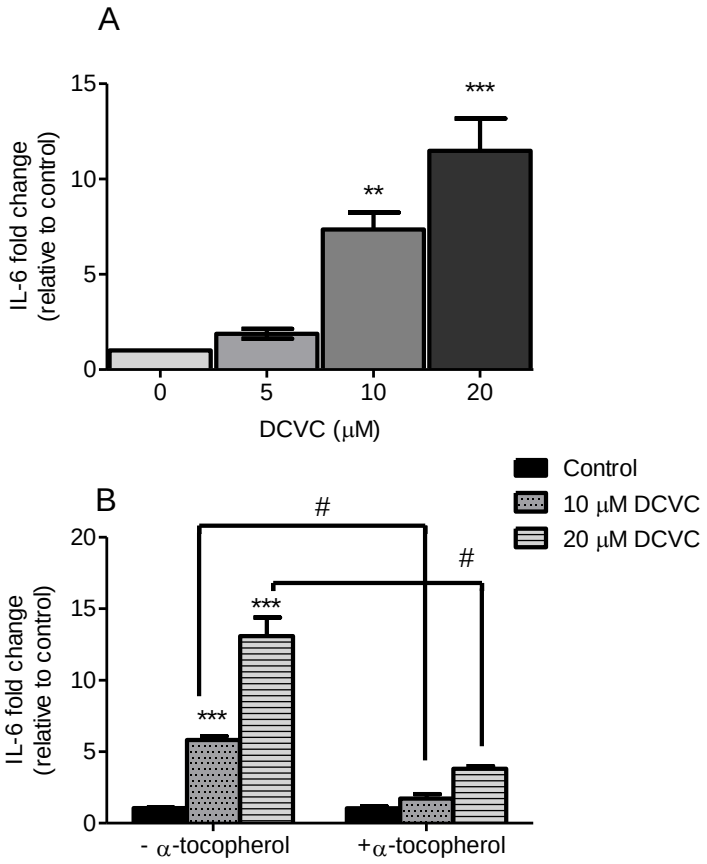

Figure 3.10. DCVC effects on IL6 mRNA expression in HTR-8/SVneo cells. A) Concentration-response of IL6 mRNA expression to treatment with 0 (control), 5, 10 or 20 μM DCVC for 24 h. B) Effect of cotreatment with 50 μM α-tocopherol on DCVC-stimulated IL6 mRNA expression. Data are represented as mean ± SE. **P<0.01, ***p<0.001 indicates means are statistically significantly increased compared with untreated control. # p<0.05 indicates the difference is statistically significant, comparing samples treated with and without α-tocopherol at the same concentration of DCVC . N= at least 3-4 experiments and each experiment was performed in triplicate.

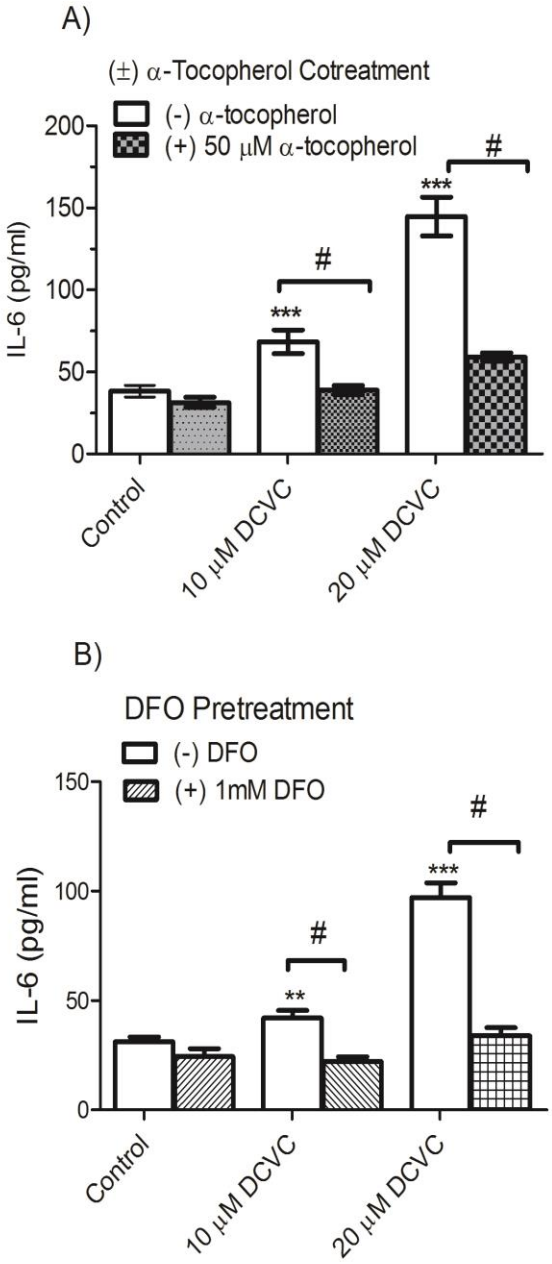

Figure 3.11. Effect of antioxidant treatments on DCVC-stimulated IL-6 release in HTR-8/SVneo cells. IL-6 concentrations were measured in culture medium of: A) cells cotreated with 50 μM α-tocopherol during a 24-h exposure to 10 or 20 μM DCVC; and B) cells pretreated with 1 mM DFO for 1 h before a 24-h exposure to 10 or 20 μM DCVC. The bars represent the means ± SE. **P<0.01 and ***p<0.001 are statistically significantly increased compared with untreated control. #P<0.05 indicates the difference is statistically significant, comparing samples treated with and without antioxidant at the same concentration of DCVC. N=3 experiments and each experiment was performed in triplicate.

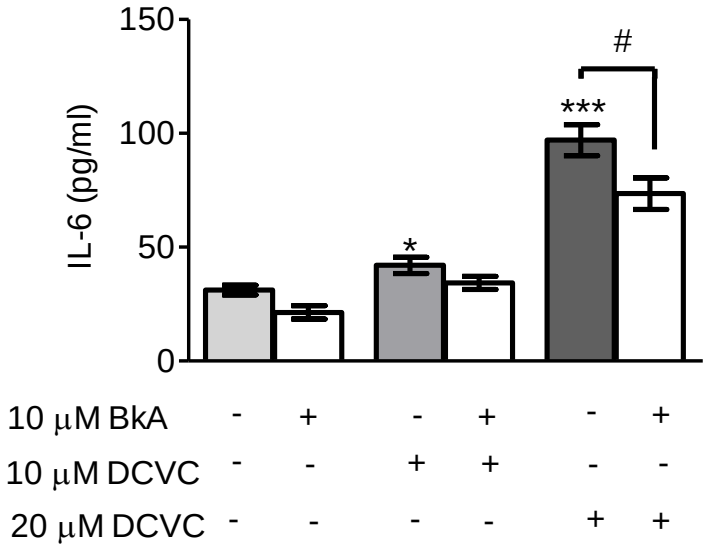

Figure 3.12. Treatment effects of bongkrekic acid (BkA) on DCVC-stimulated IL-6 release. IL-6 concentrations were measured in culture medium of HTR-8/SVneo cells pretreated with 10 μM BkA for 1 h followed by culture without or with 10 or 20 μM DCVC for 24 h. The bars represent the means ± SE. *P<0.05, ***p<0.001 is statistically significantly increased compared with untreated control. # P<0.05 is statistically significantly different. N=3 experiments and each experiment was performed in triplicate.

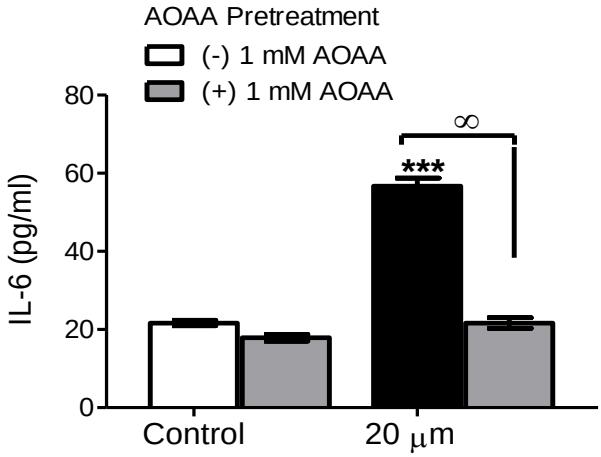

Figure 3.13. Effect of treatment with the cysteine conjugated β-lyase inhibitor aminooxyacetic acid (AOAA) on DCVC-stimulated IL-6 release. IL-6 concentrations were measured in culture medium of HTR-8/SVneo cells pretreated with 1 mM AOAA for 1 h before a 24-h exposure without or with 20 μM DCVC. The bars represent the means ± SE. ***P<0.001 is statistically significantly increased compared with untreated control. ∞ p<0.05 is statistically significantly different. N=3 experiments and each experiment was performed in triplicate.

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CHAPTER 4. PROFILE OF TRICHLOROETHYLENE METABOLIC ACTIVATION IN THE WISTAR RAT PLACENTA

Abstract

TCE is a widespread environmental contaminant, and its toxicity primarily occurs through metabolism, which is species and sex dependent. However, TCE metabolism in the placenta has not been investigated. The goal of the present study is to investigate TCE metabolism by the glutathione conjugation pathway in the rat placenta. Our hypothesis is that TCE is metabolized in the placenta to the bioactive metabolite S-(1,2-dichlorovinyl)-L- cysteine (DCVC), which our previous study showed induces oxidative stress-mediated activation of inflammatory pathways in human placental cells. Maternal serum, placental tissues, and amniotic fluid samples were collected on gestational day (gd) 16 from pregnant Wistar rats following daily exposure to 480 mg/kg TCE or vehicle (controls) from gd 6-16. Immunohistochemical analysis of the placenta showed increased expression of glutathione S-transferase (GST) in TCE-treated rats compared with controls. The activity of GST in the placenta was also significantly increased. DCVC was measured in amniotic fluid and maternal serum using HPLC/MS/MS. DCVC was detected in the amniotic fluid but the levels in maternal serum were below the limit of detection. The presence of DCVC in amniotic fluid following maternal oral exposure suggests that the placenta maybe capable of metabolizing TCE to its bioactive metabolite, DCVC, which could then potentially contribute to poor pregnancy outcomes. Further investigation on the effects of TCE metabolism on pregnancy outcomes is warranted.

Introduction

Trichloroethylene (TCE) is an organic chemical used as an industrial solvent. It is a common environmental contaminant, currently ranked 16th on the US EPA 2011 Priority List of Hazardous Substances (U.S. EPA, 2011). Exposure to TCE affects the nervous, immune, and hematopoietic systems (U.S. EPA, 2011). Additionally, TCE exhibits developmental and reproductive toxicity (Chiu *et al.*, 2013a). TCE is classified as a "human carcinogen" by IARC (IARC, 2014), and toxicity primarily occurs through metabolism (Lash *et al.*, 2000a)

Major routes of human exposure to TCE are inhalation and ingestion. With oral exposure, TCE is absorbed from the gastrointestinal tract and distributed to the liver and kidney, where it is metabolized via two separate pathways (Lash *et al.*, 2000a). The first pathway is via oxidation by cytochrome P450 (CYP) enzymes to an epoxide, then to chloral hydrate which is further metabolized to trichloroethanol or trichloroacetate, which can be further oxidized to dichloroacetate, and eventually excreted in the urine and eventually to trichloroacetate (TCA) and dichloroacetate (DCA), which are excreted in the urine (Lash *et al.*, 2000a; Lash *et al.*, 2000b). The second pathway is through conjugation with glutathione (GSH) which produces short-lived reactive metabolites (Lash *et al.*, 2000b; Lash *et al.*, 2000a). The initial GSH conjugation reaction occurs in the liver by glutathione S-transferase (GST) to S-(1,2-dichlorovinyl) glutathione (DCVG) (Lash *et al.*, 2000a; Lash *et al.*, 2000b). Subsequently, DCVG is biotransformed in the kidney by γ-glutamyl transpeptidase (GGT) to S-(1,2-dichlorovinyl)-L-cysteine (DCVC) (Lash *et al.*, 2000a; Lash *et al.*, 2000b). DCVC is further metabolized in the kidney by cysteine conjugated β-lyase to an unstable intermediate capable of forming covalent bonds with cellular nucleophiles leading to cytotoxicity (Lash *et al.*, 2000a; Lash *et al.*, 2000b). DCVC also undergoes N-acetylation and is excreted in the urine as NAcDCVC or as the

sulfoxide NAcDCVCS (Lash *et al.*, 2000b; Lash *et al.*, 2000a). According to Anders et al., metabolites generated by oxidation (TCA, DCA, choral hydrate and trichloroethanol) are chemically stable and therefore are frequently detected, whereas metabolites generated by the GSH conjugation pathway, including highly reactive intermediates generated by the cysteine conjugated β-lyase pathway, are not easily measured because they are chemically unstable (Anders *et al.*, 1986). TCE metabolism is species dependent, and studies show that human metabolism of TCE is more similar to metabolism in rats as compared with mice (Lash *et al.*, 2000a). For example, mice metabolize and eliminate TCE more rapidly than rats and humans (Lash *et al.*, 2000b; Lash *et al.*, 2000a). Epidemiologic and animals studies have shown that TCE adversely affects the reproductive and developmental systems (Chiu *et al.*, 2013b). In an epidemiologic study involving TCE and tetrachloroethylene exposure via vapor intrusion, maternal inhalation exposure to TCE was associated with fetal growth restriction, low birth weight, term low birth weight, and small-for-gestational-age (SGA) (Forand *et al.*, 2012). Another epidemiological study found an association between maternal exposure to TCE and SGA (Bove *et al.*, 2002). However, a biological explanation underlying epidemiologic associations of TCE exposure and adverse pregnancy outcomes is unknown. Knowledge of TCE metabolism is critical for understanding target organ susceptibility and toxicity (U.S. EPA, 2011). Although the placenta, like the liver, is capable of metabolizing many endogenous compounds and xenobiotics, and expresses key TCE metabolizing enzymes including cytochrome P450 and glutathione-S-transferase (Campisi *et al.*, 1994), TCE bioactivation in the placenta has yet to be explored.

The goal of the present study is to investigate TCE metabolism by the glutathione conjugation pathway in rat placenta. This study focused on the glutathione conjugation pathway

because in our earlier vitro experiments with the human placental cell line HTR-8/SVneo, we found that DCVC was much more potent for stimulating ROS generation and cytokine release compared to TCA and DCA, key metabolites generated via the CYP450 pathway (Chapter 3). Our hypothesis is that TCE is metabolized in the placenta to the bioactive metabolite DCVC. To test the hypothesis, we conducted placental enzyme activity assays, determined the expression of GST in placenta, and assayed for DCVC in amniotic fluid and maternal serum of rats exposed to TCE during pregnancy.

Materials and Methods

Animals

Time-pregnant Wistar rats between 60-90 days of age weighing 200-250 grams were obtained from Charles River (Portage, MI). The day after copulation was designated as day 0 of pregnancy. Rats were shipped at gestational day (gd) 2 and individually housed in a controlled environment with a 12-hour light/dark cycle. Dams were fed standard rat chow (Purina 5001) and water *ad libitum*.

Exposure method

Animals were exposed to TCE orally via addition to a vanilla wafer, a method adopted from work conducted by Seegal et al. for polychlorinated biphenyl (PCB) exposure (Seegal *et al.*, 1997). Rats were assigned to control or treatment group, and fed the mini wafer once a day, between 8-9 am, from gd 6-16. First, rats were trained to eat the miniwafer by placing them in individual exposure cages and placing one vanilla miniwafer without TCE in the cage for three consecutive days. After the training period, 480 mg/kg of TCE was pipetted onto a miniwafer

once a day, between 8-9 am, and immediately offered to the rats, which they readily ate. The control rats were exposed to vanilla miniwafer without TCE. The TCE dose used is a magnitude higher than the US EPA drinking water maximum contaminant level for TCE of 5 ppb (US EPA 2011).

Dissections

Rats were euthanized at gd 16 with carbon dioxide followed by cardiac puncture. Dams were euthanized in a random fashion alternating between controls and treated. The uterine horn was removed, live fetuses were weighed individually, and numbers of resorbed and dead fetuses were recorded. Dissections were conducted within a 4-h period in the morning. The collected blood was centrifuged at 2000 x g at 4°C for 30 min, and then stored at -80°C until assayed. Placental tissues were rinsed with PBS to remove red blood cells, snap frozen in liquid nitrogen, and stored in the -80°C freezer for future analysis. Amniotic fluid samples were pooled per litter, then snap frozen in liquid nitrogen and stored in the -80°C freezer for future analysis.

Enzyme activity assays

Activity levels of GST and GGT, key enzymes in TCE glutathione conjugation pathway, were measured in rat placental extracts. TCE-treated rat placenta (N=12 placentas) were harvested, rinsed in phosphate buffer solution (PBS), pooled per litter, and homogenized in 2 mL cold buffer containing PBS with 2 mM EDTA. The homogenized tissues were then centrifuged at 10,000 x g for 15 min at 4°C. The tissue extracts were collected and stored at -80°C and assayed the next day.

GST enzyme activity was assayed according to Habig et al. (Habig *et al.*, 1974), as modified by the Glutathione S-Transferase Assay Kit (Cayman Chemical) for 96-well plates. GST activity was determined by adding 10 µl 1-chloro-2,4-dinitrobenzene (CDNB), 20 µL glutathione (GSH), and 20 µL of placenta tissue extract to 150 µL GST buffer (containing 100 mM potassium phosphate and 0.1% Triton X-100). A SpectraMax M2e Multi-Mode Microplate Reader (Molecular Devices) was used to quantify formation of 2,4-dinitrophenylglutathione kinetically at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$). GST activity was calculated according to manufacturer's guidelines. One unit of enzyme activity is defined as the amount of enzyme that forms 1.0 nmol of S-2,4-dinitrophenyl GSH/min at 25ºC. The level of protein was determined by bicinchoninic acid protein assay (BCA assay) (Thermo Scientific), using bovine serum albumin as a standard.

GGT enzyme activity was measured according to the method by Orlowski et al. (Orlowski and Meister, 1963) as modified for the Gamma Glutamyl Transferase (GGT) Activity Colorimetric Assay Kit (GenWay Biotech) for 96-well plates. Briefly, 90 µL of γ-glutamyl-p-nitroanilide substrate was added to 10 µL of supernatant from placental tissue homogenates in a 96-well plate. The plate was then incubated at 37 °C. Absorbance was measured kinetically using a spectrofluorometer at 410 nM for appearance of p-nitroanilide (pNA) (molar extinction =8800 M-1cm-1). GGT activity was calculated according to the manufacturer's guidelines. One unit of GGT was defined as the amount of enzyme that generated 1.0 µmole of pNA per minute at 37 °C. Protein amount was quantified using BCA assay (Thermo Scientific), with bovine serum albumin as a standard.

HPLC-MS/MS analysis

Sample Preparation

Levels of DCVC and DCVG in serum and amniotic fluid were determined based on a method by Kim et al. (Kim *et al.*, 2009a) with some modifications. Because isotope-labelled DCVC and DCVG were not available to use as internal standards in the present study, pFF (parafluorophenylalanine monohydrate), was chosen as an internal standard. pFF has a very similar structure and retention time as that of DCVC and also ionized well in positive ion mode. DCVC, DCVG and the pFF internal standard were spiked into 95 µL of amniotic fluid or serum from pregnant rats. Then, serum and amniotic fluid protein was precipitated by adding 200 µL acetonitrile, with occasional vortexing over 10 min at room temperature. The mixture was centrifuged at 14,000 x g for 10 min at room temperature. Subsequently, 250 µL of the supernatant was transferred into limited volume (300 µL) vial and dried in a SpeedVac for approximately 1.5-2 h. The residue was reconstituted with 200 µL HPLC grade water prior to solid phase extraction. The solid phase extraction (SPE) cartridges (StrataTMX-AW) were prepared by wetting with 600 µl of methanol followed by 600 µL of XPLC grade water, then applying 200 µL of sample in XPLC grade water. After the sample flowed to the head of the SPE column, a second 100 µL portion of HPLC grade water was added to the sample vial, and that portion was applied to the SPE column. The SPE manifold collection tubes were replaced with limited volume vials (Waters Total Recovery) and the analytes were eluted with two 500-µL aliquots of methanol ammonium hydroxide (5% v/v). No vacuum was required for the SPE manifold. At each SPE step the solvent flow was stopped as the solvent reached the top of the bed. The final eluate was dried in a SpeedVac for 2 h before reconstitution with 55 µL of a water:methanol (80:20) solution. It was important to perform the entire sample preparation and analysis on the same day in order to detect 1 nM levels in the analytes.

A Waters 2695 HPLC coupled to a Thermo Finnigan TSQ Quantum Ultra AM triple-quadrupole mass spectrometer was used to determine DCVC and DCVG concentration in rat amniotic fluid and serum by HPLC/MS/MS. Sample injection volume was 20 µL. A Phenomenex Gemini C18 analytical column (150×2mm, 5 µm) plus a 4-mm guard was operated with a linear gradient of 2% methanol and 0.2% acetic acid for 0.5 min, then to 60% methanol and 0.2% acetic acid for 8 min, and subsequently to 98% methanol and 0.2% acetic acid for 9.5 min, then held for 3 min and finally returned to initial conditions from 12.6 to 23 min. A flow rate of 400 µL/min was used with a post-column split of 100 µL/min to the MS. The effluent from 0.3 to 1.02 min was diverted to waste to prevent salts from entering the MS. Analytes were detected in MRM mode, monitoring the transition of the m/z 216→127 (collision energy (CE) 25V, tubelens 102V) for DCVC, m/z 402→273 for (CE 19V, tubelens 108V) DCVG and m/z 184 -> 118 (CE 39V, tubelens 90V) for pFF. MS settings were as follows: electrospray voltage (3.5 kV), capillary temperature (300 ◦C), sheath and auxiliary gas settings (nitrogen; 50 and 30 arbitrary units), and Q2 collision gas pressure (1.0mTorr). The MS and electrospray ionization parameters were optimized by direct infusion of standards using Xcalibur® software. Standard curve samples were prepared by spiking 95 µL of blank amniotic fluid or serum with 2.5 µL of a 40 X stock of DCVC and DCVG in water and 2.5 µL of pFF stock (1400 nM) in water. TCE-exposed samples only had pFF internal standard spiked into them.

Calibration curves and quality controls were prepared with serum and amniotic fluid from pregnant Wistar rats exposed to vanilla miniwafers without TCE. Standards were prepared by spiking amniotic fluid and serum with 4 nM pFF as the internal standard and with 1, 2, and 4 nM DCVC and DCVG. Area ratios of analyte relative to the pFF internal standard were used as the basis for quantification. The limits of detection (LODs) were determined by a signal-to-noise

ratio of 3:1 based on the 20-µl injection volume. Recovery rates and matrix effects were estimated using 1, 2, 4, and 8 nM of DCVC and DCVG. The stock solutions for DCVC and DCVG were stable in -20ºC storage for 6 months with repeated freeze thaw (Kim *et al.*, 2009b). In addition, during mass spectrometry analysis, the autosampler operated at room temperature for 24-h period without sample degradation.

Matrix suppression and relative recovery rates

The matrix effect for amniotic fluid was estimated using acetonitrile precipitation and StrataXAW with 14.5 nM DCVC and DCVG spiked into rat amniotic fluid samples. Three samples were prepared. The first sample was of a solvent standard ("non-extracted neat sample"). The second sample was of a fully prepared amniotic fluid (95 µL aliquot) spiked with pFF (36.4 nM), DCVC and DCVG (each at 14.5 nM;"post-prep" spike; N=2) during the final reconstitution step after the sample extraction process. Finally, DCVC and DCVG (each at 14.5 nM), and pFF (36.4 µL) were spiked into 95 µL amniotic fluid prior to sample preparation ("pre-prep" spike; N=4). The matrix effect (ME) was calculated as the measure of the signal increase or decrease observed for an analyte in solvent vs. the signal observed in the presence of matrix. Relative recovery (RE) is a true measure of the sample recovery that takes into account matrix effect (which takes into account sample that has been through the extraction process in matrix compared with an analyate spiked into extracted matrix after extraction procedure). Matrix effect (ME) and Relative Recovery (RE) were calculated using the equations below (Chambers *et al.*, 2007):

Matrix Effect (ME) = 100 x [(B/A) -1]

Relative Recovery (RE) = 100 x C/B

where A= non-extracted neat sample, B=post-extracted spiked sample, C=extracted sample

Immunohistochemistry

Placentas were harvested and fixed in 10% formalin (Fisher) for a minimum of 48 h. Immunohistochemistry was performed by the University to Michigan Histology Core. Briefly, samples were fixed in 70% ethanol, and then embedded in paraffin, mounted on slides, and stained. Placenta glutathione S-transferase (GST) detection was performed using a commercially available primary rabbit polyclonal antibody against glutathione S-transferase pi (GST-pi) (GWB-BBP465, GenWay Biotech). GST-pi levels were analyzed because it is the GST isoform which is predominantly expressed in the placenta; the other GST isoforms are either not expressed in the placenta (GST-alpha) or are expressed in low concentrations (GST-theta and GST-mu) (Raijmakers *et al.*, 2001). To analyze expression levels of GST-pi in the placenta, heat-induced antigen retrieval was performed with citrate buffer (pH 6) for 10 min. Immunoperoxidase staining was completed on a DAKO AutoStainer at room temperature using LSAB + System-HRP kit from DAKO. Peroxidase block was followed by 30 min incubation with primary antibody at dilution of 1:1500 rabbit polyclonal. Samples were then incubated with biotinylated LINK (DAKO LSAB kit produced in goat with dilution of 1:500) for 30 min, followed by streptavidin-HRP LINK incubation for 20 min, and 3, 3′-diaminobenzidine (DAB) chromogen solution for 10 min. Microscopy imaging was conducted using Nikon Elements Software (N=5 rats per group; one placenta per litter was analyzed). For each placenta, 5-6 images were analyzed, with one image selected in each of the four quadrants and one or two in the middle of the image field.

Results

Immunohistochemical detection of GST-pi in rat placental tissues

Because TCE is metabolized to reactive intermediates including DCVC by glutathione S-transferase (GST), we determined the expression of GST-pi in TCE-treated rat placenta compared with controls using immunohistochemistry. Although GST-pi antibody stained throughout the tissues in both the labyrinth and the basal zones of the placenta. The staining was darker and visibly increased within the labyrinth as well as the basal zones in the TCE-treated rat placenta (Figure 4.1A). In contrast, the staining was less abundant in the labyrinth zone and in the basal zone of placental tissue from control rats (Figure 4.1B).

Enzyme activity assay

The catalytic activity of GST and GGT are shown in Figure 2. Activity for GST in TCE-treated rat placenta tissue was significantly increased 1.8-fold compared with controls (N=4 rats per group, $p<0.05$), but the activity for GGT was not significantly increased (Figure 4.2).

HPLC/MS/MS

A method was developed that could detect 1 nM DCVC and DCVG in spiked amniotic fluid and serum. Quantification was based on peak areas relative to the internal standard. Standard calibration curves for DCVC and DCVG show a linear response over the range of concentrations expected with high coefficients of determination. Based on a volume of 95 µL serum or amniotic fluid, 20 µL injection volume, and a signal to noise ratio of 3:1, DCVC was detected in amniotic fluid samples from two TCE-exposed rats with means of 1.14 and 3.07 nM, which were calculated from duplicate analysis using the standard curve (2.14 min retention time)

(Table 4.1); however, DCVC levels in maternal serum of TCE-exposed rats were below the limit of detection in all samples. DCVC analysis for serum and amniotic fluid were not conducted in the same rats because some samples were used up during method development. Figures 4.3A and 4.3C show the standard curves used to calculate concentrations of DCVC in amniotic fluid and maternal serum of TCE-treated rats, respectively. Figure 4.3B is a representative chromatogram indicating DCVC (retention time 2.14 min) detected in amniotic fluid of a TCE-treated rat and Figure 4D is a representative chromatogram indicating DCVC (retention time 2.13 min) in maternal serum. Figure 4.3E is a representative chromatogram image of a rat amniotic fluid spiked with 14 nM of the internal standard pFF, and a representative chromatogram showing the peak for DCVC in a rat amniotic fluid sample spike with 1 nM DCVC is shown in Figure 4.3F. For solvent standards, relative standard deviations (RSD) were less than 20% for 1 nM DCVC and DCVG. TCE-treated samples analyzed in duplicate yielded RSD's of 20-30%. For the amniotic fluid standard curve, back calculated values were within 20% of expected values even at 1 nM. Based on this accuracy, 1 nM is also our limit of quantification. Because we observed an interfering peak for DCVG, our results remain inconclusive for measuring this TCE metabolite (Supplement Figure 4.1).

Discussion

Toxicants such as TCE can be delivered to the placenta through maternal circulation where they are either metabolized to more toxic substances or are detoxified (Sastry, 1995). Moreover, TCE has been shown to cross the placenta during childbirth (Beppu, 1968; Laham, 1970). Although there is a paucity of research regarding TCE metabolism by the glutathione conjugation pathway in the placenta, studies have reported the ability of human placental GST to metabolize other toxicants (Sastry, 1995). The present study reports for the first time detection of

DCVC in TCE-treated rat amniotic fluid, suggesting that rat placenta may bioactivate TCE via DCVC. We decided to limit our investigation to the glutathione conjugation pathway because in vitro experiments with a human placental cell line showed that DCVC was much more effective at stimulating ROS generation and proinflammatory cytokine release compared with the major oxidative metabolites TCA and DCA, which are generated by the CYP450 pathway.

Glutathione S-transferase is an important enzyme for glutathione-dependent metabolism of TCE (Lash et al., 2014). In the current study, we found increased protein expression of GST-pi, the placental isoform of the GST enzyme and the most abundantly expressed GST isozyme outside the liver. Our detection of GST-pi protein in rat placentas are consistent with previous studies that found GST-pi in human tissues, including placenta, kidney, and the digestive tract (Terrier et al., 1990). Furthermore, our findings of increased expression of GST-pi protein in placentaas of TCE-treated rats are consistent with a report that occupational exposure to TCE was associated with increased levels of GST-pi in the urine (Vermeulen *et al.*, 2012). In contrast, Bruning and colleagues found a positive association between exposure to TCE and urinary concentrations of GST-alpha but not GST-pi in a retrospective study (Bruning *et al.*, 1999). In rat kidney cells, GST-alpha was the primary isoform responsible for GST conjugation of TCE (Cummings *et al.*, 2000) ; however, GST-alpha is not abundantly expressed in the placenta (Raijmakers *et al.*, 2001). Our results show that the placenta expresses GST-pi, which is capable of metabolizing TCE to more toxic compounds, and that the placental expression of GST-pi increases with TCE exposure.

Consistent with increased protein expression of GST-pi, we observed increased GST activity in the placenta of TCE-exposed rats. These results are consistent with previous studies that showed increased GST activity in the liver and lung of rats exposed to TCE (Kumar *et al.*,

2002). In addition, we observed that TCE exposure increased activity of placental GGT. Because GGT metabolizes the first step converting DCVG to DCVC, the presence of GGT activity implies that the placenta has the metabolic capability to initiate metabolic bioactivation of TCE via the glutathione pathway. Furthermore, these results are consistent with prior studies showing that TCE exposure increases GGT activity in the kidneys of mice, rats, and humans (Lash *et al.*, 1998). GGT has been found in many other tissues including in the liver, intestines, placenta, and the amniotic fluid in rodents and humans (Hanigan and Frierson, 1996). Together, the activity assay results suggest that the placenta possesses key enzymes in the glutathione conjugation pathway for TCE bioactivation via DCVC in a manner similar to that in the kidneys.

Our method for measuring the TCE metabolite DCVC is based on the work of Kim and colleagues (Kim *et al.*, 2009a). Following daily oral exposure to 480 mg TCE /kg body weight via vanilla wafer, DCVC levels detected in amniotic fluid were 1.14 and 3.07 nM (LOD = 1 nM). However, some of the values were below the limit of detection due to several limitations in our study which are discussed below. DCVC concentrations in maternal serum of TCE-treated rats were also below the limit of detection (1 nM). In contrast to our study, Kim et al. administered 2100 mg TCE/kg body weight by oral gavage in male mice (n=3) and detected DCVC and DCVG levels in serum of 2.4 ±0.9 nM and 37±15 nM (mean±SD), respectively, with a 1 nM limit of detection (Kim *et al.*, 2009a). Kim and colleagues were able to detect these higher concentrations in part because they administered a dose that was approximately 4-fold higher than the dose in our study and they used isotope-labeled internal standard in their analysis which improves precision and accuracy. Moreover, Kim et al. used male mice in their study whereas we used pregnant rats. Species and sex differences in metabolism of TCE have been well described by several studies by Lash and colleagues (Lash *et al.*, 2000b; Lash *et al.*, 2001).

In addition, Kim et al. exposed the mice by oral gavage to TCE diluted in corn oil while we use vanilla miniwafer as our exposure vehicle. Bioavailability of TCE following oral exposure is an important issue to consider. For example, Withey et al., found that rats exposed to TCE through oral gavage by use of corn oil as vehicle had significantly lower TCE blood concentrations compared with doses given in water (Withey *et al.*, 1983). The vanilla wafer exposure method was used by us because it allows oral exposure, which is a relevant exposure route for humans to TCE (U.S. EPA, 2011), yet was less stressful than gavage and minimized volatilization issues associated with drinking water exposure. The different exposure methods may also help explain the discrepancy in the results. Finally, Kim et al. collected serum samples 2 h after exposure whereas we collected serum and amniotic fluid 4 to 6 h after exposure, which could also contribute to the differences between our study results.

To our knowledge, this is the first study that has reported detectable levels of DCVC in amniotic fluid following in vivo exposure to TCE. Future studies should also measure levels of DCVC and DCVG in TCE-exposed rat placenta extracts because the amounts detected in the amniotic fluid could be diffusing from the placenta and therefore the placenta may have higher concentrations of these metabolites. Extraction recovery levels were 42% for DCVC, 34% for DCVG, and 23% for pFF in the amniotic fluid. We observed a matrix enhancement effect with both DCVC and DCVG in amniotic fluid. We observed matrix enhancement of 37% for DCVC, 34% for DCVG and 50% for the internal standard pFF. Because our results for maternal serum were below the limit of detection, we did not conduct matrix effects and extraction recovery studies. Matrix enhancement observed in the amniotic fluid could be due to matrix component(s) that limits loss on the glass vial or due to an analyte in the amniotic fluid that may be co-eluting

with DCVC and DCVG. Inclusion of an isotope-labeled internal standard would improve accuracy.

This study has a few limitations. First, we did not use isotope-labeled "internal standards" in the LC/MS method for elucidating levels DCVC and DCVG in amniotic fluid and serum of TCE-treated rats, because isotope-labeled internal standards were not available. Second, DCVC levels in maternal serum of TCE-exposed rats were below the limit of detection, which could be explained by using solvent precipitation in our sample preparation method. Future studies should determine sample recovery rates and matrix effects for DCVC in serum, which might improve our current sample extraction method. Third, we did not measure expression and activity levels of cysteine conjugated beta-lyase, a critical enzyme for bioactivating DCVC to more cytotoxic compounds. Therefore, future study should elucidate expression levels and enzyme activity of cysteine conjugated beta-lyase. Fourth, because the oxidative metabolic pathway via CYP2E1 was not investigated, future studies should be conducted to determine the role of this pathway in placental toxicity. Nevertheless, because TCE toxicity is primarily due to its metabolism, our evidence that DCVC is present in the fetal compartment is important and warrants further studies on TCE metabolism to address TCE-related adverse birth outcomes observed in epidemiology studies.

In summary, we developed a method that enabled us to measure a critical metabolite of TCE, DCVC, in amniotic fluid of TCE-exposed rats using HPLC/MS/MS. Moreover, we found increased expression in placentas of TCE-exposed rats of GST, a key enzyme in the TCE glutathione conjugation pathway. Furthermore, we observed increased activity of GST and GGT in TCE-treated rat placenta compared with controls. Our results suggest a role for TCE metabolism by the glutathione pathway in the placenta, similar to that in the kidney. Because

TCE nephrotoxicity and carcinogenicity are attributed to TCE metabolic activation via DCVC, the findings of the present study suggest the potential for DCVC-mediate toxicity in the gestational compartment as a possible explanation for TCE-associated adverse birth outcomes.

Table 4.1. DCVC concentration in amniotic fluid from TCE-exposed rats to as determined by HPLC/MS/MS.*

Rat Identification Number	DCVC (nM)			
	Injection 1	Injection 2	Mean	SD
2	1.17	1.10	1.14	0.03
3	2.98	3.17	3.07	0.09
5	<LOD	<LOD	<LOD	<LOD
6	<LOD	<LOD	<LOD	<LOD
18	<LOD	<LOD	<LOD	<LOD
19	<LOD	<LOD	<LOD	<LOD

*Rats were exposed daily to 480 mg TCE/kg body weight p.o. on gd 6-16.
**Limit of detection (LOD) = 1.0 nM.

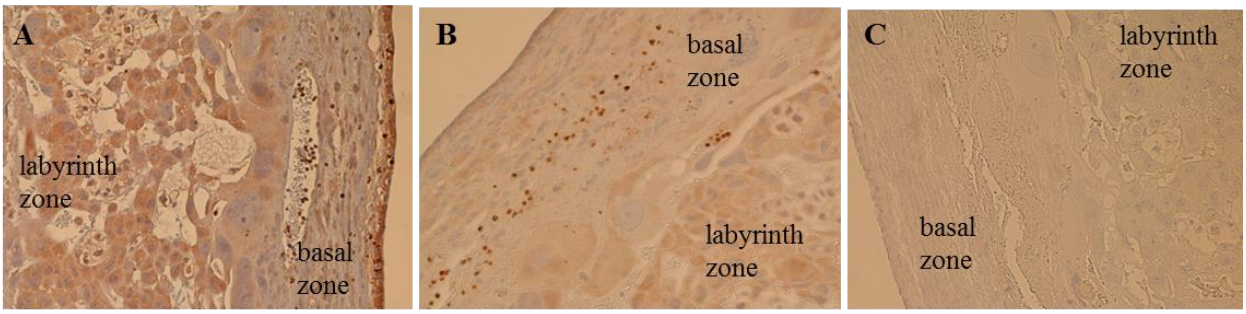

Figure 4.1. Immunohistochemical staining for glutathione S-transferase (GST-pi) in gd 16 Wistar rat placentas. Representative images of placental tissue sections from rats treated daily from gd 6-16 to A) 480 mg/kg TCE p.o. on miniwafer, or B) miniwafer without TCE (vehicle control). C) Shows a representative image of negative control placenta section incubated with secondary antibody only. (magnification 20X)

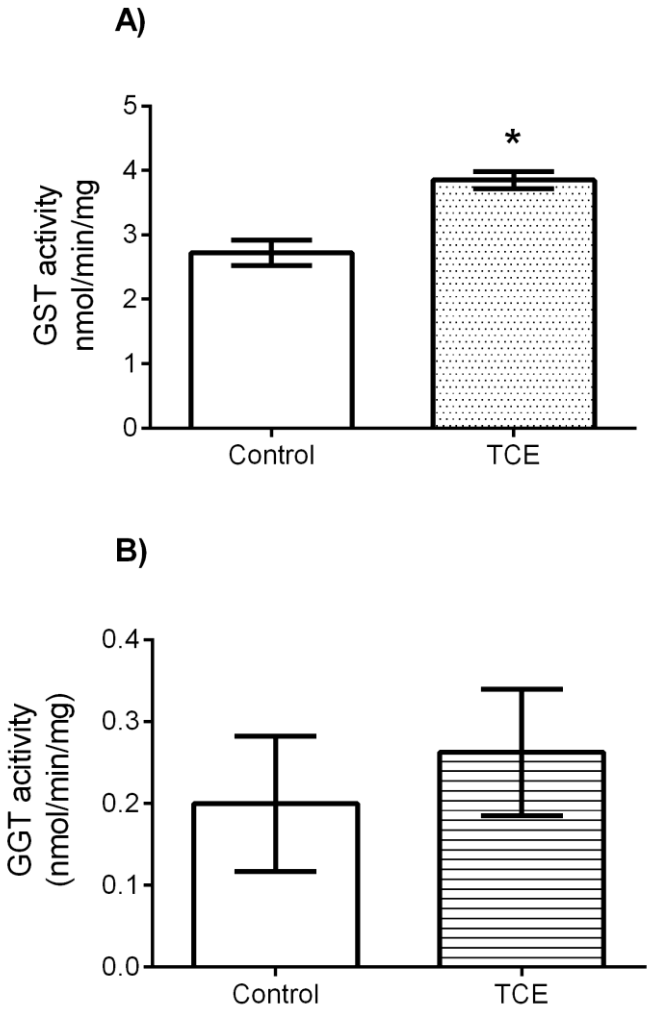

Figure 4.2. The activity of A) glutathione S-transferase (GST) activity was significantly increased (*P=0.02) compared with vehicle treated control and B) activity for gamma-glutamyltransferase (GGT) in rat placenta tissue was not statistically significantly increased P=0.6. Data are represented as means ±SE (N=4 rats per group)

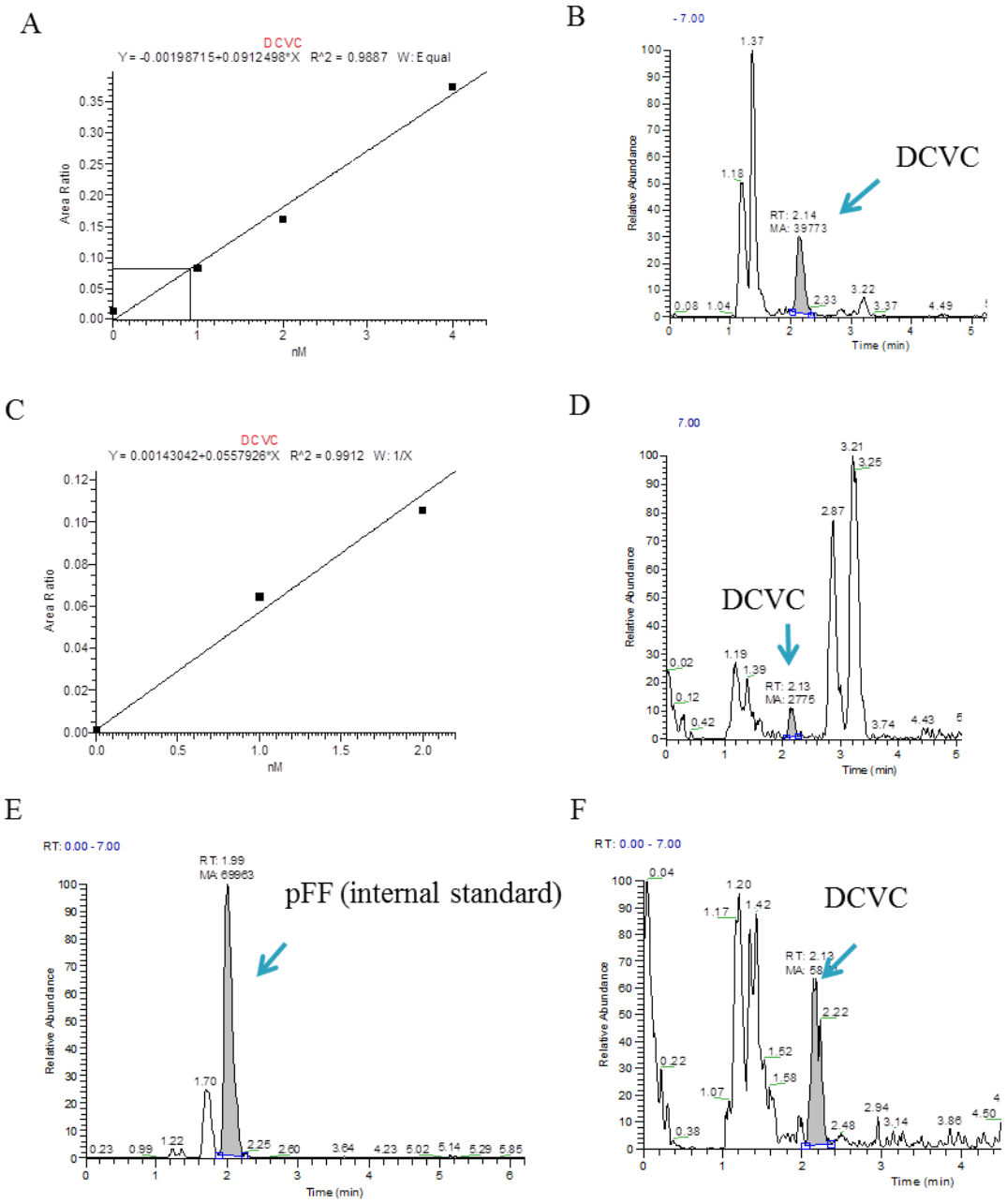

Figure 4.3. Calibration curves and representative chromatograms for detection of DCVC using HPLC/MS/MS. A) Calibration curve of DCVC added into rat amniotic fluid. B) Representative chromatogram showing DCVC (retention time 2.14 min) detected in amniotic fluid of a TCE-treated rat. C) DCVC calibration curve in rat serum. D) Representative chromatogram showing DCVC (retention time 2.13 min) detected in maternal serum of a TCE-treated rat. E) Representative chromatogram showing parafluorophenylalanine monohydrate (pFF) internal standard (retention time 1.99 min) in rat serum. F) Representative chromatogram showing 1 nM DCVC added to rat amniotic fluid.

Supplement Figure 4.1:

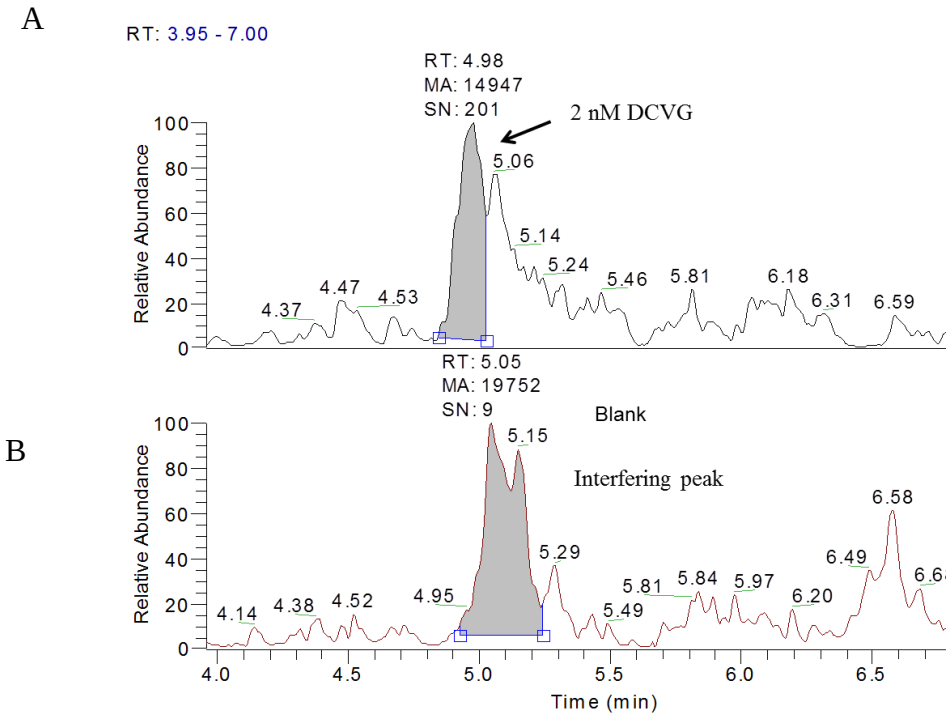

Figure 4.1S. Representative chromatogram showing A) DCVG retention time (4.98 min) with an interfering peak at 5.05 min co-eluting with DCVG in amniotic fluid from control spiked with 2 nM DCVG B) representative chromatogram of nonspiked amniotic fluid from controls. The chromatogram shows an interfering peak at retention time 5.05-5.15 min.

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CHAPTER 5. SUMMARY AND CONCLUSIONS

The research presented in this dissertation tested the hypothesis that exposure to trichloroethylene (TCE) induces oxidative stress-mediated activation of inflammatory pathways in placental cells and tissues, leading to adverse pregnancy outcomes. We report TCE-induced reduction of fetal weight in a laboratory animal model without significant changes to maternal body and organ (liver and kidney) weights. Our findings are consistent with the study of Fisher et al., who found decreased fetal weights in pregnant rats exposed to TCE (Fisher *et al.*, 1989). Similarly, our study is corroborated by two epidemiological studies that have associated TCE exposure with low birth weight, SGA, and fetal deaths (Fisher *et al.*, 1989; ATSDR, 1997).

In human gestational tissues, increased oxidative stress is associated with pathophysiology of pregnancy, including preterm labor, preeclampsia, intrauterine growth restriction, and SGA (Al-Gubory *et al.*, 2010). For example, SGA is associated with increased concentrations of lipid peroxidation byproducts of oxidative stress such as malondialdehyde (MDA) in the maternal serum and cord blood, and 4-hydroxynonenal in the placenta (Gveric-Ahmetasevic *et al.*, 2009). Similarly, higher concentrations of urinary 8-hydroxy-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage, were associated with SGA (Hsieh *et al.*, 2012). Preeclampsia is associated with increased levels of oxidative stress markers in the placenta, including F2-isoprostanes, a reliable biomarker of lipid peroxidation, nitrotyrosine, an indicator of oxidation/nitration of proteins, and 4-hydroxynonenal staining

(Raijmakers *et al.*, 2004). Similarly, preeclampsia is associated with decreased levels of antioxidants including glutathione, vitamin E, and vitamin C in the plasma (Raijmakers *et al.*, 2004).

A growing body of literature reports a link between increased reactive oxygen species (ROS) and activation of innate immune response within the gestational compartment leading to adverse birth outcomes. ROS can act as signaling molecules by stimulating prostaglandin and cytokine release in human placenta and extraplacental membranes (Lappas *et al.*, 2003). In the present study, we found that exposure to TCE induced oxidative stress and inflammation in rat placentas. Specifically, we found oxidative DNA damage in the placenta and presence of neutrophils in the decidua basalis. Moreover, microarray gene expression analysis detected increased mRNA levels in placenta of TCE-exposed rats of genes that are involved in immune system regulation. Pathway analysis showed an up-regulation of inflammation-related pathways and down-regulation of metabolic hemostasis pathways. In addition, we observed a significant increase of the proinflammatory cytokine IL-6 in maternal serum. The study presented here suggests that exposure to TCE significantly increased placental and maternal inflammation, and placental oxidative stress, in a rat model of fetal growth restriction.

Further testing showed global 5-hydroxy-methylcytosine (5-hmC was significantly increased, suggesting that exposure to TCE induces epigenetic changes in the placenta. 5-Methylcytosine can be oxidized to 5-hmC by the Tet family of enzymes, suggesting that 5-hmC is an intermediate in the DNA demethylation pathway (Wen *et al.*, 2014). Findings from recent human and rodent studies suggest that placental epigenetic and genetic profiles may serve as markers of intrauterine environment (Koukoura *et al.*, 2012). In humans, decreased placental global DNA methylation was associated with adverse birth outcomes including preterm birth and

intrauterine growth restriction (IUGR) (Nelissen *et al.*, 2011). Our findings corroborate previous reports that exposure to TCE induced global DNA hypomethylation in mouse liver, a site for TCE metabolism and a major target organ for TCE toxicity (Tao *et al.*, 1998; Blossom *et al.*, 2013).

TCE-induced intoxication is primarily dependent on its biotransformation to toxic metabolites, including S-(1,2-dichlorovinyl)-L-cysteine (DCVC) (Lash *et al.*, 2000a). DCVC is recognized as a nephrotoxic metabolite of TCE responsible for kidney tumorigenesis, but it has not been evaluated as a reproductive or developmental toxicant (Chiu *et al.*, 2013). DCVC has been shown to induce mitochondrial dysfunction, formation of reactive oxygen species, increased oxidative DNA damage, and lipid peroxidation in the kidney (van de Water *et al.*, 1993; van de Water *et al.*, 1994; Xu *et al.*, 2008). In the present study, we detected DCVC in amniotic fluid of TCE-treated rats. In placentas of TCE-treated rats compared with non-treated control rats, we found increased expression and activity of glutathione S-transferase (GST), a key-metabolizing enzyme in the TCE glutathione conjugation pathway. Because TCE toxicity is primarily due to its metabolism, our results suggest that the placenta has the capacity to bioactivate TCE through the glutathione conjugation to produce DCVC. The TCE glutathione conjugation pathway is shown in Figure 5.1.

Consistent with increased markers of oxidative stress and inflammation in placenta of TCE-treated rats, we found a direct stimulatory effect of DCVC on release of IL-6 in HTR-8/SVneo cells, an immortalized cell line derived from first trimester human placental extravillous trophoblasts. DCVC stimulated IL-6 release from HTR-8/SVneo cells to a magnitude comparable to that observed with treatment with LPS, a highly immunogenic component of Gram negative bacteria. The results with antioxidant treatments suggest that DCVC-stimulated

cytokine release in human placental cells is dependent on ROS because (±)-α-tocopherol and deferoxamine significantly decreased IL-6 release. Because dysregulation of cytokine release (Challis *et al.*, 2009) and oxidative stress (Al-Gubory *et al.*, 2010) in gestational tissues are associated with pathophysiology of pregnancy, including preterm labor, preeclampsia, and IUGR, our data suggest that DCVC activates mechanisms that may lead to adverse birth outcomes. Elevated levels of IL-6 in maternal serum and amniotic fluid are frequently found in pregnancy disorders such as recurrent miscarriage, preeclampsia and preterm delivery in humans (Prins *et al.*, 2012). Likewise, DCVC induced mitochondrial dysfunction, increased ROS, decreased glutathione, and stimulated expression of redox-sensitive genes in human placental HTR-8/SVneo cells.

In the kidney, DCVC is bioactivated by cysteine conjugated β-lyase to generate the highly reactive intermediate S-(1,2-dichlorovinyl)thiol (DCVT) (Lash *et al.*, 2000b; Lash *et al.*, 2014,). DCVT is chemically unstable, rapidly rearranging to form chlorothiolketene (CTK) and chlorothionoacetyl chloride (CTAC), two highly reactive species that are thought to be responsible for formation of covalent adducts to DNA, protein, and phospholipids (Lash *et al.*, 2000b). Although not directly demonstrated, the current study suggests that β-lyase is present in human placental cells because treatment with AOAA, a renal cysteine conjugated β-lyase inhibitor, almost completely inhibited DCVC-induced IL-6 release in HTR-8/SVneo cells. Furthermore, the present study suggests that DCVC-stimulated increase in IL-6 is due to a reactive metabolite of DCVC generated via cysteine conjugated β-lyase. We recognize, however, the importance of measuring the expression and activity of cysteine conjugated beta-lyase, a key enzyme in metabolizing DCVC to more toxic compounds, in TCE-treated rat placenta tissues.

Therefore, future studies should include measurements of cysteine conjugated beta-lyase enzyme in the rat placenta tissues.

While contributing new information on the effects of TCE and DCVC on the placenta, our study has some limitations. Firstly, we used HTR-8/SVneo, an immortalized cell line transfected with SV40, in some experiments. 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 similar phenotype to primary human trophoblasts (Graham *et al.*, 1993; Biondi *et al.*, 2006), 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 (Nicola *et al.*, 2005; Khan *et al.*, 2011), HTR-8/SVneo cells have a different epigenetic profile compared with primary extravillous trophoblast cells (Novakovic *et al.*, 2011). Nevertheless, HTR-8/SVneo cells have been a useful model in other studies to examine placentation and physiologically invasive extravillous trophoblast cells (Liu *et al.*, 2012; Wang *et al.*, 2012; Weber *et al.*, 2013; Xu *et al.*, 2014).

To address these limitations, we used a whole animal model to support mechanistic experiments conducted in HTR-8/SVneo cell culture. However, a limitation in our in vivo study is that we evaluated only one dose of TCE, 480 mg/kg-d: future studies are needed to characterize the full dose-response relationship for TCE toxicity in pregnant rats. Although the US EPA maximum contaminant level for public drinking water is 5 µg/L, TCE has been found at levels of 1400 µg/L in in municipal drinking water (ATSDR, 2003). It is also important to note limitations in our animal exposure model. Firstly, in the current study, rats were exposed once a day while humans would likely be exposed many more times during the day and through different exposure routes. Furthermore, health risk assessment shows that in addition to the

amount ingested, TCE exposure via inhalation and dermal routes during showers are major source of risk to human health (Lee *et al.*, 2002). Secondly, results from the current study provides evidence of association between exposure to TCE and increased oxidative stress and inflammation: therefore, future research should also include pretreatment with an antioxidant such as N-acetyl cysteine in order to test hypothesis that oxidative stress is an important mediator of TCE toxicity to the placenta and for inducing fetal growth restriction.

To confirm a role for epigenetic dysregulation in the observed effects, future research should investigate the relevance of global methylation changes in rat placenta to human and gene-specific DNA methylation. Candidate genes could include genes that we observed to be increased in the rat placenta with exposure to TCE. Moreover, future research could assess TCE-induced alterations to epigenetic mechanisms other than DNA methylation, including microRNA expression and histone modification, in relation to adverse pregnancy outcomes.

Despite these limitations, our findings suggest potential adverse impacts of TCE exposure during pregnancy. By using time pregnant Wistar rats, we have established an experimental model to study potential mechanisms by which exposure to environmental pollutants contributes to adverse outcomes. We chose to work with rat because TCE metabolism in rat is more similar to that of humans compared with mouse: e.g., metabolism in rat and human liver microsomes is biphasic while it is monophasic in mouse liver microsomes (Lash *et al.*, 2000a) and mice metabolize and eliminate TCE more rapidly than do rats and humans (Lash *et al.*, 2000b). Also we conducted whole animal experiment in order to support molecular mechanistic experiments in cell culture. An additional strength of our study is the use of oral exposure in food vehicle, which is a more relevant route of exposure to TCE than oral gavage (typically in vegetable oil vehicle). Moreover, in this work we used both in vivo rat studies and in vitro cell culture models

to investigate TCE exposure and adverse health outcomes. Utilizing a cell line allowed us to employ molecular approaches such as using redox-sensitive probes that are not feasible in vivo, and conducting experiments in whole animal models supported the mechanisms observed in vitro in relation to adverse pregnancy outcomes.

Findings from this dissertation identified mechanisms that may help explain associations between TCE exposure and adverse birth outcomes observed in epidemiology studies, summarized in Figure 5.2. We chose to examine toxicant-induced placental toxicity because the placenta is a critical organ for pregnancy. The placenta has multiple functions during pregnancy including nutritional transport to the fetus, xenobiotic metabolizing activity, waste transport from the fetal compartment, and endocrine action (Prouillac *et al.*, 2010). As a result, the placenta is a highly susceptible target organ for drug- or chemical-induced adverse effects, and many placental toxicants have been reported (Juchau, 1973; Leazer *et al.*, 2003).

Our findings demonstrate that TCE can activate inflammatory pathways and oxidative stress in the placenta which may lead to fetal growth restriction in an animal model. Moreover, exposure to TCE induced maternal systemic inflammation, which is associated with adverse pregnancy outcomes. Adverse birth outcomes including intrauterine growth restriction, SGA, and preterm birth constitute a significant public health problem. By identifying potential mechanisms of TCE-induced toxicity, we can inform future epidemiology studies by providing plausible biological explanations for environmental exposures associated with adverse pregnancy outcomes. Furthermore, the current research provides a rationale for future epidemiologic studies of adverse birth outcomes utilizing biomarkers of oxidative stress such as 8-OHdG, proinflammatory cytokines including IL-6, and analysis of antioxidant levels such as vitamin E, with levels of TCE metabolites in the urine and plasma of pregnant women. Finally, this thesis

provides new information for potential interventions to reduce adverse obstetrical outcomes associated with poor placentation by demonstrating that antioxidants such as α-tocopherol can act as anti-inflammatory agents.

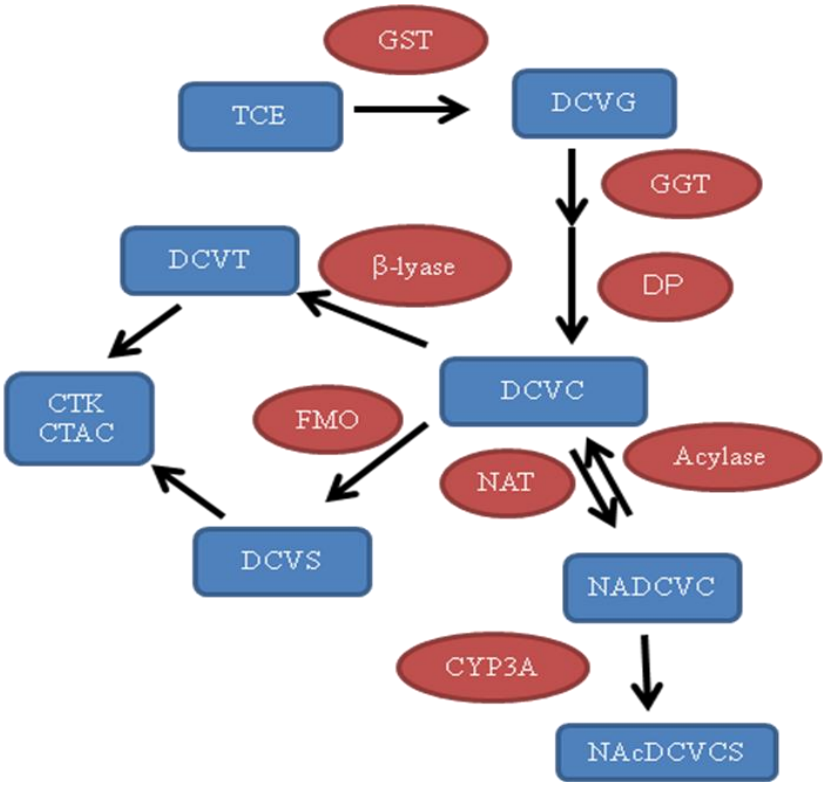

Figure 5.1. Glutathione-dependent metabolism of TCE. Enzymes are depicted in brown and metabolites in blue. Modified from Lash et al. (Lash *et al.*, 2014). Abbreviations: cytochrome P-450 3A (CYP3A); chlorothionoacetyl chloride (CTAC); chlorothioketene (CTK); dipeptidase (DP); S-(1,2- dichlorovinyl)-L-cysteine (DCVC); S-(1,2-dichlorovinyl)glutathione (DCVG); DCVC sulfoxide (DCVCO); 1,2-dichlorovinylthiol (DCVT); flavin-containing monooxygenase (FMO); glutathione S-transferase (GST); gamma-glutamyltransferase (GGT); N-acetyltransferase (NAT); NAcDCVC sulfoxide (NAcDCVCS); N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine (NAcDCVC); NAcDCVC sulfoxide (NAcDCVCS) (metabolizing enzymes depicted in brown and metabolites in blue).

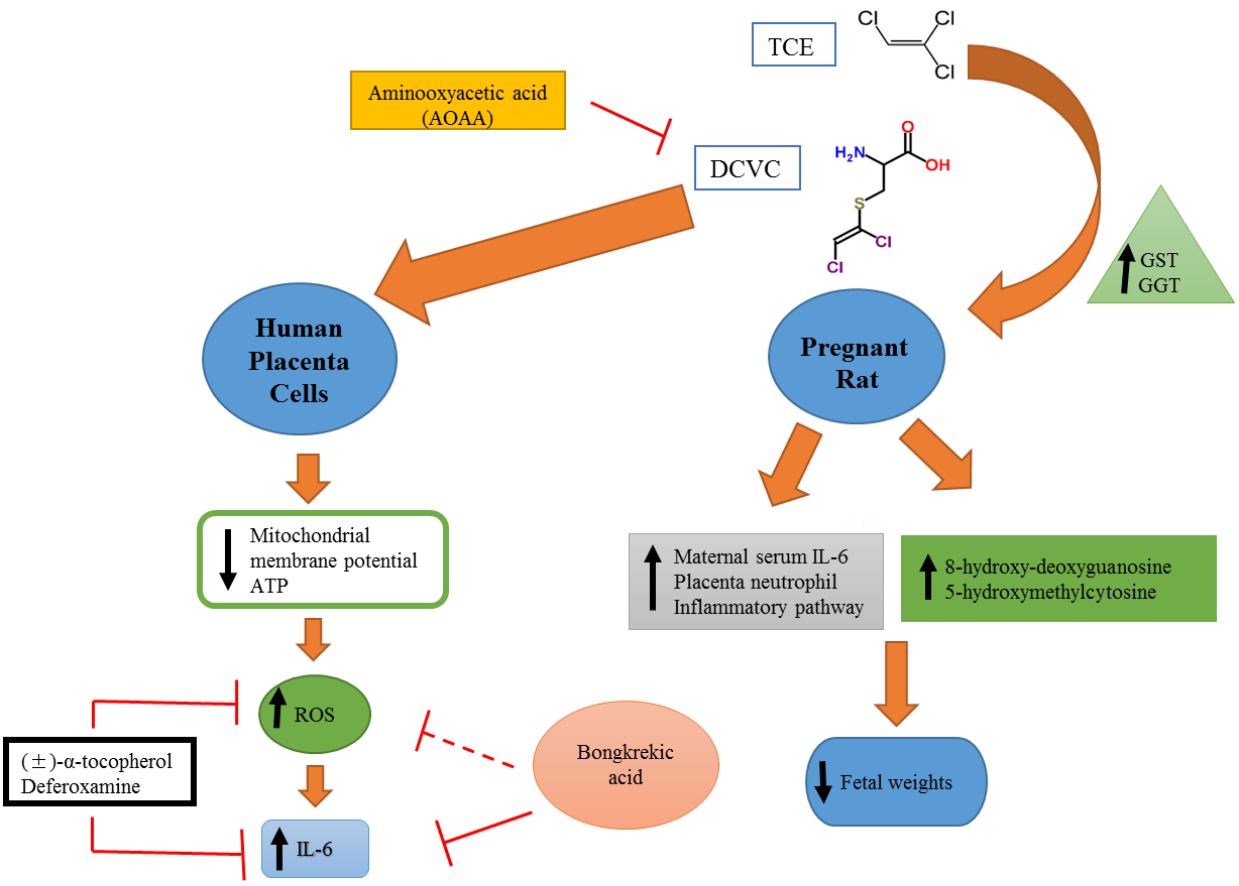

Figure 5.2. Conceptual summary of TCE effects in pregnant Wistar rat and in human placental trophoblast cells. TCE is metabolized via glutathione conjugation pathway in the placenta to DCVC. DCVC then induces oxidative stress and inflammatory response in maternal system and in the placenta. In human placenta cells, DCVC stimulated IL-6 release which was suppressed by antioxidant treatments suggesting reactive oxygen species (ROS)-mediated increase in IL-6. Treatment with AOAA, a cysteine conjugated beta-lyase inhibitor, blocked DCVC-induced IL-6 response, suggesting that DCVC toxicity is dependent on its bioactivation by cysteine conjugated beta lyase human placental cells in human placental cells. Our findings demonstrate that the placenta is a target for TCE toxicity which can lead to pregnancy complications such as fetal growth restriction through mechanisms of oxidative stress and activation of inflammatory pathways.

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