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Investigation of mechanisms of follicle depletion and the ovarian protective response to ovotoxicant exposure

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Investigation of mechanisms of follicle depletion and the ovarian protective response to ovotoxicant exposure

by

Jill A. Madden

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:
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Iowa State University
Ames, Iowa
2014

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DEDICATION

I dedicate my dissertation to my family for their continued support as well as to all the small-town kids, like me, that were told they couldn’t make it in a university setting.
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ABSTRACT

A female is born with a finite number of ovum-containing follicles, responsible for maintaining lifetime female fertility prior to menopause. Follicle quality and number can be compromised by ovotoxicant exposures; the chemotherapy drug cyclophosphamide (CPA) depletes ovarian follicles resulting in increased risks for infertility in female cancer survivors. In comparison, 7,12-dimethylbenz[a]anthracene (DMBA), liberated from burning of organic compounds, destroys all stage ovarian follicles. CPA and DMBA must be bioactivated to their active, ovotoxic, metabolites phosphoramid mustard (PM) and DMBA-1,2-epoxide, 3,4-diol, respectively. This dissertation investigated ovarian PM and DMBA exposures using a neonatal rat whole ovary culture method, to increase understanding for mechanisms driving PM- and DMBA-induced follicle depletion.

Investigations of PM metabolism demonstrated that microsomal epoxide hydrolase (Ephx1) plays a role in detoxification of PM and while increased glutathione (GSH) levels lessened PM ovotoxicity, surprisingly, depleting (GSH) had no impact, rending the role of GSH in PM metabolism as inconclusive and requiring additional studies. Generation of an ovotoxic, volatile PM metabolite, presumably chloroethylaziridine (CEZ), was identified, independent of ovarian metabolism, suggesting that PM is not the only ovotoxic metabolite of CPA.

Autophagy induction during PM-induced ovotoxicity was confirmed via identification of autophagosome formation by transmission electron microscopy and altered mRNA and protein levels of key autophagy genes. Pathway manipulations of PI3K and
mTOR to inhibit or activate autophagy, respectively, revealed that PI3K inhibition had no effect on PM-induced ovotoxicity while, mTOR activation prevented PM-induced follicle depletion.

Depletion of large preantral follicles along with altered mRNA levels of autophagy genes were also demonstrated with low-dose acute DMBA exposure. Induction of additional ovarian responses, including xenobiotic metabolism, oxidative stress and PI3K signaling were also observed in these experiments.

Taken together, this dissertation supports that ovotoxicant exposure induces ovarian chemical biotransformation and autophagy in addition to a number of other protective responses. A thorough understanding of the detrimental effects of PM, CEZ and DMBA exposures are required before advancements towards preservation of the ovarian follicle reserve can progress.
Chapter 1. INTRODUCTION

The ovary

The major mammalian female reproductive organ is the ovary, which is primarily comprised of structures called follicles. These follicles contain the oocyte or “egg” that can be surrounded by up to two somatic cell types, at initial development stages by granulosa cells and at later stages of follicular development by theca cells. The granulosa and theca cells cooperate to produce the sex steroid hormones, the estrogens (17β-estradiol; E2) and progesterone, as well as communicate with the oocyte to maintain viability. At birth, the ovarian follicle reserve is established and generally accepted as a non-replenishing pool, thus this finite number of follicles is essential to support fertility throughout the lifespan of a woman via production of a fertilizable gamete (Hirshfield, 1991).

Ovarian folliculogenesis

The earliest follicular stage is the primordial follicle, which is arrested in prophase I of meiosis, potentially being maintained through the lifetime of a woman, until receiving a signal to become activated and recruited to the growing follicle pool. At this time, the granulosa cells transition from a squamous to cuboidal state and begin dividing while the oocyte starts to grow. As shown in Figure 1, the follicle will progress through the primary stage, one granulosa cell layer surrounding the oocyte, and further onwards to the secondary stage, two layers of granulosa cells encasing the oocyte. Subsequently, a fluid-filled cavity develops, namely the antrum, and the theca cells are recruited from the ovarian stroma to comprise the outermost layer of the follicle. At this stage, the antral follicle is now luteinizing hormone (LH) and follicle stimulating
hormone (FSH) responsive and generating E2, which are key factors to the induction of ovulation, the expulsion of the oocyte from the follicle and ovary into the fallopian tube. The remaining follicular cells will luteinize to form the corpus luteum (CL), which produces progesterone to maintain a pregnancy or degenerates to complete the menstrual cycle.

Although ovulation is the ultimate physiological endpoint for an oocyte, the natural fate for the majority of follicles is atresia (follicle death). Many primordial follicles will be activated from the follicular reserve, but only one percent will complete the cycle to ovulation, thus the other 99% will die, because once activated, they cannot remain indefinitely in the growing pool or retreat to the quiescent ovarian reserve (Hirshfield, 1991). On average, by the age of 51 (Jick and Porter, 1977), all the follicles are depleted and ovarian failure, or menopause, ensues, reducing circulating E2 levels and increasing the risk of osteoporosis and heart disease (Hirshfield, 1991; Greendale et al., 1999). Unfortunately, this innate depletion of follicles can be accelerated by chemical exposures that target and kill follicles ultimately resulting in premature ovarian failure (POF) (Jick and Porter, 1977; Hoyer and Sipes, 1996; Borman et al., 2000).

**Chemical-induced ovotoxicity**

Ovotoxicants range from compounds found in plastics, pesticides, industrial and environmental pollutants to even Federal Drug Administration (FDA) approved drugs such as chemotherapeutics. Chemicals that deplete all follicular stages include the environmental toxicant 7,12-dimethylbenz[a]anthracene (DMBA), while other ovotoxicants, such as the industrial compound 4-vinylcyclohexene diepoxide (VCD), can target specific follicular stages; in the case of VCD the targets for destruction are the primordial and small primary follicles (Flaws et al., 1994; Devine et al., 2002; Rajapaksa et al., 2007; Igawa et al., 2009). An
ovotoxicant’s target follicle type is important in determining the ultimate reproductive outcome. VCD for instance, will cause POF, but will do so by eliminating the smallest follicles, which will initially allow some follicles to ovulate and fertility to continue until the ovarian reserve has been irreversibly depleted. Thus, the individual will have limited and delayed signs of decreased fertility, although the ovarian reserve is actively being reduced. In contrast, compounds targeting the larger follicles will elicit rapid signs of reduced fertility, evidence by altered menstruation or infertility. However, once the female is removed from this exposure, fertility can resume via recruitment of follicles out of the ovarian reserve which was not targeted, thus these compounds cause a reversible, temporary form of infertility (Hoyer and Keating, 2014).

Another important aspect to consider in reproductive toxicology is the age of the individual at the time of the exposure. A younger age does not equate a higher risk of chemical-induced infertility. As depicted in Figure 2, at birth, a female will have about two million follicles, a number that will only decline over time, yielding roughly 300,000 follicles remaining at the age of puberty (menarche) (Faddy et al., 1992; Wallace and Kelsey, 2004). This large pre-pubertal ovarian reserve can hinder the detrimental reproductive effect of ovotoxicants, simply due to the higher number of primordial follicles present in the ovary at the time of exposure. For example, a twelve-year-old girl can receive up to 18 Gy of radiation before being considered sterile, while a 45-year-old woman can withstand only 9 Gy before sterilization occurs (Wallace et al., 2005).

Of additional concern is the fact that there is sometimes a latent impact of endocrine disrupting and reproductive toxicants, a classic example of this is diethylstilbestrol (DES), which was FDA approved to prevent miscarriages in women in the 1950s (Veurink et al., 2005). Although no adverse effects were reported in the women administered DES, it was discovered
during the 1970s that the female offspring of those DES-exposed mothers were at an increased risk of developing a rare vaginal cancer (Herbst et al., 1971; Veurink et al., 2005). In addition, a trans-generational impact of DES continues to be evident with reproductive complications now being discovered in the granddaughters and grandsons of DES exposed women (Blatt et al., 2003).

**Ovarian chemical biotransformation**

Although the effects of ovotoxicant are staggering, fortunately, to some extent, the ovary is capable of defending itself by eliciting protective responses such as xenobiotic metabolism, autophagy, DNA repair and activation of cell survival pathways. For example, many biotransformation enzymes are present in the ovary to combat the effects of ovotoxic metabolites produced by hepatic metabolism or those that may have traveled through the bloodstream to access the ovary directly. The parent compound, or initial compound of exposure, is often not the active, ovotoxic form. 4-vinylcyclohexene (VCH), released during the manufacturing of rubber, pesticides, plastics and flame-retardants (Rappaport and Fraser, 1977), is bioactivated by enzymes from the cytochrome P450 family (CYPs) to form the active and ovotoxic metabolite VCD (Fontaine et al., 2001a; Fontaine et al., 2001b; Cannady et al., 2003; Rajapaksa et al., 2007). Notably, however, VCH has also been shown to be detoxified in the ovary by microsomal epoxide hydrolase (EPHX1), thus demonstrating the ability of an enzyme to elicit a protective response in the ovary (Flaws et al., 1994; Cannady et al., 2002).

Xenobiotic metabolism genes, like EPHX1, and their transcription factors have demonstrated their key roles in the ovarian response to chemicals. The transcription factor nuclear factor erythroid 2-related factor 2 (NRF2), for example, is bound to Kelch-like ECH-
associated protein 1 (KEAP1) until a stress occurs causing its release and translocation to the nucleus where it is then able to bind the antioxidant response elements (ARE) of genes (Itoh et al., 1999; McMahon et al., 2003; Ma et al., 2004). In mice deficient for NRF2, liver expression of the glutathione-S-transferase (GST) genes, a family of enzymes known for catalyzing the conjugation of GSH to xenobiotics for their excretion, was reduced to only 3-20% when compared to their wild-type littermates (Chanas et al., 2002). GST isoform pi (GSTP), suggested to play a direct role in DMBA detoxification, has been shown to be transcriptionally activated by the binding of NRF2 (Ikeda et al., 2004). Another important xenobiotic metabolism transcription factor is aryl hydrocarbon receptor (AHR), which activates genes by binding to xenobiotic response elements (XRE) found in the promoter of target genes (Hankinson, 1995).

**Phosphatidylinositol-3 kinase signaling**

One important player in the ovarian xenobiotic response is the phosphatidylinositol-3 kinase (PI3K) signaling pathway, which plays a vital role in primordial follicle viability (Yoshida et al., 1997; Parrott and Skinner, 1999) and control of recruitment of follicles out of the ovarian reserve (Yoshida et al., 1997; Kissel et al., 2000; Castrillon et al., 2003; Reddy et al., 2005; John et al., 2008). Initiation of the PI3K pathway in the primordial follicles occurs when the granulosa-cell-expressed kit ligand (KITLG) binds to the oocyte-expressed receptor, cKit (Manova et al., 1990; Orr-Urtreger et al., 1990; Horie et al., 1991; Ismail et al., 1996). The resulting autophosphorylation of cKit drives the activation of the PI3K pathway (Roskoski, 2005) leading to phosphorylation and nuclear translocation of AKT (pAKT), thereby regulating pro-survival activities such as growth, survival and cell cycle entry (Datta et al., 1999).
In addition to promoting follicle survival, the PI3K pathway governs primordial activation and recruitment into the growing follicle pool, which also contributes to the overall protective response of the ovary. Current evidence suggests this is achieved via pAKT actions on forkhead transcription factor family member FOXO3 (Reddy et al., 2005). When Foxo3 was knocked out in mice, the lack of FOXO3 resulted in POF, which was determined to be a result of accelerated recruitment of primordial follicles into the growing pool, followed by inevitable atresia of the over-abundantly activated follicles (Castrillon et al., 2003). Complementary to the results from the Foxo3 knockout mice, oocyte-specific FOXO3 overexpression halted activation of primordial follicles (Castrillon et al., 2003), thus suggesting a pivotal role for FOXO3 and the PI3K pathway in the determination of follicle growth and survival as well as the maintenance of the ovarian reserve.

Programmed cell death in the ovary

Although the importance of the ovarian protective response to xenobiotic exposure is apparent, the ovary must maintain a balance between pro-survival and pro-death, presumably, to ensure that the best quality oocytes are ovulated. Historically, apoptosis was the pro-death process attributed to causing atresia; however, studies now suggest that autophagy may be a key alternate or concurrent process in follicular death (Escobar et al., 2008; Rodrigues et al., 2009; Gannon et al., 2012). The process of autophagy occurs through the identification and encapsulation of cytoplasmic contents and organelles that are destined for degradation. The autophagosome, a double-membraned vesicle, transports the unwanted material to the lysosome where the two structures merge to form the autolysosome and digest the constituents (de Duve,
In yeast, 35 genes have been found to be involved with autophagy, and being an evolutionary conservation process, many of these genes have been found to have mammalian homologues, including but not limited to beclin-1 (Becn1), autophagy-related 7 homolog (Atg7) and microtubule-associated proteins 1A/1B light chain (LC-3) (Muller and Reichert, 2011).

At birth, a time of large follicle depletion, ovarian expression of pro-apoptotic genes are unchanged and the expression of genes involved with autophagy are increased in mice (Rodrigues et al., 2009). Another study reported finding classic markers of both apoptosis, (cleaved caspase-3 expression and TUNEL staining) and autophagy (increased LAMP1 expression) in atretic ovaries from 1-28 day old rats (Escobar et al., 2008). In addition to atretic activity, interestingly, and demonstrative of the complexity of this process, autophagy has also been suggested as a mode for ovarian follicle survival (Gawriluk et al., 2011). PND1 homozygous Atg7 knockout mice lacked any apparent germ cells while Becn1+/− mice had a 56% reduction in germ cells compared to control (Gawriluk et al., 2011). Taken together, these previous studies support that autophagy is an active ovarian process with the potential to be a fundamental regulator of the important balance of follicle life and death.

**Polycyclic aromatic hydrocarbons**

For decades, smoking cigarettes has been recognized to decrease the age of menopause onset (Jick and Porter, 1977), and this effect has since been accredited to the ovotoxic properties of polycyclic aromatic hydrocarbons (PAHs) like DMBA, benzo(a)pyrene (BaP), and 3-methylcholanthrene (3MC) (Mattison et al., 1983; Vahakangas et al., 1985). These ovotoxicants
are produced by the burning of any organic material, thus evoking widespread exposure via cigarette smoke, car exhaust, and charred food (Gelboin, 1980). Murine studies of these PAHs have clearly indicated the ability of these compounds to kill follicles (Mattison and Schulman, 1980; Mattison et al., 1983; Borman et al., 2000) with the following order of ovotoxic potency in mice being DMBA > 3MC > BaP (Mattison and Thorgeirsson, 1979). Co-administration of alpha-naphthoflavone, an AHR inhibitor, during exposure to each PAH prevented primordial follicle depletion in mice, suggesting that these PAHs must be bioactivated in order to elicit ovotoxic effects (Mattison and Thorgeirsson, 1979).

Ovarian bioactivation of DMBA, in particular, has been well characterized. Using both in vivo and in vitro methods, follicle depletion was observed with lower concentrations of the DMBA metabolite, DMBA-3,4-diol, than DMBA itself, thus indicating the metabolite’s higher ovotoxic potency (Igawa et al., 2009). Furthermore, by competitively inhibiting EPHX1 using cyclohexene oxide (CHO), DMBA-induced follicle depletion was prevented, while DMBA-3,4-diol-induced ovotoxicity was unaffected, indicating that EPHX1, the same enzyme that metabolizes VCH to a lesser toxic metabolite, was required for the bioactivation of DMBA in the ovary (Rajapaksa et al., 2007; Igawa et al., 2009). DMBA biotransformation begins with metabolism by CYP1B1 and EPHX1 to form the more potent DMBA-3,4-diol metabolite, which undergoes further biotransformation via epoxidation by CYP1A1 or CYP1B1 to form the ultimate ovotoxic metabolite DMBA-3,4-diol-1,2-epoxide (Bengtsson et al., 1983; Miyata et al., 1999; Shimada et al., 2003; Kleiner et al., 2004; Rajapaksa et al., 2007; Igawa et al., 2009).

Follicle destruction by DMBA is thought to be achieved by damaging the oocyte first, followed by rapid somatic cell loss (Morita and Tilly, 1999), ultimately resulting in follicle loss by apoptosis (Tsai-Turton et al., 2007). Investigation of the role of glutathione (GSH) and the
production of reactive oxygen species (ROS) in DMBA-induced ovotoxicity revealed that ROS generation was increased by DMBA exposure despite the lack of effect on ovarian GSH concentration within the ovary (Tsai-Turton et al., 2007) However, when GSH levels were decreased in cultured preovulatory follicles prior to DMBA exposure an increase in apoptosis was observed and complementary to study, when supplemented with GSH, DMBA-induced apoptosis was reduced (Tsai-Turton et al., 2007).

Despite considerable knowledge about the bioactivation of DMBA, less is known about its detoxification. The protective effect observed when supplementing follicles with GSH prior to DMBA exposure suggests GSH may play a role in the detoxification of DMBA. One study supporting this hypothesis utilized mice deficient in GSTP, which in turn increased their sensitivity to the tumorigenic effects of DMBA, therefore, suggesting GSTP as detoxifying enzyme for DMBA (Henderson et al., 1998). This role was further investigated by evaluating the ovarian activity of GSTP and other GST isoforms mu (GSTM) and alpha (GSTA) following DMBA exposure in neonatal rat ovaries. While no increase in mRNA levels were found in Gstm or Gsta following DMBA exposure, mRNA and protein levels of GSTP increased in DMBA treated ovaries relative to control, adding further support to GSTP’s role in ovarian DMBA detoxification 2012 (Bhattacharya and Keating, 2012).

In the neonatal rat ovary, during LY294002-induced PI3K inhibition, EPHX1 levels are increased and subsequently, likely due to increased bioactivation by EPHX1, DMBA follicle depletion is worsened (Keating et al., 2009; Bhattacharya et al., 2012). The results from this study suggest that PI3K may have a regulatory role for EPHX1, which was investigated further by evaluating the effect of PI3K inhibition on other xenobiotic genes. Using the same PI3K inhibition conditions, the levels of GSTM, GSTP and AHR were also increased, supporting PI3K
as a regulator of these xenobiotic metabolism genes (Bhattacharya and Keating, 2012). This role was further supported by an observed increase in genes downstream of PI3K following DMBA exposure (Sobinoff et al., 2011), therefore providing additional evidence of the PI3K pathway having a direct impact on the ovotoxicity of DMBA.

**Chemotherapeutics**

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It is estimated that over 1.6 million people in the United States will be diagnosed with cancer in 2012 (Siegel et al., 2012). Compared to 1977 when the 5-year survival rate was 49%, 67% of cancer patients will now survive at least 5 years post-diagnosis (Siegel et al., 2012). Early detection and advanced medical treatments are likely attributable to factors responsible for these enhanced survival rates. However, as cancer survival rates increase, a growing number of survivors are facing the consequences of their treatment, which unfortunately for many women includes POF and infertility.

A number of antineoplastic agents can cause female infertility, including busulfan, cisplatin, and CPA. In 1956, the first observed ovotoxic chemotherapy effects were induced by busulfan, which caused all patients (n = 4) to be premenopausal three months into therapy (Louis et al., 1956). Busulfan is an alkylating agent, which acts by cross-linking DNA to interfere with cell replication (Jackson et al., 1959).

Cisplatin (cis-diaminedichloroplatinum (II): CDDP), a nonalkylating agent, is also an ovotoxic antineoplastic chemical. CDDP is a platinum-based chemotherapy that, similar to
alkylating agents, reacts with DNA, inhibiting DNA synthesis and causing apoptosis. CDDP is used to treat a variety of cancers including breast, ovarian, lung, and head and neck cancers with side effects that include nephrotoxicity and ovotoxicity (Dobyan et al., 1980; Nakai et al., 1982; Wallace et al., 1989). Studies in rats have demonstrated that a single dose of CDDP decreased primordial follicles by 25%–35% and increased follicular cysts and apoptosis (Borovskaya et al., 2004). Repeated CDDP exposure in rats depleted the total number of follicles, increased postimplantation loss rates, and decreased litter number (Matsuo et al., 2007; Nozaki et al., 2009). There is evidence that CDDP causes DNA laddering, chromatin condensation and arrest of the cell cycle at the G2 phase (Barry et al., 1990; Boersma et al., 1996; Schmitt et al., 2004). Additionally, in vivo and in vitro studies reported that CDDP induced the production of ROS (Kopke et al., 1997; Satoh et al., 2003), which may also contribute to CDDP-induced ovarian damage.

The metabolism of CDDP is slowly being deciphered, and while it remains unclear if CDDP is enzymatically biotransformed, it is hypothesized that CDDP-containing chloride ligands are displaced by water yielding a positive charged platinum compound that reacts with DNA, thus cross-linking the strands and inhibiting DNA synthesis (McEvoy, 1992), which ultimately leads to cell apoptosis involving the Bcl-2 and Bax pathways (Barry et al., 1990; Boersma et al., 1996; Schmitt et al., 2004).

CPA is an antineoplastic prodrug that acts as an alkylating agent to treat, alone or in combination, a variety of cancers and autoimmune disorders. CPA was FDA approved in 1959 and is also known by its brand names: Cytoxan, Clafen, and Neosar. CPA is administered orally or intravenously to patients of all ages and has a wide range of side effects including alopecia, nausea, immunosuppression, and infertility (Fraiser et al., 1991). Infertility is particularly
concerning for females because CPA destroys primordial follicles (Plowchalk and Mattison, 1991).

In a similar manner as that of DMBA, CPA is inactive until it is metabolized, primarily by hepatic CYP enzymes, inducing production of a number of active and inactive metabolites. The bioactivation step that contributes greatly to CPA induced ovotoxicity is the C-4 oxidation of CPA to form 4-hydroxycyclophosphamide (4-HC), followed by interconversion to the opening form, aldophosphamide, which fragments to phosphoramidate mustard (PM) and acrolein (Ludeman, 1999; Pinto et al., 2009). Previous research has found 4-HC to be an ovotoxic metabolite of CPA, because 4-HC can easily pass through the cell membrane and produce PM intracellularly (Ludeman, 1999; Desmeules and Devine, 2006). PM is known to be the antineoplastic metabolite of CPA (Shulman-Roskes et al., 1998), and its formation is required for ovotoxicity in vitro (Plowchalk and Mattison, 1991) and in vivo (Desmeules and Devine, 2006). However, PM can undergo another spontaneous reaction to form the volatile, cytotoxic metabolite chloroethylaziridine (CEZ) (Rauen and Norpoth, 1968; Shulman-Roskes et al., 1998, 1998), which, due to its higher cell permeability (Hata and Watanabe, 1994) and longer half-life compared to PM (Lu and Chan, 2006), may also contribute to CPA-induced ovotoxicity (Desmeules and Devine, 2006).

Previous studies have demonstrated that in vitro CPA and/or PM exposures target primordial follicles in mice (Plowchalk and Mattison, 1991) and antral follicles in rats (Desmeules and Devine, 2006) at concentrations relevant to human exposures (Struck et al., 1987; Desmeules and Devine, 2006). The specific ovarian cell-type targeted depends on the follicle type, such that in smaller follicles, oocytes are being targeted, while in larger follicles, it is the granulosa cells (Desmeules and Devine, 2006). Granulosa cells of primary follicles are
stained positively for caspase-3 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (evidence of apoptosis) following PM exposure; thus, it was concluded that apoptosis is responsible for those cells’ death (Desmeules and Devine, 2006). However, it is acknowledged that due to the close interaction and communication between the granulosa cells and the oocyte, it is difficult to determine exactly which cell type is the initial ovotoxic target (Desmeules and Devine, 2006).

Evidence suggests that the detoxification of CPA and its metabolites occur by NADPH oxidation via aldehyde dehydrogenases and GSH conjugation via the GSTs, with the precise mechanisms remaining to be determined (Pinto et al., 2009). Further, these mechanisms may vary between follicle types (Desmeules and Devine, 2006). Studies also suggest that a spontaneous GSH conjugation to PM can occur, thus lessening both the toxic and therapeutic effects of CPA (Colvin et al., 1993; Colvin, 1994; Dirven et al., 1994; Shulman-Roskes et al., 1998). The stability of CEZ has also been demonstrated to decrease with excess GSH (Shulman-Roskes et al., 1998), supporting detoxification roles for GSH conjugation to CPA, PM and CEZ.
Figure 1. Ovarian follicular development. (Keating and Hoyer, 2009; With Copyright Permission)
Figure 2. Natural follicle number decline over time.
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Chapter 2. INVOLVEMENT OF A VOLATILE METABOLITE DURING PHOSPHORAMIDE MUSTARD-INDUCED OVOTOXICITY

A paper published by Toxicology and Applied Pharmacology

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Contribution Statement:
I performed all analyses on ovarian tissue in this paper, interpreted data and wrote the paper; Hoyer, P.B., was post-doctoral mentor for Keating, A.F. at the time of ovary collection for follicle counts in Figure 3; Devine, P.J. co-designed the experimental exposures for Figure 3 and edited the manuscript; Keating, A.F. designed experiments, aided in data interpretation, edited the manuscript and served as corresponding author.

Abstract
The finite ovarian follicle reserve can be negatively impacted by chemical exposures including the anti-neoplastic agent, cyclophosphamide (CPA). CPA requires bioactivation to phosphoramidate mustard (PM) to elicit its therapeutic effects however; in addition to being the tumor-targeting metabolite, PM is also ovotoxic. In addition, PM can break down to a cytotoxic, volatile metabolite, chloroethylaziridine (CEZ). The aim of this study was initially to characterize PM-induced ovotoxicity in growing follicles. Using PND4 Fisher 344 rats, ovaries were cultured for 4 days before being exposed once to PM (10 or 30 µM). Following eight
additional days in culture, relative to control (1% DMSO), PM had no impact on primordial, small primary or large primary follicle number, but both PM concentrations induced secondary follicle depletion ($P < 0.05$). Interestingly, a reduction in follicle number in the control-treated ovaries was observed. Thus, the involvement of a volatile, cytotoxic PM metabolite (VC) in PM-induced ovotoxicity was explored in cultured rat ovaries, with control ovaries physically separated from PM-treated ovaries during culture. Direct PM (60 µM) exposure destroyed all stage follicles after 4 days ($P < 0.05$). VC from nearby wells depleted primordial follicles after 4 days ($P < 0.05$), temporarily reduced secondary follicle number after 2 days, and did not impact other stage follicles at any other time point. VC was determined to spontaneously liberate from PM, which could contribute to degradation of PM during storage. Taken together, this study demonstrates that PM and VC are ovotoxicants, with different follicular targets, and that the VC may be a major player during PM-induced ovotoxicity observed in cancer survivors.

**Introduction**

Cyclophosphamide (CPA) is used, alone or in combination, to treat an array of cancers and autoimmune disorders, and elicits numerous undesirable health side effects including alopecia, nausea, immunosuppression and infertility (Fraiser *et al.*, 1991). As a prodrug, CPA requires bioactivation to the metabolite phosphoramid mustard (PM) in order to induce anti-neoplastic effects (Shulman-Roskes *et al.*, 1998). Bioactivation of CPA to PM occurs through a cascade of reactions (Fig. 1) that are initiated primarily by the hepatic cytochrome P450 enzymes (CYPs) isoforms 2B and 3A (Philip *et al.*, 1999). These CYPs hydroxylate CPA to form 4-hydroxycyclophosphamide, which is non-enzymatically transformed to the open-ring metabolite,
aldophosphamide. Aldophosphamide fragments, producing the compounds acrolein and PM (Ludeman, 1999).

Interestingly, PM can further transform into a volatile, cytotoxic compound chloroethylaziridine (CEZ; Rauen and Norpoth, 1968; Lu and Chan, 1996). CEZ was first identified as a volatile metabolite of CPA in 1968, when approximately 2% of the CPA administered was shown to be exhaled as CEZ from the lungs of exposed rats (Rauen and Norpoth, 1968). CEZ was also found to be responsible for the airborne cytotoxicity, observed in untreated cells cultured adjacent to cells exposed to CEZ-producing compounds, termed the “neighboring well effect” (Flowers et al., 2000). Although the generation of CEZ has been demonstrated both in vivo and in vitro, determining the relative contribution of CEZ to CPA-induced cytotoxicity has proven difficult due to the compound’s volatility and the instability of its precursor, PM. CEZ plasma concentrations were shown to peak 5 minutes after intravenous PM administration in rats (Lu and Chan, 2006). An additional study demonstrated that following complete degradation of PM in solution, 85% of the solution’s cytotoxicity remained due to the generation and continued presence of CEZ (Chan et al., 1994). Taken together, these studies support that CEZ is a major degradation product of CPA/PM and suggests that CEZ may play a key role in the toxicity of CPA.

CPA-induced side effects are of growing concern as cancer survival rates continue to improve. In particular, increased cancer survival rates have resulted in a greater number of female cancer survivors affected by CPA-induced infertility (Linet et al., 1999; Sklar et al., 2006; Pulte et al., 2008). Female fertility is dependent on the quality of oocytes, which are maintained within the ovary as follicular structures, consisting of a meiotically-arrested oocyte surrounded by granulosa cells. The ovarian follicular reserve is established at birth and
progressively declines until menopause or ovarian failure, the point at which no follicles remain (Hirshfield, 1991). Chemical exposures, including CPA, accelerate follicular death and subsequent decline of the follicular reserve leading to premature ovarian failure (POF), thus causing permanent infertility (Plowchalk and Mattison, 1991). Aside from sustaining fertility, the survival of these follicles is important to overall female health; POF increases the risk of various health conditions including osteoporosis and heart disease (Greendale et al., 1999).

CPA-induced infertility is attributed to the generation of PM because, in addition to being the anti-neoplastic metabolite, PM is also recognized as the ovotoxic CPA metabolite (Plowchalk and Mattison, 1991; Desmeules and Devine, 2006). However, CEZ has also been suggested to be ovotoxic due to observed loss of primordial follicles induced by a volatile breakdown product of PM in a neonatal rat ovarian culture model (Desmeules and Devine, 2006). Therefore, in addition to PM, CEZ may be at least partially responsible for both the anti-neoplastic and ovotoxic properties of CPA. The purpose of the current study was initially to determine the impact of acute exposure to PM on large growing follicles, which led to the characterization of the ovotoxicity of a volatile compound (VC), presumably CEZ, liberated from PM using a neonatal rat ovary culture system. Specifically, the temporal pattern of PM- and VC-induced follicle loss was characterized, the requirement of ovarian tissue for VC liberation was determined and the impact of storage on PM-induced ovotoxicity at -20°C was evaluated.

**Materials and Methods**

**Reagents:**

Bovine serum albumin (BSA), ascorbic acid, transferrin, formaldehyde, dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich Inc. (St. Louis, MO). Dulbecco’s Modified Eagle
Medium: nutrient mixture F-12 (Ham) 1x (DMEM/Ham’s F12), Albumax, penicillin (5000U/ml) Hank’s Balanced Salt Solution (without CaCl$_2$, Mg Cl$_2$, or MgSO$_4$) were obtained from Invitrogen Co. (Grand Island, NY). Millicell-CM filter inserts and 48-well cell culture plates were obtained from Millipore (Billerica, MA) and Corning Inc. (Corning, NY), respectively. Phosphoramide mustard was obtained from the National Institutes of Health National Cancer Institute (Bethesda, MA).

Animals:
Fisher 344 (F344) rats were housed one per plastic cage and maintained in a controlled environment (22 ± 2°C; 12h light/12h dark cycles). The animals were provided a standard diet with *ad libitum* access to food and water and allowed to give birth. The University of Arizona and Iowa State University Institutional Animal Care and Use Committee approved all experimental procedures.

*Ex vivo ovarian cultures:*
Ovaries were collected from female postnatal day (PND) 4 F344 rats and cultured as described by Devine *et al.*, 2002. Ovaries were removed, trimmed of oviduct and other excess tissue, and placed onto a Millicell-CM membrane floating on 250 µl of previously 37°C equilibrated DMEM/Ham’s F12 medium containing 1 mg/ml BSA, 1 mg/ml Albumax, 50 µg/ml ascorbic acid, 5 U/ml penicillin and 27.5 µg/ml transferrin per well in a 48-well plate. A drop of medium was placed on top of each ovary to prevent dehydration and maintained at 37°C and 5% CO$_2$.

Effect of single exposure PM on growing follicles: Ovaries (n = 3/treatment) were cultured for four days to allow large primary and secondary follicles to develop in culture before
being treated once with vehicle control media (1% DMSO), PM (10 µM or 30 µM) and maintained in culture for an additional eight days. These concentrations were based on those previously described (Petrillo et al., 2011).

Ovotoxicity time course:
Ovaries (n = 3-4/treatment) were treated on alternate days with vehicle control DMSO (1%), PM (60 µM) or VC for 2, 4 or 6 days. These PM concentrations were chosen to achieve a phenotypic endpoint of approximately 50% primordial follicle loss and to achieve sufficient VC liberation for ovotoxicity evaluation. All PM-treated ovaries were maintained in a separate incubator from the control-treated ovaries (CT). VC-exposed ovaries were cultured in control media in the same incubator as the PM treated ovaries, but on separate plates from the PM samples, thus the ovaries were exposed to the volatile metabolite liberated from PM-treated wells.

Evaluation of ovarian-required VC generation:
Ovaries (n = 4) were cultured for six days in control media adjacent to wells that were treated on alternate days with PM (60 µM) but did not contain an ovary (Fig. 2).

Evaluation of PM degradation during storage: Cultured ovaries (n = 4) were treated on alternate days with freshly suspended PM (“New”) or with previously suspended PM, which had been stored for two years in DMSO at -20°C (“Old”). Ovaries were cultured for six days in separate incubators to reduce VC-induced ovotoxicity as a confounding factor.
Histological evaluation of follicle numbers:
Following treatment, ovaries were placed in 4% paraformaldehyde fixative for 2 hours, washed and stored in 70% ethanol, paraffin embedded, and serially sectioned (5 µM) at the histology laboratory in the Department of Veterinary Pathology (Iowa State University). Every 6th section was mounted and stained with hematoxylin and eosin. Healthy oocyte-containing follicles were identified and counted in every 6th section. Unhealthy follicles were identified from healthy follicles by the appearance of pyknotic bodies and intense eosinophilic staining of oocytes. Healthy follicles were classified and enumerated according to Flaws et al., 1994. Slides were blinded to prevent counting bias.

Statistical analysis:
Comparisons were made between treatments for follicle count experiments using Analysis of Variance (ANOVA) and t-tests compared treatment with control data at each individual time-point. All statistical analysis was performed using Prism 5.04 software (GraphPad Software). Statistical significance was defined as $P < 0.05$.

Results
Effect of single PM exposure on growing ovarian follicles.
To gain an understanding of the impact of PM on growing ovarian follicles, PND4 rat ovaries were cultured for four days in control media in order for larger follicles to develop prior to exposure. Ovaries were treated with vehicle control, 10 µM PM or 30 µM PM and maintained in culture for eight additional days. Ovaries were histologically evaluated, follicles classified and enumerated. Neither concentration of PM induced loss of primordial (Fig. 3A), small primary
(Fig. 3B) or large primary (Fig. 3C) follicles. However, PM exposure caused secondary follicle loss ($P < 0.05$) at both concentrations (Fig. 3D). This experiment demonstrates that even single acute exposure of PM can deplete ovarian follicles.

**Temporal pattern of PM- and VC-induced follicle loss.**

To determine the temporal pattern of PM-induced ovotoxicity, as well as investigate the liberation of VC and evaluate the ovotoxicity of VC relative to PM, PND4 rat ovaries were cultured in medium containing vehicle control (Fig. 4A), PM (60 µM; Fig. 4B) or VC (Fig 4C) for 2, 4, or 6 days. The plate containing control ovaries was removed to a separate incubator. The VC-exposed ovary was placed onto a membrane floating on control medium, maintained on a separate culture plate but in the same incubator as the culture plate containing an ovary floating on PM-treated media. Media was replaced on alternate days and culture was maintained for 2, 4 or 6 days. Following culture, ovaries were sectioned, stained with hematoxylin and eosin and healthy follicles were classified and counted.

After two days of exposure, there was no impact of PM on follicle number (Fig. 4D-G). However, following four days of culture, PM induced primordial (Fig. 4D), small primary (Fig. 4E) and large primary follicle (Fig. 4F) loss ($P < 0.05$). After six days of PM exposure, primordial (Fig. 4D), small primary (Fig. 4E) and large primary (Fig. 4F) follicles were depleted ($P < 0.05$).

Primordial (Fig. 4D) follicle numbers were reduced by VC exposure ($P < 0.05$) after four days, however with the exception of loss ($P < 0.05$) of secondary follicles after two days of exposure (Fig. 4G), VC did not impact the number of small primary, large primary, or secondary
follicles, indicating that while PM can deplete all stage follicles, that the primary target of VC is likely the primordial follicle.

Compared to control, ovaries exposed to either PM or VC had a distinct empty ring between the oocyte and the granulosa cells, which occurred most obviously in the larger follicles, but also occurred in the small follicle types. It appears the oocyte has shrunk away from the granulosa cells resulting in an observable “gap” induced by PM (Fig. 4B) and VC (Fig. 4C) exposure.

**Biotransformation requirement for CEZ liberation from PM**

To determine whether VC generation requires ovarian biotransformation, PND4 rat ovaries were cultured for six days in control media in Incubator A (Fig. 2). In Incubator B ovaries were plated adjacent to wells lacking an ovary. The wells that did not contain an ovary were treated on alternate days with PM (60 µM) to allow for potential spontaneous, ovarian-independent generation of VC. The neighboring, untreated ovaries were evaluated for ovotoxicity via follicle classification and counting. Relative to control, primordial (Fig. 5A) and small primary follicles (Fig. 5B) were depleted ($P < 0.05$) as a result of ovarian-independent generation of VC. Also, there were very few large primary and complete absence of secondary follicles in the VC exposed ovaries, though variation in the CT-treated ovaries negated statistical significance. Thus, there is no requirement for ovarian enzyme-mediated biotransformation of PM to VC.

**Impact of storage condition on PM degradation**

We observed an apparent loss of potency in terms of ovotoxicity of PM aliquots stored for approximately two years in DMSO. To confirm this observation and evaluate PM degradation
under -20 °C storage conditions, PND4 ovaries were cultured with either two-year old PM (“Old”; 60 µM) or newly dissolved PM (“New”; 60 µM) on alternate days for six days. Following hematoxylin and eosin staining, follicles were classified and counted. Equal primordial follicle numbers (Fig. 6A) were observed between new and old PM aliquots. However, new PM caused loss ($P < 0.05$) of small primary (Fig. 6B) and large primary (Fig. 6C) follicles, compared to old PM aliquots. Additionally, there were no secondary follicles present in the ovaries exposed to new PM (Fig. 6D). These data indicate the importance of proper storage conditions when evaluating cytotoxicity of PM.

**Discussion**

The primordial follicle pool encompasses the ovarian follicle reserve and the maintenance of these irreplaceable structures is essential for female fertility. If activated to grow and mature out of the primordial follicle pool, follicles will either progress toward ovulation or die by atresia. The balance of these processes is vital to preserving fertility throughout the lifespan of a female. Chemical exposures can disturb this balance leading to increased activation out of the follicle reserve (Keating *et al.*, 2009) as well as increased atresia (Hu *et al.*, 2001a; Hu *et al.*, 2001b), which if continued, can ultimately result in infertility or POF. If an ovotoxicant targets the large pre-ovulatory follicles, once the exposure ceases, the primordial follicles can still be recruited and develop to ovulation, thus only inducing temporary infertility (Keating and Hoyer, 2009). However, if the primordial follicles are depleted, permanent infertility will ensue.

Our initial goal of this study was to investigate the impact of a single PM exposure on the growing follicle pool, which revealed that a single exposure to PM depleted secondary follicles. Interestingly, the control follicle numbers in this experiment were lower than expected compared
to our previous experiments using the same culture system (Keating et al., 2009; Bhattacharya and Keating, 2012). PM is capable of partitioning to generate a volatile, cytotoxic compound, CEZ, which occurs within the same pH range as our ovarian culture system (Colvin et al., 1976; Lu and Chan, 1996; Shulman-Roskes et al., 1998). The potential ovotoxicity of a volatile metabolite of CPA was suggested in a study due to an observed “neighboring well effect” (Flowers et al., 2000) in cultured neonatal rat ovaries (Desmeules and Devine, 2006). Ovotoxicity of this volatile PM metabolite, however, remains uncharacterized. This present study evaluated the generation of a volatile compound from PM, which we strongly believe is CEZ and designate as VC, and determined the ovotoxicity of this compound relative to that of PM using a neonatal rat ovary culture system.

To confirm our hypothesis that VC was being generated in the culture system, PND4 control ovaries were cultured in a separate incubator from those exposed to PM and VC to eliminate contamination from the volatile compound. Thus, in another incubator, PM exposed ovaries were in one culture plate and a second culture plate contained ovaries in control media that were being evaluated for the volatile compound exposure. Histological evaluation demonstrated that VC depletes primordial follicles, while PM is ovotoxic to primary and secondary follicles ultimately leading to depletion of all follicle types. This could potentially be due to increased recruitment from the primordial follicle pool to replace depleted, more developed follicles, as has been shown previously with CPA (Kalich-Philosoph et al., 2013) and another ovotoxic compound, 4-vinylcyclohexene diepoxide (Keating et al., 2009). VC exposure reduced the number of secondary follicles after 2 days of exposure, which could indicate that either the VC depleted the secondary follicles or that their development was hindered. The numbers of secondary follicles at this stage were very low (~2 per ovary in controls) and there
was a lot of variability within the PM-treated ovaries, thus whether this observed VC-induced reduction of secondary follicles is physiologically relevant is unclear. It is important to note that PM exposed ovaries are exposed simultaneously to VC thus complicating compound-specific analysis. Nonetheless, this experiment confirmed that a volatile compound, which literature strongly supports is CEZ, is ovotoxic and liberated from PM in this culture system.

Our next objective of this study was to determine if ovarian tissue is required for the biotransformation of PM to VC. This was achieved by culturing an ovary that did not receive any chemical treatment adjacent to a PM-treated well that lacked an ovary. We hypothesized that if ovarian tissue is indeed required for the volatile compound formation that no ovotoxicity would be observed in the untreated ovary. Follicle depletion was observed in the untreated ovary, further confirming that an ovotoxic and volatile compound, was liberated spontaneously from the well that contained PM. This experiment clearly demonstrates that VC generation is independent of ovarian tissue, which agrees with a prior study that reported that the reaction of PM to CEZ can occur spontaneously (Colvin et al., 1976; Engle et al., 1979; Watson et al., 1985; Shulman-Roskes et al., 1998), thus providing additional evidence that CEZ is likely the volatile compound produced. It is also probable that the ovarian tissue plays a role in detoxification of CEZ, however, this was outside the scope of the current study, but an avenue that we are pursuing.

Following completion of these experiments, PM, which had been suspended in DMSO and stored at -20°C, was used for additional histological studies. We noticed observably less ovotoxicity induced by PM. In order to confirm this suspicion, we determined the impact of storage on PM-induced ovotoxicity. While this may seem just a routine laboratory analysis, we thought it important to highlight for future investigators using PM. Follicle enumeration
revealed that the “old” PM was less ovotoxic compared to the “new” PM, therefore, suggesting that PM degraded while stored at -20°C. Although the short half-life of PM is known, the apparent degradation of frozen PM was unexpected but noteworthy.

Many studies question the toxicity of PM due to its inability to enter cells because of the molecules polarity, and although this study does not directly address this question, the results again suggest that the volatile compound, likely CEZ, could be the primary or sole active metabolite of CPA. Separating the effects of VC from PM is challenging since the ovaries treated with PM are also receiving the highest VC exposure. When VC exposed ovaries are maintained on a separate plate from PM, VC appears to primarily target the primordial follicles, however, when VC ovaries were cultured in wells adjacent to PM, all follicular stages were depleted by VC similarly to those directly treated with PM. In addition, whether the exposure is acute compared to chronic is worthy of consideration since a single exposure of PM depleted only secondary follicles, but not other follicular stages, although the “controls” were likely receiving concurrent exposure to the VC in this experiment. Thus, proximity to VC (i.e. the concentration of VC), stage of follicular development, and length of exposure and storage conditions are important considerations in these studies.

Overall, these studies depict both the complexity and ovotoxicity of PM and VC, which reinforces the need to gain a better understanding of the health impacts and chemical characteristics of these CPA metabolites. This study’s novel characterization of VC ovotoxicity is particularly concerning because, if, like rats (Rauen and Norpoth, 1968), it is being exhaled in the form of CEZ from patients receiving CPA treatment, individuals in close proximity to the patient would then be at risk of CEZ exposure. Previous studies have shown that healthcare workers have trace amounts of chemotherapy metabolites in their urine with one study detecting
a high level of airborne CPA in the outpatient clinics compared to the pre-treatment preparation rooms (Odraska et al., 2011), thus further supporting that CEZ may be expired from the lungs of patients and exposing individuals in the vicinity. Whether the level of CEZ expired would be clinically relevant is as yet unknown, but this study strongly supports that the VC liberated from PM metabolism is an ovotoxicant worthy of further investigation.

Conflict of Interest Statement
The project described was supported by the National Institutes of Environmental Health Sciences [R00ES016818 to AFK and R01ES09246 to PBH]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.
Figure 1. CPA metabolism.
CPA metabolism is initiated by CYP2B and CYP3A to form 4-hydroxycyclophosphamide, which is non-enzymatically transformed to aldophosphamide. Fragmentation of aldophosphamide forms PM and further partitioning of PM forms CEZ. Adapted from Hong and Chan, 2006.
Figure 2. Experimental design to evaluate VC exposure. Control (1% DMSO)-treated PND4 rat ovaries (indicated in yellow) were cultured for 6 days in Incubator A. In a separate incubator, Incubator B, a PM-treated well that lacked an ovary was plated adjacent to a well containing an ovary floating above control media.
Figure 3. Effect of single PM exposure on growing ovarian follicles.
PND4 rat ovaries were cultured for 4 days in control media and exposed to a single 1% DMSO (vehicle control) or PM (10 µM or 30 µM). Following eight additional days of culture, follicles were classified and counted: (A) Primordial Follicles; (B) Small Primary Follicles; (C) Large Primary Follicles; (D) Secondary Follicles. Values (A-D) are mean ± SE total follicles counted/ovary, n=3; * = different from control in each follicle type, $P<0.05$. 
Figure 4. Temporal pattern of PM- and VC-induced ovotoxicity.
Cultured PND4 rat ovaries were exposed to (A) control treatment (1% DMSO; CT), (B) PM (60 µM) or (C) VC on alternate days for 2, 4 or 6 days (Example shown on day 6). Follicles were classified and counted: (D) Primordial Follicles; (E) Small Primary Follicles; (F) Large Primary Follicles; (G) Secondary Follicles. Values (D-G) are mean ± SE total follicles counted/ovary, n=3-4; * = different from control, $P < 0.05$; † = different from control, $P < 0.10$; # = different from PM, $P < 0.05$. 
Figure 5. Ovarian-mediated biotransformation requirement for VC liberation from PM. PND4 rat ovaries were cultured for 6 days in control media adjacent to wells that lacked an ovary but contained PM (60 µM). Control-treated ovaries (CT) were cultured in a separate incubator. Follicles were classified and counted: (A) Primordial Follicles; (B) Small Primary Follicles; (C) Large Primary Follicles; (D) Secondary Follicles. Values (A-D) are mean ± SE total follicles counted/ovary, n=4; * = different from CT, $P < 0.05$. 

Figure 5 (continued):
Figure 6. Impact of storage on PM degradation.
Cultured PND4 rat ovaries were exposed on alternate days to stored (“Old”) PM or freshly resuspended (“New”) PM for 6 days. Follicles were classified and counted: (A) Primordial Follicles; (B) Small Primary Follicles; (C) Large Primary Follicles; (D) Secondary Follicles. Values (A-D) are mean ± SE total follicles counted/ovary, n=4; * = different from control in each follicle type, $P < 0.05$. 
References


Chapter 3. INVESTIGATING THE INVOLVEMENT OF EPHX1 AND GLUTATHIONE ON PHOSPHORAMIDE MUSTARD-INDUCED OVOTOXICITY, BIOTRANSFORMATION AND LIBERATION OF ITS VOLATILE METABOLITE

A paper to be submitted for publication to *Toxicological Sciences*

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Contribution Statement:
I performed all ovarian tissue cultures and analysis, data interpretation, aided in experimental design and wrote the manuscript; Keating, A.F. aided in the design of experiments, assisted in data interpretation and figure construction, edited the manuscript and served as corresponding author.

Abstract

The anti-neoplastic prodrug, cyclophosphamide, requires biotransformation to phosphoramide mustard (PM), which can partition to the volatile chloroethylaziridine (CEZ). PM and CEZ are ovotoxic, but their ovarian biotransformation remains unclear. Thus, this study investigated mechanisms driving PM and CEZ metabolism through utilization of cultured postnatal day 4 (PND4) Fisher 344 (F344) rat whole ovaries exposed to vehicle control (1% DMSO) or PM (60 µM) on alternate days for 2 or 4 days. Quantification of mRNA levels via an RT² profiler PCR array and target specific RT-PCR found increased mRNA levels of key
xenobiotic metabolism genes including *microsomal epoxide hydrolase* (*Ephx1*) and *glutathione S-transferase isoform pi* (*Gstp*). Compared to control, Western blot analysis determined increased protein abundance of GSTP and EPHX1 after 2 or 4 d, respectively, of PM exposure. PND4 ovaries were treated with (1% DMSO), PM (60 µM), cyclohexene oxide to inhibit EPHX1 (CHO; 2 mM) or PM + CHO for 4 d. PM-induced follicle loss was greater in ovaries exposed to PM with CHO, suggesting a detoxification role for EPHX1 in PM metabolism. PND4 ovaries were also treated with (1% DMSO), PM (60 µM), BSO (Glutathione (GSH) depletion; 100 µM), GEE (GSH supplementation; 2.5 mM), PM ± BSO or PM ± GEE for 4 d. Results suggest that addition of GSH prevented PM-induced follicle loss, while GSH depletion had no effect on PM ovotoxicity. Lastly, the effect of GSH levels on CEZ liberation and ovotoxicity was evaluated. Both untreated and GEE-treated PND4 ovaries were plated adjacent to ovaries receiving PM + GEE or PM + BSO treatments. Less CEZ-induced ovotoxicity was observed with both GEE and BSO treatments indicating reduced CEZ liberation from PM. Collectively, this study supports that the ovary is capable of xenobiotic metabolism, which can influence the ovotoxicity of CPA-derived metabolites.

**Introduction**

As cancer survival rates increase, concern is growing for the survivor’s quality of life post-treatment. In the U.S. alone, there are an estimated 7.2 million female cancer survivors and 360,000 of these women are under the age of 40 (Siegel *et al.*, 2012). In addition, the National Cancer Institute estimates that by 2015, 1 in every 250 adults will be survivors of childhood cancer. For female cancer survivors, a major concern that can affect the quality of life post-
treatment is the increased risk for premature ovarian failure (POF), which similar to menopause, renders the female infertile.

The anti-neoplastic drug, cyclophosphamide (CPA), when included in the chemotherapy regimen of breast cancer patients, led to a significant increase in the number of women experiencing POF (Bines et al., 1996). POF is a result of the depletion of the finite ovarian follicle pool (Hirshfield, 1991). Follicles are the structures encompassing the oocyte, or “egg”, with granulosa cells (small preantral follicles) and theca cells (antral follicles) surrounding the gamete. Primordial follicles are the most premature follicle type and due to their inability to be replenished, it is critical that primordial follicles remain viable to ensure fertility of the female. CPA induces POF via the targeting of these primordial follicles for atresia (follicle death) (Plowchalk and Mattison, 1991).

Similar to women, studies in rodents have shown that CPA exposure targets the primordial follicles in mice (Plowchalk and Mattison, 1991; Desmeules and Devine, 2006) and antral (the developmental stage prior to ovulation) follicles in rats (Jarrell et al., 1991) at concentrations relevant to human exposures (Struck et al., 1987; Desmeules and Devine, 2006). The results from these studies coincide with the human side effects, such as amenorrhea, premature menopause and infertility, reported by women and young girls who have undergone CPA treatment (Sanders et al., 1988; Suarez-Almazor et al., 2000). Each of these side effects is a direct result of follicle loss which alters steroid production and regulation of hormones required to maintain normal menses and fertility.

Although CPA exposure results in these detrimental effects, when administered, CPA is not in its active form. CPA is a prodrug that must be bioactivated by the patient’s hepatic metabolism in order for the chemotherapeutic effects to occur. Cytochrome P450 enzymes
(CYP), primarily in the liver, initiate CPA biotransformation, which then continues non-
enzymatically to form the active, antineoplastic metabolite phosphoramide mustard (PM) (Shulman-Roskes et al., 1998; Ludeman, 1999). In addition to targeting cancer cells, PM is also the ovotoxic metabolite of CPA, both in vivo (Plowchalk and Mattison, 1991) and in vitro (Desmeules and Devine, 2006). Interestingly, PM is capable of partitioning further to form the volatile metabolite, CEZ, (Rauen and Norpoth, 1968; Shulman-Roskes et al., 1998) that is also ovotoxic (Desmeules and Devine, 2006; Madden et al., 2014).

Although the bioactivation of CPA is well established, information on the mechanism of detoxification of this drug is lacking. The evidence available suggests that CPA and metabolites are detoxified by NADPH oxidation and glutathione (GSH) conjugation (Pinto et al., 2009), with variation in mechanisms occurring among follicle types (Desmeules and Devine, 2006). GSH conjugation to PM can occur spontaneously or non-spontaneously (Yuan et al., 1991; Dirven et al., 1994). Non-spontaneous GSH conjugation to PM likely occurs via the glutathione S-transferase (GST) family of enzymes, which are the predominate enzymes involved in GSH conjugation and detoxification of xenobiotics. Specifically, GSH, in excess, has been shown to decrease the stability of CEZ (Shulman-Roskes et al., 1998) and, furthermore, GSH has been shown to reduce the generation of CEZ from PM by promoting an alkylation reaction, not the CEZ-forming hydrolysis reaction (Shulman-Roskes et al., 1998).

The conjugation of GSH to PM has been reported to replace one or both of the chlorides of PM (Dirven et al., 1994); however, it is unclear whether this reaction leads to a less toxic, detoxified PM metabolite. Thus, this study investigated the role of GSH in PM-induced ovotoxicity through the manipulation of the GSH levels present during PM exposure via glutathione ethyl ester (GEE), a cell-permeable GSH derivative able to supplement the amount of
GSH present, and DL-buthionine-(S,R)-sulfoximine (BSO), an inhibitor of the synthesis of GSH, to deplete the GSH concentrations in the ovary.

In addition to GSH conjugation, which is a phase II detoxification reaction, PM likely also undergoes phase I biotransformation, such as hydroxylation. This has been demonstrated to be a key reaction in the biotransformation of other ovotoxicants. 7,12-dimethylbenz[a]anthracene (DMBA), for example, is bioactivated to its ovotoxic form by hydroxylation via the action of the enzyme microsomal epoxide hydrolase (EPHX1) (Rajapaksa et al., 2007; Igawa et al., 2009). However, EPHX1 activity results in the formation of an inactive tetrol form of 4-vinylcyclohexene diepoxide (VCD), thereby providing a detoxification role for VCD, in contrast to its role in DMBA bioactivation (Flaws et al., 1994; Cannady et al., 2002). In congruence with the DMBA and VCD examples, we hypothesize that EPHX1 is active in the ovarian biotransformation of PM and in order to gain insight as to which role, bioactivation or detoxification, EPHX1 performs in the presence of PM, we used the competitive inhibitor of EPHX1 cyclohexene oxide (CHO).

Briefly, to test the hypotheses presented, we utilized a neonatal rat ovarian culture system to characterize the biotransformation of PM in the absence of hepatic contribution, using qRT-PCR and Western blotting to evaluate enzymes that are involved in PM metabolism. In addition, manipulation of ovarian GSH and EPHX1 levels were achieved using chemical approaches in order to gain insight into the role of these classic metabolism players and the impact they could have with respect to the reproductive outcome.
Materials and Methods

Reagents:

Bovine serum albumin (BSA), ascorbic acid, transferrin, 2-β-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, ammonium persulphate, glycerol, N’ N’ N’ N’-tetraethylethylenediamine (TEMED), Tris base, Tris HCL, sodium chloride, Tween-20, phosphatase inhibitor, protease inhibitor, BSO, CHO, and GEE were purchased from Sigma Aldrich Inc. (St. Louis, MO). Dulbecco’s Modified Eagle Medium: nutrient mixture F-12 (Ham) 1x (DMEM/Ham’s F12), Albumax, penicillin (5000U/ml) Hank’s Balanced Salt Solution (without CaCl₂, Mg Cl₂, or MgSO₄) were obtained from Invitrogen Co. (Grand Island, NY). Millicell-CM filter inserts, anti-GSTP and anti-GSTM were obtained from Millipore (Billerica, MA). RNeasy Mini kit, QIA Shredder kit, RNeasy Mini Elute kit, Quantitect ™ SYBR Green PCR kit RT² First Strand kit, RT² SYBR Green Mastermix, and the Drug Metabolism RT² Profiler PCR arrays were purchased from Qiagen Inc. (Valencia, CA). RNAlater and 48-well cell culture plates were obtained from Ambion Inc. (Grand Island, NY) and Corning Inc. (Corning, NY), respectively. PM was acquired from the National Institutes of Health National Cancer Institute (Bethesda, MA). All primers were obtained from the DNA facility of the Iowa State University office of biotechnology (Ames, IA). Ponceau S was purchased from Fisher Scientific (Waltham, MA). SignalFire™ ECL Reagent and anti-rabbit, HRP-link secondary antibody was purchased from Cell Signaling Technology® (Danvers, MA). Anti- EPHX1 antibody was purchased from Detroit R&D (Detroit, MI) and goat anti-rabbit and donkey anti-goat HRP-labeled secondary antibodies were obtained from Southern Biotech (Birmingham, AL) and Santa Cruz (Dallas, TX), respectively.
Animals:
Fisher 344 (F344) rats were housed one per plastic cage and maintained in a controlled environment (22 ± 2°C; 12h light/12h dark cycles). The animals were provided a standard diet with *ad libitum* access to food and water, and allowed to give birth. The Iowa State University Institutional Animal Care and Use Committee’s approved all experimental procedures.

*In vitro* ovarian cultures:
Ovaries were collected from female postnatal day (PND) 4 F344 rats (Devine *et al.*, 2002) after euthanasia. Both ovaries were removed, trimmed of excess tissue and placed onto a Millicell-CM membrane floating on 250 µl of previously 37°C equilibrated DMEM/Ham’s F12 medium containing 1 mg/ml BSA, 1 mg/ml Albumax, 50 µg/ml ascorbic acid, 5 U/ml penicillin and 27.5 µg/ml transferrin per well in a 48-well plate. A drop of medium was placed on top of each ovary to prevent dehydration. Ovary cultures containing PM treatments were cultured in a separate incubator from other treatments to eliminate contamination from the ovotoxic, PM-generated volatile metabolite (Madden *et al.*, 2014).

**RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR):**
Ovaries were stored in RNA*Later* at -80°C following 2 or 4 d of *in vitro* culture. Ovaries (n=3; 10 ovaries per pool) were homogenized and added to a QIAshredder column before proceeding to total RNA isolation using an RNeasy Mini kit. RNA concentration was performed with RNeasy Mini Elute kit and total RNA isolated was quantified with a NanoDrop (λ = 260/280 nm; ND 1000; Nanodrop Technologies Inc., Wilmington, DE).
**RT² PCR array**

According to the manufacturer’s protocol, total RNA (250 ng) was reverse transcribed to cDNA using the RT² first-strand kit and pipetted into Drug Metabolism RT² Profiler PCR array, which contains 96-wells, each containing a gene-specific primer set, therefore one plate tested 96 genes per each sample. The PCR protocol utilized was a 10 min hold at 95ºC and 40 cycles of denaturing at 95ºC for 15 s and a combined annealing and extension for 1 minute at 60ºC. Each gene was normalized to both housekeeping genes **hoxanthine phosphoribosyltransferase (Hprt)** and **ribosomal protein, large, P1 (Rplp1)**, as recommended by the company-provided analysis software. There was no effect of PM on mRNA levels of either of these two housekeeping genes. The online SABiosciences RT² Profiler™ PCR Array Data Analysis software quantified the changes in mRNA levels using the $2^{-\Delta\Delta Ct}$ method.

**Individual gene target RT-PCR**

Total RNA (250 ng) was reverse transcription to cDNA with the Superscript III One-Step RT-PCR System (Invitrogen). Using an Eppendorf mastercycler (Hauppauge, NY) and Quantitect™ SYBR Green PCR kit (Qiagen Inc. Valencia, CA), PCR amplification of the following genes was performed: Ephx1 (forward: 5’-GGCTCAAGCCATCAGGCA-3’; reverse: 5’-CCTCCAGAGGACACCACTTT-3’) Gsp (forward: 5’-GGCATCTGAAGCCTTTTGAG-3’; reverse: 5’-GAGCCACATAGGCAGAGAGC-3’) and Gstm (forward: 5’-TTCAAGCTGGCCTGGAC-3’; reverse: 5’-CAGGATGGCATTGCTCTG-3’). The RT-PCR protocol used was a 15 min hold at 95ºC and 40 cycles of denaturing at 95ºC for 15 s, annealing temperature of 58ºC, and extension at 72ºC for 15 s. Gapdh was used as the housekeeping gene for normalization as no change in its mRNA levels was observed between treatments. PM-
induced changes in mRNA levels were quantified using the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001).

**Protein isolation and Western blot analysis:**

PND4 ovaries (n=3; 10 ovaries per pool) were homogenized in 200 µl of ice-cold tissue lysis buffer and protein quantified using a standard BCA protocol on a 96-well assay plate. Total protein (15 µg) was separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Prior to 1 hr blocking in 5% milk, equal protein loading was confirmed by Ponceau S staining. Membranes were then incubated at 4 ºC with their respective, specific primary antibodies [Rabbit Anti-GSTM, Rabbit Anti-GSTP, Goat Anti-EPHX1]. Following 48 - 72 hrs of incubation, donkey anti-rabbit or porcine anti-goat secondary antibody was added and membranes were incubated with shaking for 1h at room temperature. Autoradiograms were visualized on X-ray films in a dark room after 10 min incubation of membranes with 1X SignalFire™ ECL reagent. Densitometry of the appropriate sized bands was measured using Image Studio Lite Version 3.1 (LI-COR Biosciences, Lincoln, NE) which eliminates background noise. Values were normalized to Ponceau S staining.

**Competitive inhibition of EPHX1:**

On alternate days, ovaries (n=4-6) were treated with vehicle control media (1% DMSO), PM (60 µM), CHO (2 mM) or PM (60 µM) + CHO (2 mM) for 2 or 4 d. The concentration of CHO was determined by a previous study (Igawa et al., 2009).
GSH depletion and supplementation:
Ovaries were treated on alternate days with DMSO (1%), PM (60 µM), GEE (2.5 mM), BSO (100 µM), PM (60 µM) ± GEE (2.5 mM), PM (60 µM) ± BSO (100 µM) or left untreated to evaluate the effect of the neighboring well CEZ liberation from PM. BSO and GEE concentrations and the addition of BSO 8 hrs prior to PM treatment were based on conditions described in a previous study (Tsai-Turton and Luderer, 2006). As previously reported, CEZ exposed ovaries were cultured in control media in the same incubator as PM-containing treatment, thus the ovaries were exposed to the volatile metabolite, presumably CEZ, liberated from PM (Madden et al., 2014).

Histological evaluation of follicle numbers:
Following treatment, ovaries were placed in 4% paraformaldehyde for 2 hours, washed and stored in 70% ethanol, paraffin embedded, and serially sectioned (5 µM). Every 6th section was mounted and stained with hematoxylin and eosin. Healthy oocyte-containing follicles were identified and counted in every 6th section. Unhealthy follicles were distinguished from healthy follicles by the appearance of pyknotic bodies and intense eosinophilic staining of oocytes. Healthy follicles were classified and enumerated according to Flaws et al., 1994. Slides were blinded to prevent counting bias.

Statistical analysis:
Treatment comparisons for follicle count experiments were performed using Analysis of Variance (ANOVA). Quantitative RT-PCR and Western blot data were analyzed by t-test comparing treatment with control raw data at each individual time point. All statistical analysis
was performed using Prism 5.04 software (GraphPad Software). Statistical significance was defined as \( P < 0.05 \), with a trend for a difference considered at \( P < 0.1 \).

**Results**

**Investigation of xenobiotic biotransformation genes involved in the ovarian response to PM exposure**

To gain insight into the ovarian genes that are activated during PM exposure, a drug metabolism RT\(^2\) Profiler PCR array was performed on PND4 rat ovaries (n=3, 10 ovaries per pool) cultured 2 or 4 d with vehicle control (1% DMSO) or PM (60 \( \mu \)M) exposure on alternate days. After 2 d of PM exposure, of the 89 genes tested, 4 were undetectable, 7 genes had altered (\( P < 0.05 \)) gene expression and 1 gene, \( Chst1 \), was trending towards significance (\( P < 0.10 \)) compared to the control (Table 1). Following 4d of PM exposure, 6 genes were undetected, 24 genes had altered (\( P < 0.05 \)) gene expression and 4 genes had a tendency for altered (\( P < 0.10 \)) mRNA abundance in response to PM, relative to control (Table 1). The results of this PCR array are summarized in Table 1.

**Effect of PM exposure on xenobiotic biotransformation gene expression**

To both validate gene expression changes observed in the RT\(^2\) PCR array data and to determine changes in genes of interest that were not present on the array, primers were designed for candidate ovarian xenobiotic metabolism genes in PM metabolism. PND4 ovaries (n=3, 10 ovaries per pool) were cultured for 2 or 4 d in control media (1% DMSO) or PM (60 \( \mu \)M) to capture the molecular changes occurring prior to PM-induced follicle depletion (Madden *et al.*, 2014). Following 2 d of PM exposure, there was no effect of treatment on \( Gstp \) or \( Gstm \) mRNA
level compared to CT (Fig. 1B and C). However, after 4 d of PM exposure, $Gstp$ was increased ($P < 0.05$; 0.86-fold), relative to control (Fig. 1C). At 2 d, $Ephx1$ mRNA expression showed a tendency toward being increased ($P < 0.1$; 1.17-fold), followed by a large PM-induced increase in $Ephx1$ mRNA expression after 4 d ($P < 0.05$; 4.63-fold) (Fig. 1A).

**PM-induced changes in protein levels of xenobiotic metabolism genes**

In order to confirm the mRNA changes observed after PM exposure, protein levels were quantified in cultured PND4 ovaries (n=3, 10 ovaries per pool) exposed to control media or media containing PM for 2 and 4 d. Compared to control, following 2 d of PM exposure, GSTP protein levels were increased ($P < 0.05$), but there was no effect of PM on GSTP protein level after 4 d of exposure (Fig. 1F). GSTM protein levels also increased ($P < 0.05$) following 2 days of PM exposure, but decreased ($P < 0.05$), relative to control, after 4 d (Fig. 1E). There was a trend for a PM-induced increase ($P < 0.10$) in EPHX1 protein levels after 2 d, followed by a large increase in EPHX1 after 4 days (Fig. 1D).

**Evaluation of the role of EPHX1 in PM metabolism**

EPHX1 was competitively inhibited by CHO in the neonatal culture system to evaluate the role of the enzyme in PM metabolism. PND4 ovaries (n = 4-6) were cultured for 4 d with vehicle control (1% DMSO), PM (60 µM), CHO (2 mM), or PM (60 µM) + CHO (2 mM). PM and PM + CHO treated ovaries were cultured at separate times, in a separate incubator from the control ovaries to prevent control treated ovaries from being exposed to the volatile compound liberated from PM in our culture system (Madden et al., 2014).
There was no effect of CHO treatment on primordial or large primary follicle number (Fig. 2A and C), however, CHO treatment alone lowered small primary and secondary follicles when compared to control (Fig. 2B and D). As previously reported (Madden et al., 2014), PM induced \(P < 0.05\) primordial (Fig. 2A), small primary (Fig. 2B), large primary (Fig. 2C) and secondary follicle loss compared to control (Fig. 2D). When EPHX1 was inhibited by CHO, in conjunction with PM exposure, greater \(P < 0.05\) primordial and small primary follicle loss was observed relative to PM alone (Fig. 2A and B).

**Impact of ovarian GSH level manipulation on PM ovotoxicity**

In order to gain an understanding for the impact of GSH manipulation during PM ovotoxicity, PND4 ovaries \((n=4-6)\) were cultured for 4 d with DMSO (1%), PM (60 µM), GEE (2.5 mM), BSO (100 µM), PM (60 µM) ± GEE (2.5 mM) or PM (60 µM) ± BSO (100 µM). All PM-containing treatments were cultured in a separate incubator from other treatments to eliminate CEZ contamination (Madden et al, 2014).

As previously reported, compared to control, PM depleted all follicle types after 4 d of culture (Madden et al, 2014). The addition of GSH alone, via GEE, depleted \(P < 0.05\) primordial, small primary and large primary follicles (Fig. 3A-C), relative to control, with the number of small primary follicles being reduced to PM levels (Fig 3B). The supplementation of GSH via GEE in the presence of PM, reduced PM-induced follicle loss in all follicle types (Fig 3).

When GSH levels were depleted \(P < 0.05\) via the inhibition of GSH synthesis with BSO, BSO alone reduced \(P < 0.05\) the number of primordial and small primary follicles (Fig. 4A and B), while there was no effect on large primary follicles (Fig. 4C) and a trend \((P < 0.1)\) for
an increase in the number of secondary follicles (Fig. 4D) was observed relative to control. Similar to PM, compared to control, the inclusion of BSO with PM resulted in depletion ($P < 0.05$) of all follicle stages, with no difference in follicle loss observed relative to PM (Fig 4).

**Impact of altered GSH levels on CEZ liberation and ovotoxicity**

To evaluate the effect of altered ovarian GSH level on the generation of CEZ from PM and also to investigate the effect of GSH supplementation on CEZ ovotoxicity, cultured PND4 ovaries ($n = 4-6$) were treated for 4 d with DMSO (1%), PM (60 µM), GEE (2.5 mM), BSO (100 µM). In addition, ovaries were cultured in wells that received no treatment but were placed adjacent to wells containing and ovary that was exposed to PM + BSO or PM + GEE to evaluate the impact of GSH manipulation on CEZ generation from PM. These treatments were named Liberation (Lib.) PM + BSO or Lib. PM + GEE, respectively. Lastly, ovaries were treated with GEE (2.5 mM) or BSO (100 µM) in wells that were placed beside those that received PM + BSO (100 µM) or PM + GEE (2.5 mM) treatments in order to evaluate the effect of altered GSH on CEZ ovotoxicity. These treatments were designated CEZ + GEE and CEZ + BSO, respectively. This scheme is depicted in Figure 5. All ovaries receiving PM-containing treatments were cultured in a separate incubator from other treatments to eliminate CEZ contamination (Madden *et al.*, 2014).

As previously reported, the volatile compound liberated from PM, presumably CEZ, depletes ($P < 0.05$) all follicle stages after 4 d of exposure (Madden *et al.*, 2014). Relative to control, there was no primordial, large primary or secondary follicle loss induced by the GEE + CEZ or Lib PM + GEE treatments (Fig 6A, C and D). Small primary follicle depletion, however, was impacted by the GEE + CEZ treatment and further worsened to PM levels by the
Lib. PM + GEE treatment (Fig 6B). BSO + CEZ and Lib PM + BSO exposures had no effect on follicle number at any stage of development, compared to control (Fig 7).

Discussion

In 2012, an estimated 1.6 million people were diagnosed in the U.S. with cancer and 67% of these individuals will now survive at least five years post-diagnosis, compared to a 49% in 1977 (Siegel et al., 2012). Increasing the cancer survival rate is a great medical feat, yet coincides with an increasing concern for patient quality of life post-cancer treatment. For the patients receiving chemotherapy and/or radiation for their cancer treatment, the risk of reduced fertility and sterility is heightened (Byrne et al., 1992; Sklar et al., 2006). This is especially true for women, because unlike the testes, the ovary is unable to replenish its gamete pool. The ovarian follicle reserve established at birth is responsible for sustaining the fertility of a woman throughout her lifespan, thus if depleted, the woman will undergo POF (Hirshfield, 1991).

One chemotherapy drug demonstrated to increase the risk of POF is CPA (Bines et al., 1996). However, to elicit the anti-neoplastic effects, CPA must be bioactivated to PM, which is ovotoxic (Plowchalk and Mattison, 1991; Desmeules and Devine, 2006) and potentially further to the volatile compound CEZ (Flowers et al., 2000; Madden et al., 2014). This bioactivation of CPA to PM has been well characterized in various tissues. The detoxification of CPA and its metabolites, on the other hand, is lacking clarity. Previous studies have demonstrated that GSH conjugation of PM occurs (Dirven et al., 1994), which is assumed to represent a detoxification reaction, but this has not been confirmed. Therefore, this study used a PND4 rat ovary culture system to investigate the role of GSH in PM metabolism and CEZ liberation, as well as
involvement of EPHX1, which has previously demonstrated to have an important role in the biotransformation of other ovotoxicants.

In the current study, following PM exposure, EPHX1 was found to be increased at both transcriptional and translational levels suggesting its involvement in PM metabolism. Previous reports have found that EPHX1 bioactivates DMBA to its ovotoxic form (Rajapaksa et al., 2007; Igawa et al., 2009), while detoxifying the ovotoxicant VCD (Flaws et al., 1994; Cannady et al., 2002). Thus, in order to evaluate the role of EPHX1 in PM metabolism, a competitive inhibitor of EPHX1, CHO, was used to block the action of EPHX1 during PM-induced follicle depletion. The lack of EPHX1 activity worsened PM-induced follicle loss therefore suggesting that EPHX1 is involved in the detoxification of PM.

As an epoxide hydrolase, EPHX1 action adds a hydrogen to the epoxide group of a chemical, which to our knowledge, is not included in the chemical structure of PM and its metabolites. Interestingly, however, CEZ, the volatile and ovotoxic metabolite of PM (Madden et al., 2014) is an aziridine compound capable of hydroxylation and may be the metabolite being detoxified by EPHX1 (Watabe and Suzuki, 1972), and this is an area for future investigation but outside the scope of the experiments described herein.

In addition to EPHX1, the mRNA encoding several other genes implicated in GSH conjugation catalysis and usage were altered by PM exposure. At a time prior to PM-induced follicle loss (Madden et al., 2014), the protein levels of GSTM and GSTP were increased. Interestingly, we observed a subsequent decrease in GSTM levels after 4d of PM. We have previously demonstrated that GSTM negatively regulates ovarian apoptosis signal-regulating kinase 1 (ASK1) through protein interactions (Bhattacharya et al., 2013), thus a decline in GSTM protein could indicate that this negative regulation is being relieved and the cell being
pushed towards an apoptotic fate as has been observed with ovarian DMBA exposure (Bhattacharya et al., 2013). These data support that the \textit{Gstp} and \textit{Gstm} genes are responsive to and involved in the ovarian response to PM exposure.

By manipulating the levels of GSH present in the ovary, the impact of GSH on PM ovotoxicity was investigated. The supplementation of GSH with GEE during PM exposure restored follicle numbers to the GEE alone levels, with the exception of the large primary follicles where PM + GEE completely restored follicle loss to control levels. Surprisingly, GEE alone caused follicle loss in nearly all the follicle stages investigated indicating that an excess in GSH may be detrimental to follicles. Unexpectedly, in the opposing experiment, when GSH levels were depleted by BSO, which blocks an enzyme essential to the synthesis of GSH, there was no impact on PM-induced follicle loss. These results question the involvement of GSH conjugation in lessening the toxicity of PM, since lack of GSH did not alter PM-induced follicle loss. Our data are in agreement with a study that found that depleting glutathione levels in rat embryo did not have an impact on PM-induced embryotoxicity, but did increase the toxicity of another CPA metabolite, acrolein (Slott and Hales, 1987). Similarly, using rat hepatocytes and K562 human chronic myeloid leukemia cell in culture, another study found that PM did not impact the level of cellular GSH in either cell type, again, in contrast to acrolein, which depleted GSH levels.

Interestingly, the ovulated murine oocyte has an 8-10 mM GSH concentration (Calvin et al., 1986; Perreault et al., 1988), which is amongst the highest of any cell-type (Zuelke et al., 2003), and a prior study found that GSH concentration increased with age in mice in association with an decrease in CPA sensitivity (Mattison et al., 1983), but literature review failed to provide the GSH concentrations in small primary follicle and, of particular interest, for the primordial
pool. Knowing the GSH level in the primordial follicle would aid in interpretation of our results as well as improve our general understanding of primordial follicles and the mechanisms protecting this irreplaceable pool.

Another important aspect of the present study was the evaluation of the effect of ovarian GSH levels on CEZ generation from PM. Previous studies report that excess GSH will reduce the generation and stability of CEZ (Shulman-Roskes et al., 1998), thus we hypothesized that increasing GSH levels during PM exposure would reduce CEZ liberation, subsequently leading to reduced CEZ-induced follicle loss. When PM exposure was supplemented with GSH, our hypothesis was confirmed. However, surprisingly, when the levels of ovarian GSH were depleted during PM exposure, CEZ-induced follicle loss was also prevented. The physiological relevance of this result is unclear. It may be that BSO is not depleting ovarian GSH to a great enough extent to affect CEZ liberation. It could also be that a direct chemical reaction between BSO and PM is occurring to block the release of CEZ, but both scenarios render further investigation.

If, in the case of GSH supplementation and depletion, CEZ liberation is inhibited, this may partly explain the reduced follicle loss observed in the PM + GEE compared to PM treatments. The ovotoxicity of CEZ has been demonstrated and lack of CEZ liberation is suggested by our data. Taken together, continued investigation of CEZ is required to understand the ovotoxic activity of this volatile, PM-generated metabolite is required.

In summary, the results from this study suggest that EPHX1 is involved in the detoxification of PM. Interestingly, excess GSH by itself impaired follicle viability, but GSH supplementation did reduce PM-induced follicle depletion. Depletion of GSH did not affect PM-induced ovotoxicity, thus the role of GSH in PM metabolism and ovotoxicity remains unclear.
The prevention of PM-induced follicle loss, potentially by the overexpression of EPHX1, could have profound implications for fertility preservation following cancer treatment with CPA, leading to an improved quality of life for millions of female cancer survivors.
## Figures

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<th>Gene Name</th>
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Table 1. Effect of PM exposure on ovarian expression of drug metabolism genes.
PND4 rat ovaries (n=3; 10 ovaries per pool) were treated with 1% DMSO (vehicle control) or PM (60 μM) for 2 or 4d. Following RNA isolation, mRNA levels were quantified with an RT² Profiler PCR array. Values represent fold-change ± SEM relative to a control value of 1, normalized to Hprt1 and Rplp1. * = different from control, P < 0.05; † = P < 0.10.

<table>
<thead>
<tr>
<th>Enzyme Name</th>
<th>Symbol</th>
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<th>P-value</th>
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<td>Rplp1</td>
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Figure 1. Effect of PM on ovarian abundance of Ephx1, Gstm and Gstp mRNA and protein. PND4 rat ovaries (n=5; 10 ovaries per pool) were treated with 1% DMSO (vehicle control, CT) or PM (60 µM). Following 2 or 4 d of culture, mRNA and protein were isolated and (A and D) Ephx1, (B and E) Gstm, and (C and F) Gstp, levels evaluated by quantitative RT-PCR or Western blot, respectively. mRNA values represent fold-change ± SEM relative to a control value of 1, normalized to Gapdh. Protein values represent signal intensity ± SEM normalized to Ponceau S. * = different from control, P < 0.05; # = P < 0.1.
Figure 2. Impact of EPHX inhibition during PM exposure on follicle number.
PND4 rat ovaries were cultured and treated on alternate days to 1% DMSO (vehicle control, CT), PM (60 µM), CHO (2 mM) or PM (60 µM) + CHO (2 mM). Following 4 d of culture, follicles were classified and counted: (A) Primordial Follicles; (B) Small Primary Follicles; (C) Large Primary Follicles; (D) Secondary Follicles. Values represent mean ± SE total follicles counted/ovary, n = 4-6. Different letters represent significant difference between treatments $P < 0.05$. 
Figure 3. Impact of GSH supplementation during PM exposure on follicle number. PND4 rat ovaries were cultured and treated on alternate days to 1% DMSO (vehicle control, CT), PM (60 µM), GEE (2.5 mM) or PM (60 µM) + GEE (2.5 mM). Following 4 d of culture, follicles were classified and counted: (A) Primordial Follicles; (B) Small Primary Follicles; (C) Large Primary Follicles; (D) Secondary Follicles. Values represent mean ± SE total follicles counted/ovary, n = 4-6. Different letters represent significant difference between treatments $P < 0.05$. * = $P < 0.10$ difference from CT.
Figure 4. Impact of GSH depletion during PM exposure on follicle number.
PND4 rat ovaries were cultured and treated on alternate days to 1% DMSO (vehicle control, CT), PM (60 µM), BSO (100 µM) or PM (60 µM) + BSO (100 µM). Following 4 d of culture, follicles were classified and counted: (A) Primordial Follicles; (B) Small Primary Follicles; (C) Large Primary Follicles; (D) Secondary Follicles. Values represent mean ± SE total follicles counted/ovary, n = 4-6. Different letters represent significant difference between treatments P < 0.05. * = P < 0.10 difference from CT.
Figure 5. Plate layout for evaluation of the impact of ovarian GSH levels on CEZ liberation.

Each well contained one PND4 rat ovary that was treated accordingly for 4 d to evaluate the following effects: (A) Untreated – ovaries cultured in control media were exposed to the CEZ liberated from the adjacent PM + GEE, thus named Liberated (Lib.) PM + GEE; (B) PM + GEE – ovaries treated with PM and GEE to evaluate the effect of GEE (GSH supplementation) on PM-induced ovotoxicity; and (C) GEE – ovaries were treated with GEE and exposed to the CEZ liberated from the adjacent PM + GEE, thus named CEZ + GEE. Control treated ovaries were maintained in a separate incubator.
Figure 6. Impact of GSH supplementation on CEZ liberation and ovotoxicity.

PND4 rat ovaries were cultured and treated as described in Figure 5. Following 4 d of culture, follicles were classified and counted: (A) Primordial Follicles; (B) Small Primary Follicles; (C) Large Primary Follicles; (D) Secondary Follicles. Values represent mean ± SE total follicles counted/ovary, n = 4-6. Different letter represent significant difference between treatments *P < 0.05. * = *P < 0.10 difference from CT.
Figure 7. Impact of GSH depletion on CEZ liberation and ovotoxicity.
PND4 rat ovaries were cultured and treated as described in Figure 5. Following 4 d of culture, follicles were classified and counted: (A) Primordial Follicles; (B) Small Primary Follicles; (C) Large Primary Follicles; (D) Secondary Follicles. Values represent mean ± SE total follicles counted/ovary, n = 4-6. Different letter represent significant difference between treatments P < 0.05. * = P < 0.10 difference from CT (a*) or PM (b*).
References


Chapter 4. INVOLVEMENT OF AUTO PHAGY DURING PHOSPHOR AMIDE MUSTARD-INDUC ED OVOTOXICITY

A paper to be submitted for publication to Biology of Reproduction

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Contribution Statement:
I performed the ovarian tissue cultures and molecular experiments, data analysis and interpretation, assisted in experimental design and wrote the manuscript; Keating, A.F. assisted in the design of experiments, in data interpretation and figure construction, edited the manuscript and served as corresponding author.

Abstract
Phosphoramide mustard (PM) is an ovotoxic metabolite of the chemotherapeutic agent cyclophosphamide, and although PM-induced follicle depletion is observed, the mechanism driving this cell loss is unknown. To gain insight into PM-induced ovotoxicity, a postnatal day 4 (PND4) Fisher 344 rat whole ovary culture system was used. Treatment of these cultured ovaries occurred on alternate days with vehicle control (1% DMSO) or PM (60 µM) for 2 or 4 d. Upon ovary collection, mRNA or protein were isolated and quantified by RT² profiler PCR array, target-specific RT-PCR and/or Western blot. Analysis determined altered mRNA levels of
genes involved in autophagy such as *Beclin 1 (Becn1)* following PM exposure. Western blot analysis determined BECN1 protein levels to also be increased after 2 d of PM exposure suggesting the induction of autophagosome formation. To confirm autophagy formation histologically, transmission election microscopy (TEM) was performed over a time course of PM exposure, and at time points prior to PM-induced follicle loss, both the presence of autophagosomes and the degradation of mitochondria were observed. To investigate a functional role for PM-induced autophagy, ovaries were treated with PM under with either autophagy inhibition, via LY294002 (LY); or activation, via rapamycin. Thus, ovaries were cultured with vehicle control (1% DMSO), PM (60 µM), LY (20 µM), rapamycin (1 µM), PM + LY or PM + rapamycin for 4 d. LY had no effect on PM-induced ovotoxicity, while rapamycin treatment prevented PM-induced follicle loss. These data identify that autophagy is involved in PM-induced follicle loss, and that the mammalian target of rapamycin, mTOR, may be a gatekeeper during regulation of PM-induced ovotoxicity.

**Introduction**

The female ovarian reserve is established at birth and represents a finite number of follicles, which are comprised of the oocyte surrounded at initial stages of development by granulosa cells. Throughout the lifespan of a woman, follicles gradually progress through development beginning at the primordial follicle stage and continuing through the primary and secondary follicle stages to eventually become surrounded by theca cells, form an antrum and ultimately be ovulated. However, less than 1% percent of follicles will complete the cycle to be ovulated. Any follicles that are not ovulated will die, thus resulting in over 99% of mammalian
follicles dying by the process of atresia (Hirshfield, 1991). Once the ovarian reserve is depleted of follicles, ovarian failure, or menopause, occurs. On average, the natural phenomenon of menopause occurs at the age of 51 (Jick and Porter, 1977), however, chemical exposures can deplete follicles leading to premature ovarian failure (POF).

Up until the last decade, apoptosis was accepted as the type of programmed cell death (PCD) occurring in ovarian follicles undergoing atresia (follicular death). However, previous studies now suggest that autophagy may be an important process in follicles depletion, working independently or in tandem with apoptosis (Escobar et al., 2008; Rodrigues et al., 2009; Gannon et al., 2012). Autophagy genes Beclin 1 (BECN1), autophagy-related 7 homolog (S. cerevisiae) (ATG7) and lysosome associated membrane protein (LAMP1) are involved in key steps of autophagosome and autolysosome assembly (Kim et al., 1999; Liang et al., 1999; Eskelinen, 2006). An autophagosome is the double-membraned structure that forms around the material targeted for destruction, transports that unwanted material, and merges with the lysosome for digestion forming the autolysosome (de Duve, 2005; Mizushima, 2007).

A study evaluating PCD at birth, a time of significant follicle loss, found in mice that at the time of parturition, autophagy is the active PCD pathway (Rodrigues et al., 2009). Here, autophagy is thought to be the result of the nutrient deprivation experienced at birth due to the removal of the fetus from the placenta, a phenomenon that has also been observed in other tissues (Kuma et al., 2004; Rodrigues et al., 2009). Nutrient deprivation is sensed by the key response element mammalian target of rapamycin (mTOR) leading to the induction of autophagy via initiation of the autophagosome-forming cascade (Schmelzle and Hall, 2000). mTOR lies downstream of the phosphatidylinositol-3 kinase (PI3K) signaling pathway and the interaction of
these two pathways mediates cell survival and viability as well as negatively regulating autophagy (Jung et al., 2010).

In addition to the natural occurrence of autophagy in the ovary, chemical exposures, such as cigarette smoke, have been shown to induce autophagy-mediated follicular death in mice (Gannon et al., 2012; Gannon et al., 2013). Compared to unexposed controls, cigarette smoke-exposed mice had a reduced number of follicles, but did not have a higher level of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), DNA laddering or caspase activity - all of which are classic signs of apoptosis (Tuttle et al., 2009). However, cigarette smoke-exposed mice did have a higher number of autophagosomes in granulosa cells, increased BECN1 and LC3 protein levels and mitochondrial dysfunction (Gannon et al., 2013). Also, through the use of a neonatal rat ovary culture method, single low exposures to 7,12-dimethylbenz[a]anthracene (DMBA), a component of cigarette smoke, reduced follicle number and altered BECN1 mRNA and protein levels (Madden et al., 2014a). Collectively, these studies suggest autophagy is an active process during ovarian response to xenobiotics.

The chemotherapy drug cyclophosphamide (CPA) exposure targets and depletes primordial follicles in mice (Plowchalk and Mattison, 1991; Desmeules and Devine, 2006) and antral (the developmental stage prior to ovulation) follicles in rats (Jarrell et al., 1991; Hoyer and Devine, 2002) at concentrations relevant to human exposures (Struck et al., 1987; Desmeules and Devine, 2006). The results from these studies coincide with the human side effects, such as amenorrhea, premature menopause and infertility, reported by women and young girls who have undergone CPA treatment (Sanders et al., 1988; Suarez-Almazor et al., 2000).

CPA is a prodrug and must be metabolized in order to have anti-cancer effects. Hepatic metabolism by cytochrome P450 (CYP) enzymes initiates the biotransformation of CPA, which
ultimately forms the active, anti-cancer and ovotoxic metabolite phosphoramide mustard (PM). Notably, PM can further biotransform to a volatile and ovotoxic metabolite, presumably chloroethylaziridine (CEZ), which also likely contributes to PM overall toxicity (Madden et al., 2014b). Previous studies suggest that oocytes are the main PM target, while in larger follicles, the granulosa cells are the targeted cell type (Desmeules and Devine, 2006). Using a neonatal rat ovary culture method, this study also found that following PM exposure, the granulosa cells of primary follicles stained TUNEL positive following PM exposure (Desmeules and Devine, 2006). Interesting, caspase-3 staining in PM-exposed ovaries was not different from control and no primordial follicles stained positive for this apoptotic indicator (Desmeules and Devine, 2006). Additionally, a study investigating the effects of varying doses of CPA in vivo found no TUNEL or caspase-3 positive primordial follicle, despite finding a loss of primordial follicles induced by CPA (Kalich-Philosoph et al., 2013), which again, encourages the possibility that another PCD form is occurring, especially in the primordial follicles, in response to PM exposure.

Therefore, in this study we hypothesized that autophagy is an active ovarian response following PM exposure and likely contributes to PM-induced follicle loss. We evaluated mRNA and/or protein abundance of the autophagy related genes (Becn1, Atg7, LC3-II and Lamp) along with the presence of autophagosomes via transmission electron microscopy (TEM). To further evaluate a functional role for autophagy in follicle death and survival, the mTOR targeted autophagy activator, rapamycin, and the PI3K targeted inhibition of autophagy, LY294002, were utilized in combination with PM to evaluate the effect on PM-induced follicle loss.
Materials and Methods

Reagents:

Bovine serum albumin (BSA), ascorbic acid, transferrin, 2-β-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, ammonium persulphate, glycerol, N’ N’ N’ N’-tetramethylethylenediamine (TEMED), Tris base, Tris HCL, sodium chloride, Tween-20, phosphatase inhibitor, protease inhibitor and rapamycin were purchased from Sigma Aldrich Inc. (St. Louis, MO). Dulbecco’s Modified Eagle Medium: nutrient mixture F-12 (Ham) 1x (DMEM/Ham’s F12), Albumax, penicillin (5000U/ml) Hank’s Balanced Salt Solution (without CaCl₂, MgCl₂, or MgSO₄) were obtained from Invitrogen Co. (Grand Island, NY). Millicell-CM filter inserts and 48-well cell culture plates were obtained from Millipore (Billerica, MA) and Corning Inc. (Corning, NY), respectively. RNeasy Mini kit, QIA Shredder kit, RNeasy Mini Elute kit, Quantitect™ SYBR Green PCR kit, RT² First Strand kit, RT² SYBR Green Mastermix, and the Stress and Toxicity RT² Profiler PCR arrays were purchased from Qiagen Inc. (Valencia, CA). RNAlater was obtained from Ambion Inc. (Grand Island, NY). 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) was purchased from A.G. Scientific, Inc. (San Diego, CA). PM was obtained from the National Institutes of Health National Cancer Institute (Bethesda, MA). All primers were obtained from the DNA facility of the Iowa State University office of biotechnology (Ames, IA). Ponceau S was purchased from Fisher Scientific (Waltham, MA). SignalFire™ ECL Reagent, anti-LAMP1, and the HRP-linked secondary anti-rabbit antibody was purchased from Cell Signaling Technology® (Danvers, MA). Anti-Becn1 antibody was purchased from Santa Cruz (Dallas, TX) and donkey anti-rabbit FITC labeled secondary antibody was obtained from Southern Biotech (Birmingham, AL).
Animals:
The Iowa State University Institutional Animal Care and Use Committee’s approved all experimental procedures. Fisher 344 (F344) rats were housed one per plastic cage and maintained in a controlled environment (22 ± 2°C; 12h light/12h dark cycles). The animals were provided a standard diet with *ad libitum* access to food and water, and allowed to give birth.

*In vitro ovarian cultures:*
Following euthanasia, ovaries were removed from female postnatal day (PND) 4 F344 rats (Devine *et al.*, 2002). Each ovary was removed, trimmed of oviduct and excess tissue, and placed onto a Millicell-CM membrane floating on 250 µl of previously 37°C equilibrated DMEM/Ham’s F12 medium containing 1 mg/ml BSA, 1 mg/ml Albumax, 50 µg/ml ascorbic acid, 5 U/ml penicillin and 27.5 µg/ml transferrin per well in a 48-well plate. To prevent dehydration, a drop of medium was placed on top of each ovary. In order to eliminate CEZ as a confounding factor, any PM-treated ovaries were cultured separately from other treatments (Madden *et al.*, 2014b)

*RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR):*
Following 2 or 4 d of *in vitro* culture, ovaries were stored in RNAlater at -80°C. Total RNA was isolated from ovaries (n=3; 10 ovaries per pool) using an RNeasy Mini kit according to the manufacturer’s instructions, concentrated via RNeasy Mini Elute kit and quantified with a NanoDrop (λ = 260/280 nm; ND 1000; Nanodrop Technologies Inc., Wilmington, DE).
According to the company-provided protocol, total RNA (250 ng) was reverse transcribed to cDNA using the RT² first-strand kit and applied to the Stress and Toxicity Profiler PCR Array, which contains 96-wells, each well containing a specific primer set, thus each plate tested 96 genes per one sample. The performed PCR protocol was a 10 min hold at 95°C and 40 cycles of denaturing at 95°C for 15 s and a combined annealing and extension for 1 minute at 60°C. The genes of interest were normalized to the housekeeping genes recommended by company-provided analysis software, hypoxanthine phosphoribosyltransferase (Hprt) and ribosomal protein, large, P1 (Rplp1). The mRNA level of these two genes did not change across treatments. The online SABiosciences RT² Profiler™ PCR Array Data Analysis quantified the changes in mRNA levels using the \(2^{-\Delta\Delta Ct}\) method.

**Individual gene target RT-PCR**

Reverse transcription of total RNA (250 ng) to cDNA was performed using the Superscript III One-Step RT-PCR System (Invitrogen). Using an Eppendorf mastercycler (Hauppauge, NY) and Quantitect™ SYBR Green PCR kit (Qiagen Inc. Valencia, CA), the autophagy genes Atg7 (forward: 5′-CTTCCCTGGCAAGGTGTTTA-3′; reverse: 5′-GTTGCTAGACGGTCTCCTC-3′) and Becn1 (forward: 5′-TAATGTGGGAAGGACAAG-3′; reverse: 5′-AAATCCTTCCACATCTCAAACA-3′) were amplified. The PCR protocol used was a 15 min hold at 95°C and 40 cycles of denaturing at 95°C for 15 s, annealing temperature of 58°C, and extension at 72°C for 15 s. The genes of interest were normalized to the housekeeping gene Gapdh as no change in its mRNA levels were observed between treatments. Changes in mRNA levels were quantified using the \(2^{\Delta\Delta Ct}\) method (Livak and Schmittgen, 2001; Pfaffl, 2001).
Protein isolation and Western blot analysis:

PND4 ovaries (n=3; 10 ovaries per pool) were homogenized in 200 µl of ice-cold tissue lysis buffer and protein quantified using a standard BCA protocol on a 96-well assay plate. Total protein (15 µg) was separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Prior to 1 hr blocking in 5% milk, membranes were stained with Ponceau S to confirm equal protein loading. The membrane was then incubated at 4 ºC with the Rabbit Anti-BECN1 (1:250; Santa Cruz). Following ~60 hrs of incubation, donkey anti-rabbit secondary antibody was applied and rocked with membrane for 1h at room temperature. Autoradiograms were visualized on X-ray films in a dark room following 10 min incubation of membranes with 1X SignalFire™ ECL reagent. Densitometry of the appropriate sized bands was measured using Image Studio Lite Version 3.1 (LI-COR Biosciences, Lincoln, NE) which eliminates background noise. Values were normalized Ponceau S staining.

Immunofluorescence staining:

Following treatment, ovaries were placed in 4% paraformaldehyde for 2 hours, washed and stored in 70% ethanol, paraffin embedded and serially sectioned (5 µM). Two sections per ovary (n = 3) were deparaffinized, blocked with 5% BSA and incubated with anti-LAMP1 primary antibody (1:50 dilution) at 4ºC overnight. Secondary FITC-labelled antibody was applied for 1 h, followed by incubation with the nuclear stain, Hoechst (30 minutes; 1:1000 dilution/5mg/ml; Invitrogen). Slides were repeatedly rinsed with PBS, cover-slipped, and stored in the dark (4ºC) until visualization. Primary antibody was not added to immunonegative ovarian sections. Immunofluorescence was visualized on a Leica DMI300B fluorescent microscope at λ = 488 and
633 nm for FITC and Hoechst, respectively. All images were captured using a 10× objective lens. Protein staining was quantified using Image J software. LAMP1 protein abundance was compared via quantification of signal intensity of foci (n=10) present in each section (n=2/ovary).

**Histological evaluation of autophagy-like morphology:**

For light and transmission electron microscopy (TEM), PND4 ovaries (n=5) were exposed on alternate days to PM (60 µM) and collected from culture after 1, 2, 3 or 4 days. The ovaries were fixed with 2% glutaraldehyde (w/v) and 2% paraformaldehyde (w/v) in 0.1M sodium cacodylate buffer, pH 7.2, for at least 48 hrs at 4°C. Samples were washed in buffer and then fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr at room temperature The samples were then dehydrated in a graded ethanol series, cleared with ultra-pure acetone, infiltrated and embedded using a modified EPON epoxy resin (Embed 812; Electron Microscopy Sciences, Ft. Washington, PA). Resin blocks were polymerized for 48 hours at 70°C. Thick and ultrathin sections were made using a Leica UC6 ultramicrotome (Leeds Precision Instruments, Minneapolis, MN). Thick sections were stained with epoxy resin stain (Electron Microscopy Sciences, Ft. Washington, PA) and imaged with a Zeiss Axioplan II light microscope with a MRC AxioCam and Axiovision software (Carl Zeiss INC, Thornwood, NY). Ultrathin sections were collected onto copper grids and images were captured using a JEM 2100 200kV scanning and transmission electron microscope (Japan Electron Optic Laboratories Inc., Peabody, MA).
Autophagy induction by rapamycin:

Ovaries (n=6) were treated with one of: vehicle control media (1% DMSO); PM (60 µM); rapamycin (1 µM); or PM (60 µM) + rapamycin (1 µM) on alternate days for 4d. The rapamycin concentration administered was adapted from a prior study (Wang et al., 2008a).

Inhibition of autophagy by LY294002:

For 4d, ovaries (n=4-6) were treated on alternate days to one of: vehicle control media (1% DMSO); PM (60 µM); LY294002 (20 µM); or PM (60 µM) + LY294002 (20 µM). The concentration of LY294002 using the PND4 ovary culture method was previously determined to be effective in the ovarian culture system (Keating et al., 2009).

Histological evaluation of follicle numbers:

Following treatment, ovaries were placed in 4% paraformaldehyde for 2 hours, washed and stored in 70% ethanol, paraffin embedded, and serially sectioned (5 µM). Every 6th section was mounted and stained with hematoxylin and eosin. Healthy oocyte-containing follicles were identified and counted in every 6th section. Unhealthy follicles were distinguished from healthy follicles by the appearance of pyknotic bodies and intense eosinophilic staining of oocytes. Healthy follicles were classified and enumerated according to Flaws et al., 1994. Slides were blinded to prevent counting bias.

Statistical analysis:

Treatment comparisons for follicle count experiments were performed using Analysis of Variance (ANOVA). Quantitative RT-PCR, Western blot and immunofluorescence staining data
were analyzed by t-test comparing treatment with control raw data at each individual time point. All statistical analysis was performed using Prism 5.04 software (GraphPad Software). Statistical significance was defined as $P < 0.05$ and a trend for a significant difference from control was designated at $P < 0.1$.

Results

Evaluation of changes in genes involved in stress and toxicity pathways following PM exposure

A stress and toxicity RT$^2$ Profiler PCR array was used to investigate the mRNA profile of PND4 rat ovaries (n=3, 10 ovaries per pool) which had been exposed to PM (60 µM) for 2 and 4 days. As described in Table 1, following 2d of PM exposure, $B2m$ mRNA levels decreased ($P < 0.05$), as well as a trend for a decrease ($P < 0.10$) in $Tnfa$ and a trend for an increase in $Hspa1b$ and $Sqstml$, relative to control treated ovaries. After 4d of PM exposure, $Bbc3$, $Cdkn1a$, $Cftr$, $Edn1$, $Gstp1$, $Nqo1$, $Tlr4$, $Tnfrsf1a$, $Txnrd1$ mRNA levels increased ($P < 0.05$) compared to control, while decreases ($P < 0.05$) after 4d PM exposure were found in $Casp1$ and $Il1b$ mRNA levels. In addition, 4d PM exposure induced a trend ($P < 0.10$) for increased mRNA levels for the following genes: $Cd40lg$, $Fas$, $Gadd45a$ and $Mre11a$ as well as a trend ($P < 0.10$) for a decrease in levels of $Adm$, $Epo$, $Mmp9$, $Tnf$, and $Vegfa$. The results of this experiment are summarized in Table 1.

PM-induced mRNA expression changes of autophagy genes

PND4 rat ovaries (n=3, 10 ovaries per pool) were cultured and collected after 2 and 4 days of PM (60 µM) exposure. PM-induced changes in mRNA levels of the autophagy genes $Atg7$ and
Becn1 were quantified by qRT-PCR. Following 2 days of PM exposure, compared to control, there was a trend \((P < 0.10)\) for decreased Atg7 mRNA abundance but there was no impact of PM on Atg7 mRNA after 4 days of exposure (Fig. 1A). There was no effect of PM on Becn1 mRNA levels after 2 days of PM exposure; however, there was a trend \((P < 0.10)\) for a slight increase in Becn1 after 4 days (Fig. 1B), relative to control.

**Effect of PM exposure on ovarian location and level of autophagy proteins**

To investigate post-transcriptional changes in autophagy proteins, BECN1 and LAMP1 protein levels activity was measured in PND4 rat ovaries following 2 and 4 days of PM (60 µM) exposure by protein isolation and Western blotting or fixed and sectioned for immunofluorescence \((n=3, 2\) sections per ovary), respectively. Relative to control, an increase \((P < 0.05)\) in BECN1 was found after 2d of PM exposure, but at 4 d, no difference between treatments in BECN1 levels was observed (Fig. 2). At both time points, LAMP1 foci number was unaffected by PM exposure (Fig. 3); however, the size of the LAMP foci appear larger in PM-treated ovaries versus control.

**Investigation of autophagy by TEM**

In order to determine autophagosome formation and activity, PND4 ovaries were exposed to PM (60 µM) on alternate days and collected on days 1, 2, 3 and 4 of culture for TEM. After 1 day, PM-exposed ovaries exhibited dark, electron dense structures in the oocyte cytoplasm of primordial follicles (Fig. 4C) and large double-membraned autophagosomes in the granulosa cells of the small primary follicles which appears to contain a mitochondria (Fig. 4C). Multilamellar bodies were seen in both control and PM-treated ovaries after 2d, with a greater
abundance apparent visually in PM-treated ovaries. Some oocytes of small primary follicles following 2d of PM exposure appeared dead with global vacuolization (Fig. 4F), while other small primary follicles had little sign of damage. Similar to observations after 1d of PM exposure, the granulosa cells after 2d contained large double-membraned autophagosomes with unidentified structures engulfed suggesting a more advance state of digestion compared to 1d PM exposure (Fig. 4F). After 3d of PM exposure, dead primordial follicles were evident by the lack of definition between the granulosa cell layer and the oocyte cytoplasm as well as small autophagosomes displacing the oocyte nucleus (Fig. 4G). Like the earlier time points, the cytoplasm of granulosa cells continued to have large vacuolization, increasing in electron density. Following 4d of PM exposure, in addition to apparent degradation, abnormal golgi apparatus were evident across follicle types (Fig. 4 and 5).

Large primary and secondary follicles, were evident after 3 and 4d, respectively, in control ovaries, however, these two follicle types were difficult to distinguish in PM-exposed ovaries because, although the oocyte had grown, the granulosa cell layer was minute, about 2 µm, compared to the control, which was over 10 µm across (Fig. 5). Also, in contrast to control, the mitochondrial are much darker, electron-dense in the large follicle of PM-treated ovaries (Fig. 5C and D). Analogous to the PM-treated primordial and small primary follicles, abnormal golgi apparatus and autophagosome were present in the oocyte cytoplasm and granulosa cells, respectively (Fig. 5C and D).

**Evaluation of a functional role for PM-induced autophagy**

To gain insight into the role of autophagy induced by PM exposure, PND4 ovaries (n= 5-6) were treated with vehicle control (1% DMSO), PM (60 µM), rapamycin (1 µM), PM (60 µM) +
rapamycin (1 µM), LY294002 (20 µM), or PM (60 µM) + LY294002 (20 µM) on alternate days for 4 d and then processed for histology. As previously described (Madden et al., 2014), compared to control, there were reduced numbers of all follicle stages due to PM exposure (Fig. 6). Compared to control, rapamycin treatment alone only reduced \( P < 0.05 \) secondary follicle number with no effect of treatment on any other follicle stage (Fig. 6D). Rapamycin cultured with PM prevented PM-induced primordial and small primary follicle loss (Fig. 6A and B). Interestingly, PM + rapamycin treated ovaries had a strong trend for increased \( P = 0.056 \) numbers of primordial follicles than control ovaries (Fig. 6A). The addition of rapamycin did not impact the effects of PM on large primary and secondary, relative to control treated ovaries (Fig. 6C and D).

The addition of LY294002 resulted in an increased \( P < 0.05 \) number of primordial follicles (Fig. 7A) and a trend \( P < 0.10 \) for increased small primary follicles (Fig 7B), and a reduction \( P < 0.05 \) in large primary and secondary follicles (Fig 7C and D), when compared to control ovaries. The addition of LY294002 during PM exposure caused \( P < 0.05 \) follicle depletion relative to control in all follicle developmental stages; however, this loss was not different from ovaries treated solely with PM (Fig. 7).

**Discussion**

PM is the ovotoxic and antineoplastic metabolite of the chemotherapy drug CPA (Plowchalk and Mattison, 1991; Desmeules and Devine, 2006). CPA is used to treat a variety of cancers, but due it its ability to deplete primordial follicles (Plowchalk and Mattison, 1991), CPA increases a female patient’s risk of reduced fertility or POF (Bines et al., 1996), which is a major concern for the millions of female cancer survivors. Although compromised fertility via follicle
depletion is well documented as a side effect of CPA, little is known about how this ovotoxicant elicits its detrimental effects to the ovary.

A prior study, that used the same neonatal rat ovary culture system as in our experiments, investigated apoptosis as a PCD mechanism active during PM-induced follicle loss and found that granulosa cells of primary follicles were positively identified during TUNEL and cleaved caspase-3 staining following PM exposure (Desmeules and Devine, 2006). However, the number of follicles positive for cleaved caspase-3 was not different from control and no primordial follicles stained positive for this classic apoptosis marker (Desmeules and Devine, 2006). In addition, the use of a caspase-3 inhibitor did not prevent PM-induced follicle loss leading the authors to conclude that PM-induced follicle loss occurs via a caspase-independent pathway (Desmeules and Devine, 2006).

Autophagy, a caspase-independent PCD process, represents an alternative atretic route outside of apoptosis. There is mounting evidence in favor of the involvement of autophagy in both ovarian follicle death and survival. For example, studies investigating the mechanisms responsible for follicle death at birth and puberty, times of large natural follicle loss, have found the classic markers of autophagy plentiful, with little to no signs of apoptosis (Rodrigues et al., 2009; Tingen et al., 2009). Furthermore, ovarian induction of autophagy genes was found in murine ovaries following exposure to cigarette smoke (Gannon et al., 2012; Gannon et al., 2013). Also, we have demonstrated altered mRNA levels of autophagy genes in neonatal rat ovaries exposed to low levels of DMBA (Madden et al., 2014a), suggesting autophagy as a contributing factor to both the ovotoxicity of these compounds and the removal of damaged and/or dead follicles.
Interestingly, autophagy has also been suggested to act as a pro-survival mechanism in the ovary, particularly in the perinatal ovary and its establishment of the primordial follicle pool from the female germ cells (Gawriluk et al., 2011). Specifically, PND1 Atg7−/− knockout mice had no apparent germ cells and Becn1+/− had less than half the number of germ cells compared to control, thus supporting the functional requirement of these autophagy proteins for normal follicular development to occur and the consideration of autophagy as more than just a PCD process.

The ovarian culture method is perfectly suited to the study of autophagy involvement in ovotoxicity, since there is lack of the functional circulatory system by which immune cells could remove such debris. The lack of a concomitant increase in unhealthy or dead follicles mirroring the loss of healthy follicles after PM exposure, supports that there is some mechanism in place for this removal. We observed markers indicating that autophagy is an active ovarian process following PM exposure. The increase observed in the mRNA and protein level of Becn1, an early initiator of the autophagosome formation, is suggestive that autophagy is occurring, but additional gene expression alternations were observed in support of this hypothesis. For example, relative to control, a large increase was found in Bcl-2 binding component 3 (PUMA), which has been shown to be involved in mitochondria-specific autophagy. In addition, a striking increase in cyclin-dependent kinase inhibitor 1A (p21) was found in PM-exposed ovaries compared to control, which has also been linked to the induction of autophagy (Chen et al., 2014). In congruence, a decrease in mRNA level was found in caspase-1 after PM exposure, which is in agreement with the previous study reporting PM-induced follicle depletion is caspase-independent (Desmeules and Devine, 2006).
Ovarian histology was examined in fine detail using TEM following a time course of PM exposure. Since larger primary and secondary follicles develop after approximately 3-4 days in culture, determination of the impacts of PM on all stage follicles (primordial, small primary, large primary and secondary) was possible. In small preantral (primordial and small primary) follicles, electron dense structures along with engulfed mitochondria in double-membraned structures was noted. As the time of exposure progressed, vacuolization of oocytes was observed as well as abnormal golgi apparatus were apparent. In large preantral follicles (large primary and secondary), many of the same morphological alterations were seen. A major observation incompatible with follicle viability and growth is reduction in the width of the granulosa cell layer, from about 2 to 10 µM in width. These data are in agreement with the gap between the oocyte and granulosa cell that we have previously reported (Madden et al., 2014). More importantly, these PM-induced changes in the cellular components are observed at time points prior to the observation of follicle loss (day 4; Madden et al., 2014), supporting that they are ovotoxic changes that occur due to PM exposure and contribute to ovotoxicity. Similarly, DNA double-stranded breaks by PM were detected prior to follicle loss (Petrillo et al., 2011).

Our findings coincide with a previous in vivo study, which found ovarian autophagy induced in response to whole-body cigarette exposure (Gannon et al., 2012; Gannon et al., 2013). Similarly, the majority of autophagosomes were observed in the granulosa cells and these structures appeared to also contain mitochondria (Gannon et al., 2012). Additionally, an increase in BECN1 protein level was observed. Collectively, these studies are among the first to report that autophagy is an active ovarian process that can be initiated in response to xenobiotic exposures.
Our evidence collected to this point strongly supported autophagy as an ovotoxic mechanism induced by PM, thus, a functional role for PM-induced autophagy was investigated using chemical manipulation of pathways reported in the literature to regulate autophagy (Holen et al., 1993; Blommaart et al., 1997; Petiot et al., 2000; Codogno and Meijer, 2005; Wang et al., 2008b). By administering PM in combination with LY294002, which inhibition autophagy via the PI3K pathway (Holen et al., 1993; Blommaart et al., 1997; Petiot et al., 2000), we hypothesized that if autophagy was prevented, that PM-induced follicle depletion would be at a higher level than in ovaries treated with PM alone. Surprisingly, there was no impact of the LY294002 treatment on PM-induced follicle loss. Interestingly, this result underscores the difference in mechanisms of ovotoxicity induced by chemical exposure. Increased and decreased primordial follicle loss was observed in ovaries treated with DMBA or 4-vinylcyclohexene diepoxide (VCD), respectively, during LY294002 treatment, relative to DMBA and VCD alone (Keating et al., 2009).

Our next step was to activate autophagy via the use of rapamycin, which inhibits mTOR, a negative regulator of autophagy (Chen et al., 1995; Choi et al., 1996; Jung et al., 2010). We expected that if autophagy is an ovotoxic mechanism induced by PM, accelerated PM-induced follicle loss would be evident. In fact, the opposite was the case; PM-induced follicle loss was prevented in primordial and small primary follicles in the PM + rapamycin treatment. mTOR is a downstream component of PI3K signaling and is activated and inhibited through phosphorylation at different sites (Cheng et al., 2004). If PM is working through mTOR to induce autophagy, it is unsurprising that no impact of PM during PI3K inhibition was noted, since mTOR was likely unavailable to PM in this experimental paradigm. It was interesting that rapamycin treatment prevented PM-induced follicle loss, and while this effect could be through
autophagy induction, examining autophagy marker induction during rapamycin and PM exposure will be a worthwhile next step for future experiments.

The PI3K and mTOR pathways have gained recent attention in ovarian physiology and toxicology, as two pathways suggested to play a key role in follicle activation and influence ovotoxicity (Keating et al., 2009; Adhikari et al., 2010; Sobinoff et al., 2011). An *in vivo* study found in mice that CPA increased phosphorylation of proteins in the PI3K pathway, including mTOR and when the PI3K pathway was perturbed via the drug AS101, CPA-induced follicle loss was reduced (Kalich-Philosoph et al., 2013). However, how AS101 impacts the PI3K pathway appears unknown, but similar to this study, manipulation of this pathway, mTOR specifically in this study, show promising results as a potential therapeutic to alleviate CPA/PM-induced infertility.
## Figures

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**Table 1. Effect of PM exposure on ovarian expression of stress and toxicity genes.**
PND4 rat ovaries were treated with 1% DMSO (vehicle control) or PM (60 µM) for 2 or 4 d. Following RNA isolation, mRNA levels were quantified with an RT² Profiler PCR array. Values represent fold-change to a control value of 1, normalized to Hprt1 and Rplp1. * = different from control, $P < 0.05$; † = $P < 0.1$. 


Figure 1. Effect of PM on ovarian mRNA abundance of Atg7 and Beclin.
PND4 rat ovaries (n = 3; 10 ovaries per pool) were treated with 1% DMSO (vehicle control; CT) or PM (60 µM). Following 2 or 4 d of culture, mRNA was isolated and (A) Atg7 and (B) Beclin levels evaluated by quantitative RT-PCR. mRNA values represent fold-change ± SEM relative to a control value of 1, normalized to Gapdh. # = P < 0.1 difference from CT.
Figure 2. Effect of PM on ovarian abundance of BECN1 protein. PND4 rat ovaries (n = 3; 10 ovaries per pool) were treated with 1% DMSO (vehicle control; CT) or PM (60 µM). Following 2 or 4 d of culture, protein levels of BECN1 were evaluated by (A) Representative Western blot of 2 d normalized to Ponceau S and (B) data quantification. Values represent signal intensity ± SEM. # = P < 0.1 different from control.
Figure 3. Localization and effect of PM on ovarian LAMP1 protein.
PND4 rat ovaries (n = 3; 2 sections per ovary) were treated with 1% DMSO (vehicle control; CT) or PM (60 µM). Following 2 or 4 d of culture, ovaries were fixed in formalin, and immunofluorescence staining was performed using a primary antibody directed against LAMP1: (A) 2d CT; (B) 2d LAMP; (C) 4d CT and (D) 4d LAMP1. LAMP1 is represented in red and the Hoechst nuclear stain is in blue. (E) Quantification of the number of LAMP1 foci was performed.
Figure 4. Histological evaluation by TEM in small preantral follicles following PM exposure. PND4 rat ovaries were treated with 1% DMSO (vehicle control, CT) or PM (60 µM). Following 1, 2, 3 and 4 d of culture, ovaries were fixed and processed for TEM (A) 1d CT primordial follicle; (B) 1d CT small primary follicle; (C) 1d PM primordial (D) 1d PM small primary (E) 2d PM primordial (F) 2d PM small primary (G) 3d primordial (H) 3d small primary (I) 4d primordial (J) 4d small primary; Hollow arrow = autophagosomes; G = abnormal golgi apparatus; Thin black arrow = double membrane; Thin dashed black arrow = electron dense structures; Solid thick black arrow = multilamellar structures; Arrow head = global vacuolization of oocyte.
Figure 5. Detection of autophagosomes by TEM in large preantral follicles following PM exposure.

PND4 rat ovaries were treated with 1% DMSO (vehicle control, CT) or PM (60 µM). Following 1, 2, 3 and 4 d of culture, ovaries were fixed and processed for TEM (A) 3d CT; (B) 4d CT; (C) 3d PM and (D) 4d PM. Black double-ended arrows indicate the width of the granulosa cell layer; Hollow arrows = autophagosomes; G = abnormal golgi apparatus
Figure 6. Effect of autophagy induction via rapamycin (Rapa) during PM exposure on follicle number.

PND4 rat ovaries were cultured and treated on alternate days with 1% DMSO (vehicle control, CT), PM (60 µM), Rapa (1 µM) or PM (60 µM) + Rapa (1 µM). Following 4 d of culture, follicles were classified and counted: (A) Primordial Follicles; (B) Small Primary Follicles; (C) Large Primary Follicles; (D) Secondary Follicles. Values represent mean ± SE total follicles counted/ovary, n = 4-6. Different letters represent significant difference between treatments $P < 0.05$. * = $P < 0.10$ difference from CT.
Figure 7. Effect of autophagy inhibition via LY294002 (LY) during PM exposure on follicle number.
PND4 rat ovaries were cultured and treated on alternate days with 1% DMSO (vehicle control, CT), PM (60 µM), LY (20 µM) or PM (60 µM) + LY (20 µM). Following 4 d of culture, follicles were classified and counted: (A) Primordial Follicles; (B) Small Primary Follicles; (C) Large Primary Follicles; (D) Secondary Follicles. Values represent mean ± SE total follicles counted/ovary, n = 4-6. Different letters represent significant difference between treatments $P < 0.05$. * = $P < 0.10$ difference CT.
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Chapter 5. ACUTE 7,12-DIMETHYLBENZ[A]ANTHRACENE EXPOSURE CAUSES DIFFERENTIAL CONCENTRATION-DEPENDENT FOLLICLE DEPLETION AND GENE EXPRESSION IN NEONATAL RAT OVARIIES

A paper published by Toxicology and Applied Pharmacology

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Contribution Statement:
I performed all ovarian tissue analysis and data interpretation as well as wrote this paper; Hoyer, P.B., was the post-doctoral mentor for Keating, A.F. thus provided the animals and reagents for ovary culture and exposure for follicle counts in Figure 1; Devine, P.J. assisted in the experimental design for Figure 1 and edited the manuscript; Keating, A.F. designed experiments, assisted in data interpretation and figure construction, edited the manuscript and served as corresponding author.

Abstract

Chronic exposure to the polycyclic aromatic hydrocarbon 7,12-dimethylbenz[a]anthracene (DMBA), generated during combustion of organic matter including cigarette smoke, depletes all ovarian follicle types in the mouse and rat, and in vitro models
mimic this effect. To investigate the mechanisms involved in follicular depletion during acute DMBA exposure, two concentrations of DMBA at which follicle depletion has (75 nM) and has not (12.5 nM) been observed were investigated. Postnatal day four F344 rat ovaries were maintained in culture for four days before a single exposure to vehicle control (1% DMSO; CT) or DMBA (12 nM; low-concentration or 75 nM; high-concentration). After four or eight additional days of culture, DMBA-induced follicle depletion was evaluated via follicle enumeration. Relative to control, DMBA did not affect follicle numbers after 4 days of exposure, but induced large primary follicle loss at both concentrations after 8 days; while, the low-concentration DMBA also caused secondary follicle depletion. Neither concentration affected primordial or small primary follicle number. RNA was isolated and quantitative RT-PCR performed prior to follicle loss to measure mRNA levels of genes involved in xenobiotic metabolism (Cyp2e1, Gstmu, Gstpi, Ephx1), autophagy (Atg7, Becn1), oxidative stress response (Sod1, Sod2) and the phosphatidylinositol 3-kinase (PI3K) pathway (Kitlg, cKit, Akt1) 1, 2 and 4 days after exposure. With the exception of Atg7 and cKit, DMBA increased (P < 0.05) expression of all genes investigated. Also, BECN1 and pAKTThr308 protein levels were increased while cKIT was decreased by DMBA exposure. Taken together, these results suggest an increase in DMBA bioactivation, add to the mechanistic understanding of DMBA-induced ovotoxicity and raise concern regarding female low concentration DMBA exposures.

**Introduction**

The ovary contains a finite number of oocyte-containing follicles. Appropriate maturation and survival through the primordial, small primary, large primary, secondary, and antral follicular stages is essential for both conception and female health. The primordial is most
immature follicular stage, in which the oocyte remains arrested in meiosis and waits for an appropriate signal to enter into the growing follicular pool toward ovulation. Once the ovarian primordial follicular reserve is depleted, ovarian failure occurs, rendering the female both infertile and at an increased risk for development of a variety of health conditions including osteoporosis and heart disease (Greendale et al., 1999).

Follicles are vulnerable to toxic exposures at all developmental stages. 7,12-dimethylbenz[a]anthracene (DMBA), a polycyclic aromatic hydrocarbon (PAH), is produced from the burning of organic material (Gelboin, 1980), thus cigarette smoke is an exposure source. DMBA depletes all ovarian follicle types in mice and rats (Mattison and Schulman, 1980) and is the most ovotoxic of three PAH cigarette smoke-components (DMBA, 3-methylcholanthrene and benzo[a]pyrene (Borman et al., 2000). An acute high-concentration exposure to DMBA in vivo destroyed primordial oocytes in rats and mice (Mattison, 1979; Mattison and Thorgeirsson, 1979), and use of an ovary culture system has demonstrated that repeated exposures of DMBA to F344 rat ovaries caused primordial follicle loss at concentrations of 75 nM and higher (Igawa et al., 2009). In support of the ovotoxicity of DMBA (and other cigarette smoke components), ovarian failure onset is accelerated in female cigarette smokers (Jick and Porter, 1977).

In order for ovotoxicity to ensue, DMBA must be bioactivated to an ovotoxic metabolite; DMBA-3,4-diol, 1,2-epoxide (Miyata et al., 1999; Igawa et al., 2009), by a number of enzymes including microsomal epoxide hydrolase (Ephx1). Using the in vitro postnatal day (PND) 4 ovarian culture system, competitive inhibition of EPHX1 by cyclohexene oxide reduced DMBA-induced follicle loss in ovaries from mice (1 μM DMBA; Rajapaksa et al., 2007) and rats (1 μM DMBA; Igawa et al., 2009). Furthermore, mRNA levels of Ephx1 increased after 2 days of
DMBA exposure, prior to follicle loss which occurs after 4 days, relative to control (Rajapaksa et al., 2007; Igawa et al., 2009). Additionally, cytochrome P450 isoform 2E1 (Cyp2e1)-null mice had increased sensitivity to DMBA-induced primordial and small primary follicle loss (Keating et al., 2008). This was at least in part due to increased Ephx1 levels in the Cyp2e1-null ovaries. Thus, the action of ovarian EPHX1 is critical for DMBA to impart its ovotoxicant effects, and Cyp2e1 and Ephx1 regulation are in some way interrelated (Keating et al., 2008).

Previous studies have suggested that the phosphatidylinositol 3-kinase (PI3K) pathway plays a role in Ephx1 expression regulation (Kim et al., 2003; Bhattacharya et al., 2012). Specifically, PI3K inhibition using LY294002 in cultured F344 rat ovaries resulted in increased Ephx1 mRNA and protein expression (Bhattacharya et al., 2012), while decreased EPHX1 was observed following PI3K inhibition in rat hepatocytes (Kim et al., 2003). Despite these tissue-specific responses, a link between Ephx1 gene expression and the PI3K pathway is supported.

In addition to its role in xenobiotic biotransformation via Ephx1 regulation, the PI3K pathway is vital for follicle survival and recruitment, particularly pre-antral follicles (Yoshida et al., 1997; Parrott and Skinner, 1999; Castrillon et al., 2003; Keating et al., 2009). Initiation of the PI3K signaling pathway occurs once the granulosa-derived signaling molecule Kit ligand (KITLG) binds the oocyte-expressed stem cell receptor (cKIT; Ismail et al., 1996). Following KITLG-cKIT interaction, the PI3K signaling cascade is activated leading to phosphorylation of the downstream effector molecule protein kinase B (AKT) and subsequently the forkhead transcription factor 3a (FOXO3a), both important for control of primordial follicle survival and recruitment, respectively (Castrillon et al., 2003; Reddy et al., 2005; Liu et al., 2006; John et al., 2008). Foxo3a-null mice suffer from follicular depletion and ovarian failure as a result of global follicle activation (Castrillon et al., 2003), while oocyte-specific FOXO3a overexpression
restricts primordial follicles from entering the recruitment pool, also rendering the female infertile (Liu et al., 2006).

The ovarian response to oxidative stress is also important for follicle viability. Reactive oxygen species (ROS) have been shown to increase prior to DMBA-induced follicle loss in vitro, suggesting that DMBA exposure (0.1-100 µM) induces oxidative stress within the ovary (Tsai-Turton et al., 2007). Co-treatment with DMBA (10 µM) and glutathione (GSH; which detoxifies ROS) alleviated DMBA-induced follicle loss (Tsai-Turton et al., 2007), further supporting that DMBA induces ROS generation. Reactive xenobiotics can also be detoxified through GSH conjugation catalyzed by the glutathione S-transferase (GST) family of enzymes (Reddy et al., 1983). GST isoform pi (Gstp) mRNA and protein increase following DMBA exposure in the cultured neonatal F344 rat ovary prior to the onset of follicle loss (Bhattacharya and Keating, 2012). Furthermore, mice that are deficient in GSTPI have increased levels of DMBA-induced skin tumors (Henderson et al., 1998), indicating that GSTP-catalyzed GSH conjugation to DMBA is a potential detoxification event.

In addition to ROS alleviation by GSH, superoxide dismutases (SOD1 – cytoplasmic; SOD2 - mitochondrial) can act directly on superoxide anion radicals to form water and hydrogen peroxide. Studies have shown that Sod1-null mice exhibit reduced fertility (Ho et al., 1998; Matzuk et al., 1998), while although Sod2-deficient mice die pre-pubertally, when SOD-deficient ovaries are transplanted to wild-type mice, reproductive abnormalities are not observed (Matzuk et al., 1998).

As an alternative to apoptosis (Morita and Tilly, 1999; Hu et al., 2001), another form of programmed cell death, autophagy, may contribute to follicle depletion. When activated, autophagy induces the formation of an autophagosome, which, once fused with the lysosome,
can consume internal components of a cell (Levine and Klionsky, 2004). Several proteins are involved in the process of autophagy including Autophagy-related protein 7 (ATG7) and Beclin-1 (BECN1; Kim et al., 1999; Liang et al., 1999). ATG7 and BECN1 are of particular interest in the ovary due to previous evidence suggesting their involvement in autophagy for female germ cell survival (Gawriluk et al., 2011). Further evidence supporting involvement of autophagy during DMBA-induced ovotoxicity has come from work demonstrating that cigarette smoke exposure (a source of DMBA) induced autophagy in the ovaries of exposed mice (Gannon et al., 2012; 2013). Thus autophagy could potentially be involved during DMBA-induced follicle loss.

It is difficult to estimate human DMBA ovarian exposure due to differences in hepatic bioactivation between individuals. For this reason, the ovotoxic effects of repeated DMBA exposures have been investigated using the PND4 in vitro ovary culture system at a concentration (1 µM) that causes approximately 50% primordial follicle loss after 4 days (Rajapaksa et al., 2007; Igawa et al., 2009). Additionally, these exposures have been previously administered from the onset of culture when the ovary is largely comprised of primordial follicles. The current study was designed to delay exposure to DMBA until after 4 days of culture in order to determine the impact of DMBA exposure on large primary and secondary follicles. Also, single DMBA exposures were used to determine the impact of low concentration acute exposures to DMBA, similar to that of passive cigarette smoke exposure, on ovarian function. The ovarian response to acute DMBA exposure was examined by quantifying follicle numbers and measuring the mRNA levels of genes involved in 1) xenobiotic biotransformation - Cyp2e1, Ephx1, Gstpi and Gstmu; 2) PI3K signaling - Akt1, cKit and Kitlg; 3) the oxidative stress response - Sod1 and Sod2; and 4) autophagy - Atg7 and Becn1. Also, levels of EPHX1,
BECN1, cKIT and pAKTThr308 in ovaries treated with control, low (12.5 nM) or high (75 nM) DMBA were evaluated.

**Materials and Methods**

**Reagents:**

7,12-dimethylbenz[a]anthracene (DMBA), bovine serum albumin (BSA), ascorbic acid, transferrin, 2-β-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, ammonium persulphate, glycerol, N’ N’ N’ N’-Tetrathylethylenediamine (TEMED), Tris base, Tris HCL, sodium chloride, Tween-20 were purchased from Sigma Aldrich Inc. (St. Louis, MO). Dulbecco’s Modified Eagle Medium: nutrient mixture F-12 (Ham) 1x (DMEM/Ham’s F12), Albumax, penicillin (5000U/ml) Hank’s Balanced Salt Solution (without CaCl₂, MgCl₂, or MgSO₄) were obtained from Invitrogen Co. (Grand Island, NY). Millicell-CM filter inserts and 48-well cell culture plates were obtained from Millipore (Billerica, MA) and Corning Inc. (Corning, NY), respectively. RNeasy Mini kit, QIA Shredder kit, RNeasy Mini Elute kit, and Quantitect ™ SYBR Green PCR kit were purchased from Qiagen Inc. (Valencia, CA). RNAlater was obtained from Ambion Inc. (Grand Island, NY). With the exception of cKit and Kitlg which were obtained from Integrated DNA Technologies (Coralville, IA), all primers were obtained from the DNA facility of the Iowa State University office of biotechnology (Ames, IA). Anti-EPHX1 antibody was from Detroit R&D (Detroit, MI). Anti-pAKTThr308 was purchased from Abcam Technology (Cambridge, MA) and Cell Signaling Technology (Danvers, MA). Anti-BECN1 and anti-cKIT were obtained from Santa Cruz (Dallas, TX) and Cell Signaling Technology (Danvers, MA), respectively.
Animals:
Fisher 344 (F344) rats (approximately 6 months of age) were housed in plastic cages and maintained in a controlled environment (22 ± 2°C; 12h light/12h dark cycles). The animals were provided a standard diet with *ad libitum* access to food and water, and housed with a proven male for 5 days (two females per male). Approximately 2-3 days before parturition date, females were separated and housed one per cage and allowed to give birth. The University of Arizona and Iowa State University Institutional Animal Care and Use Committee’s approved all experimental procedures.

*In vitro ovarian cultures:*
Ovaries were collected from female PND4 F344 rats and cultured as described by Devine *et al.*, 2002. The PND4 rat pups were euthanized by CO₂ inhalation followed by decapitation. Ovaries were removed, trimmed of oviduct and other excess tissue, and placed onto a Millicell-CM membrane floating on 250 µl of previously 37°C equilibrated DMEM/Ham’s F12 medium containing 1 mg/ml BSA, 1 mg/ml Albumax, 50 µg/ml ascorbic acid, 5 U/ml penicillin and 27.5 µg/ml transferrin per well in a 48-well plate. A drop of medium was placed on top of each ovary to prevent dehydration. Ovaries were cultured at 37°C and 5% CO₂ for 4 days, then treated with vehicle control media (1% DMSO), low DMBA (12.5 nM) or high DMBA (75 nM) for an additional 1, 2, 4 or 8 days. This time of exposure ensured that large primary and secondary ovarian follicles were present at time of treatment.
Histological evaluation of follicle numbers:

Following 4 or 8 days of culture, ovaries were placed in 4% paraformaldehyde for 2 hours, washed and stored in 70% ethanol, paraffin embedded, and serially sectioned (5 µM). Every 6th section was mounted and stained with hematoxylin and eosin. Healthy oocyte-containing follicles were identified and counted in every 6th section. Follicles were considered primordial if they contained an oocyte surrounded with a single layer of squamous-shaped granulosa cells; small primary if they contained an oocyte surrounded by ≤ 10 cuboidal-shaped granulosa cells; large primary if they contained an oocyte surrounded by > 10 cuboidal shaped-granulosa cells; and secondary if they contained an oocyte surrounded by multiple layers of granulosa cells. Unhealthy/atretic follicles were distinguished from healthy follicles by the appearance of pyknotic bodies and intense eosinophilic staining of oocytes. Healthy follicles were classified and enumerated according to Flaws et al., 1994. Slides were blinded to prevent counting bias.

RNA isolation and polymerase chain reaction (PCR):

Following 1, 2 or 4 days post-treatment, ovaries were stored in RNAlater at -80°C. Total RNA was isolated from ovaries (n=3; 6 ovaries per pool) using an RNeasy Mini kit according to the manufacturer’s instructions. RNA was eluted in 14 µl of RNase-free water and concentration quantified using a NanoDrop (λ = 260/280 nm; ND 1000; Nanodrop Technologies Inc., Wilmington, DE). Total RNA (150 ng) was reverse transcribed to cDNA using the Superscript III One-Step RT-PCR System. Genes of interest were amplified using an Eppendorf mastercycler (Hauppauge, NY) using a Quantitect ™ SYBR Green PCR kit (Qiagen Inc. Valencia, CA). The primers used are listed in Table 1. The PCR conditions used were a 15 min hold at 95°C and 40 cycles of denaturing at 95°C for 15 s, annealing at 58 °C for 15 s, and
extension at 72°C for 20 s. Changes in gene expression were quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001). It should be noted that DMBA exposure impacted expression of a number of housekeeping genes ($\beta$-actin, cyclophilin B, and hypoxanthine phosphoribosyltransferase 1, data not shown), with the exception of Gapdh and 18S rRNA. Gapdh was chosen as the housekeeping gene.

**Immunofluorescence staining:**

Following treatment, ovaries were placed in 4% paraformaldehyde for 2 hours, washed and stored in 70% ethanol, paraffin embedded and serially sectioned (5 μM). Two sections per ovary (n = 3) were deparaffinized and incubated with primary antibodies (1:50 dilution) directed against EPHX1, pAKT$^{Thr308}$, BECN1 or cKIT at 4°C overnight. The blocking solution used was 5% BSA. Secondary FITC antibody was applied for 1 h, followed by Hoechst (30 minutes; 5mg/ml). Slides were repeatedly rinsed with PBS, cover-slipped, and stored in the dark (4°C) until visualization. Primary antibody was not added to immunonegative ovarian sections. Immunofluorescence was visualized on a Leica DMI300B fluorescent microscope at $\lambda = 488$ and 633 nm for FITC and Hoechst, respectively. All images were captured using a 10× objective lens. Protein staining was quantified using ImageJ software by circling the follicle under analysis and measuring integrated density. An identical measurement area was maintained between follicles of a particular stage. Integrated density of EPHX1, pAKT, and cKIT protein staining was measured in ten small (primordial and small primary) follicles and five large (large primary and secondary) follicles per section. The average number of follicles analyzed per ovary was 20 small and 10 large. BECN1 protein abundance was evaluated via quantification of the total number of foci present in each section. Statistical analysis was performed by comparison
between control and treatment on day of staining. When statistical difference was observed for all three staining repetitions, an effect of treatment on the protein of interest was concluded. For ease of presentation, control treatment is presented as 100% and the DMBA treatments are expressed as a percentage of control. Statistical difference is from the raw data.

**Statistical analysis:**

Comparisons were made between treatments for follicle count experiments using Analysis of Variance (ANOVA). Quantitative RT-PCR and immunofluorescence staining data were analyzed by t-test comparing treatment with control raw data at each individual time-point. All statistical analyses were performed using Prism 5.04 software (GraphPad Software). Statistical significance was defined as $P < 0.05$.

**Results**

**Effect of acute DMBA exposure on ovarian histology and follicle number**

To investigate the effects of acute low and high DMBA exposures on follicle viability and ovarian morphology, PND4 ovaries were exposed once after 4 days of culture and the ovaries were maintained for an additional 4 (Figure 1A - D) or 8 (Figure 2A - H) days. There was no impact of a single exposure to DMBA at either concentration on primordial, small primary or large primary follicles after 4 days in culture. Interestingly, there were increased ($P < 0.05$) secondary follicle number at the low DMBA concentration. Additionally, DMBA did not induce primordial or small primary follicle loss at either concentration 8d after exposure (Figure 2A-1F). Both DMBA concentrations induced large primary follicle loss ($P < 0.05$; Figure 2G).
Interestingly, only the low DMBA concentration induced secondary follicle loss ($P < 0.05$; Figure 2H).

In addition to follicle depletion, DMBA single exposure caused morphological alterations in both large primary and secondary follicles. Shrunken, eosinophilic oocytes resulting from an apparent loss of connection between the oocyte and granulosa cells occurred at both concentrations. Intense eosinophilic staining of follicles also indicated significantly more atretic follicles in ovaries exposed to DMBA (Figure 2D).

**Effect of acute DMBA exposures on mRNA expression**

*Temporal pattern of single DMBA exposure on expression of genes involved in chemical metabolism*

*Cyp2e1* mRNA was increased ($P < 0.05$) by both DMBA treatments after 4d of exposure, and there was a trend ($P < 0.1$) for an increase after 2d. Interestingly, the low DMBA exposure induced a greater increased in *Cyp2e1* at the 4d time point (Figure 3A). *Gstpi* was increased ($P < 0.05$) in a temporal and concentration dependent pattern by the high and low DMBA exposures increasing *Gstpi* mRNA level after 1d and 4d, respectively (Figure 3B). There was no impact of DMBA exposure on *Gstmu* mRNA until 4d in the low exposure when *Gstmu* was increased ($P < 0.05$; Figure 3C). *Ephx1* mRNA was increased ($P < 0.05$) by low DMBA after 4d, with a trend ($P < 0.1$) for a lesser increase at the high DMBA concentration after 2d of exposure (Figure 3D).

*Induction of genes involved in oxidative stress response by DMBA exposure*

Both DMBA exposures increased ($P < 0.05$) *Sod1* mRNA after 2d, however, this increase was returned to control levels after 4d in the high DMBA exposure, while there was a trend ($P < 0.1$)
for increased Sod1 to be maintained after 4d at the low DMBA exposure (Figure 4A). Sod2 was increased at the high DMBA exposure after 2d, and there was a trend for an increase in Sod2 by the low DMBA (P = 0.1) after 2d (Figure 4B).

**Induction of autophagy genes by single DMBA exposure**

There was no impact of high DMBA exposure on Atg7 mRNA expression, however, the low concentration tended (P < 0.1) to reduce Atg7 mRNA after 2d with a further decrease (P < 0.05) after 4d (Figure 5A). Beclin1 mRNA was increased by low DMBA after 2d (P < 0.1) and 4d (P < 0.05) while high DMBA tended to increase Beclin1 after 2d with a return to basal levels after 4d (Figure 5B).

**Impact of DMBA exposure on PI3K gene expression**

There was a temporal concentration dependent pattern of Kitlg induction with the high DMBA exposure increasing Kitlg after 2d, while the low DMBA exposure lagged and induced Kitlg mRNA after 4d. There was also a trend for induced Kitlg after 2d in the low DMBA exposure (Figure 6A). Neither concentration of DMBA affected cKit mRNA level (Figure 6B), while there was also a temporal concentration dependent pattern of Akt1 mRNA induction (Figure 6C). The high DMBA exposure induced increased (P < 0.05) Akt1 mRNA after 1d, was at control levels after 2d but increased (P < 0.05) again after 4d of exposure. At the low DMBA concentration, Akt1 was increased (P < 0.05) after 2 and 4d of exposure (Figure 6C).
Effect of acute DMBA exposure on protein localization and level

EPHX1 protein was localized to the oocyte cytoplasm and interstitial tissue. No impact of DMBA exposure on EPHX1 protein was observed in the oocyte cytoplasm or in the ovary as a whole at either DMBA concentration (Figure 7A-D). BECN1 protein appeared as punctate foci localized mainly in the granulosa cell layer. A greater number of BECN positive foci were observed in ovaries treated with 12.5 nM DMBA, relative to control or 75 nM DMBA treated ovaries (Figure 7E-H). cKIT was localized to the oocyte cytoplasmic membrane of primordial and small primary follicles. There was a trend for decreased cKIT protein concentration in 75 nM DMBA treated ovaries but no impact of 12.5 nM DMBA related to control treated ovaries (Figure 7I-L). Finally, pAKT\textsuperscript{Thr308} was present in the oocyte cytoplasm of follicles of all stages. Quantification of pAKT\textsuperscript{Thr308} demonstrated higher levels ($P < 0.05$) in the oocytes of small and large follicles exposed to 12.5 nM DMBA relative to control and in the large follicles in 75 nM DMBA-treated ovaries (Figure 7M-P).

Discussion

DMBA is an ovotoxicant that causes follicle depletion, ultimately resulting in ovarian failure (Keating et al., 2008; Igawa et al., 2009; Nteeba et al., 2014). This study was designed to focus on effects of DMBA on large primary and secondary follicles by allowing these follicles to develop in culture before DMBA exposure. Previously, follicle loss was observed after continuous exposure (alternate days) to 75 nM DMBA but not 12.5 nM (Igawa et al., 2009). Relative to control, we observed that a single concentration of DMBA at either concentration did not deplete primordial or small primary follicles at either timepoint examined, while both DMBA concentrations depleted large primary follicles. The low DMBA concentration caused loss of
secondary follicles also. These data support that even low level acute DMBA exposures can affect and deplete the number of large primary and secondary follicles, potentially causing temporary infertility in exposed females. While previous studies have shown that all stage follicles are depleted by DMBA (Rajapaksa et al., 2007; Igawa et al., 2009), our data may suggest that the larger follicles could be the initial targets of DMBA, and that depletion of smaller follicles stages may be a consequence of loss of larger follicles, that is, increased recruitment from the pool of smaller follicles may occur to replace the larger follicles that have been lost. Alternatively, it may be that higher DMBA concentrations can directly deplete the primordial and small primary follicle pools, as has been observed previously (Igawa et al., 2009). Taken together, these data support that acute, low-level DMBA exposure represents a valid female health concern and warrants attention.

It was both interesting and surprising that there appeared to be a difference in depletion of the large primary and secondary follicles between low and high DMBA exposures, which led to the hypothesis that DMBA may induce concentration-dependent differential ovarian gene expression in the neonatal rat ovary. Over a time course of DMBA exposure, we discovered that both concentrations increased Cyp2e1 mRNA level, and this was highest at the low DMBA concentration. Though there is little data in the literature to suggest that CYP 2E1 is involved in DMBA biotransformation, our data showing that ovarian Cyp2e1 mRNA level increases in response to DMBA exposure suggests that this gene is responsive to DMBA exposure and may support a role for CYP2E1 in DMBA metabolism. In support of this possibility, Cyp2e1-null mice exposed to DMBA (0.5µM and higher) exhibited greater primordial and small primary follicle loss relative to controls (Keating et al., 2008).
The GST family of enzymes mediate xenobiotic metabolism by conjugating GSH to compounds, promoting excretion from the body (Reddy et al., 1983). In cultured rat pre-ovulatory follicles, co-treatment with GSH during DMBA exposure lessened follicle apoptosis (Tsai-Turton et al., 2007) suggesting the possible involvement of GST enzymes in catalyzing detoxification of DMBA. Gstpi has previously been identified to be involved in the ovarian response to DMBA exposure in neonatal rat ovaries (Bhattacharya and Keating, 2012), and adult mice exposed to DMBA (Nteeba et al., 2014). Gstpi-null mice are also more sensitive to DMBA-induced skin tumors (Henderson et al., 1998). GSTMU has also been demonstrated to be increased in response to DMBA in the ovaries from obese adult mice (Nteeba et al., 2014). In addition to the role of GSTPI and GSTMU in catalyzing GSH conjugation to xenobiotics, they can also play a role in inhibiting the pro-apoptotic proteins c-Jun N-terminal Kinase (JNK) (Keating et al., 2010; Bhattacharya and Keating, 2012) and apoptosis signal-regulating kinase 1 (ASK1) (Bhattacharya et al., 2013).

The current study determined that Gstmu is increased by the low DMBA exposure, with no impact of a higher exposure. Gstpi follows a temporal and concentration dependent pattern of increase with an increase in the high DMBA after 1d, but low DMBA after 4d. These data demonstrate that even low-level exposures activate the ovarian response to xenobiotic exposure, and underscore the importance of the timepoint examined in terms of an ovarian response to an ovotoxic exposure. GSTMU and GSTPI are thus likely involved in ovarian DMBA biotransformation, however, it remains unclear which role (regulation of detoxification or apoptosis) of GSTPI and GSTMU predominates during ovarian DMBA exposure. However, it is clear that these data are in agreement with those previously demonstrated in cultured rat pre-
ovulatory follicles, though the DMBA concentrations used in that study were higher than our treatments (Tsai-Turton et al., 2007).

EPHX1 is increased in response to ovarian DMBA exposure and bioactivates DMBA to the ovotoxic metabolite DMBA-3,4-diol, 1,2-epoxide (Rajapaksa et al., 2007; Igawa et al., 2009; Nteeba et al., 2014). Interestingly, although a trend for an increase in Ephx1 mRNA at the 75 nM DMBA concentration was observed, the low DMBA had a greater increase in Ephx1 after 4d of exposure. This could potentially result in increased bioactivation of DMBA to the more ovotoxic metabolite. Although increased EPHX1 protein was not observed in the current study it is possible that basal EPHX1 is depleted by low concentrations of DMBA at a higher rate than high DMBA, and that this potentially results in increased Ephx1 mRNA as a feedback mechanism, though this has not previously been reported for Ephx1. If this is the case, ovaries treated with low DMBA could therefore be exposed to higher amounts of the ovotoxic DMBA metabolite, while lack of any increase in Ephx1 mRNA in the high DMBA-treated ovaries may indicate less biotransformation of DMBA to the active form above the normal ovarian level due to basal EPHX1 contribution.

Oxidative stress contributes to DMBA-induced follicular apoptosis likely through formation of ROS (Tsai-Turton et al., 2007), and Sod1 and Sod2 play essential roles in the detoxification of ROS. Sod1 was increased by low DMBA after 2d with a trend for an increase after 4d of exposure, and after 4d by the high DMBA. Interestingly, Sod2 was increased by high DMBA with only a trend for an increase by low DMBA exposure at the same timepoint. These data suggest that ROS are formed following DMBA treatment and the ovary induces an oxidative stress response to combat the harmful oxygen molecules. Previous studies have shown that large pre-antral and antral follicles are particularly sensitive to ROS, in contrast to
primordial and small primary follicles which are more resistant, thus an increase in ROS at the low DMBA exposure may partially explain the loss of secondary follicles (Devine et al., 2002; Tsai-Turton et al., 2007). These findings provide additional evidence that DMBA induces production of ROS in the ovary, and that oxidative stress may play a role in DMBA-induced follicle depletion.

Cigarette smoke exposure increases expression of genes involved in the autophagy response in mouse ovaries (Gannon et al., 2012). We observed that, in the cultured rat ovary, compared to control, Atg7 is decreased by low but not high DMBA exposure. Becn1 mRNA levels increased after both treatments, again demonstrating temporal pattern of elevation which peaks in the high before the low DMBA. In addition, increases in BECN1 foci were observed at the low DMBA exposure. Autophagy may be activated as a means of follicle depletion and involved in DMBA-induced follicle loss, consistent with data in cigarette-smoke exposed mouse ovaries (Gannon et al., 2012). These results add to the growing number of studies generating evidence for ovarian autophagy playing an active role in follicle depletion.

PI3K signaling is essential for primordial and small primary follicle viability, regulation of follicular recruitment into the growing pool (Yoshida et al., 1997; Parrott and Skinner, 1999; Castrillon et al., 2003) and for regulation of ovarian xenobiotic metabolism gene expression (Bhattacharya and Keating, 2012). The key components of this signaling pathway investigated in this study were the granulosa expressed ligand, Kitlg; the oocyte receptor, cKit; and the signaling molecule, Akt1. It is worth noting that the earliest observed mRNA change in this study was that of Akt1. Low DMBA exposure increased mRNA levels of Kitlg and Akt1 while high DMBA exposure also increased Kitlg, earlier than the low treatment, and increased Akt1 also at an earlier timepoint. Interestingly, no impact of either DMBA exposure on cKit was
observed. However, cKIT protein was reduced, but the proxy molecule for PI3K activation, pAKTThr308 was increased by both DMBA concentrations, supporting increased PI3K activation. This increase in the PI3K pathway could partially explain why the primordial and small primary follicles were protected from DMBA exposure. PI3K inhibition increases DMBA-induced primordial and small primary follicle loss (Keating et al., 2009), thus our data supports that finding because the induction of the PI3K pathway observed may have prevented primordial and primary follicle loss.

The data herein support that acute low-level exposure to DMBA is a concern for female fertility due to depletion of large primary and secondary follicles, and is consistent with the reduced fecundity experienced by many female smokers (Baird and Wilcox, 1985; Alderete et al., 1995). While large primary were depleted by both DMBA exposures, and secondary follicles lost only during the low exposure, it is worth considering that the numbers within these follicle classes are low, thus small changes can appear significantly different, thus, we consider it premature to assume that differential follicle loss occurred. Undoubtedly however, low concentrations of DMBA depleted large ovarian preantral follicles. Also, genes involved in xenobiotic biotransformation, the oxidative stress response, autophagy and PI3K signaling are activated as part of the ovarian response to DMBA exposure.
### Figures

**Primers used in real-time PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
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<tbody>
<tr>
<td>Ephx1</td>
<td>GGCTCAAAGCCATCAGGCA</td>
<td>CCTCCAGAAGGACACCACTTT</td>
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<td>GSTp</td>
<td>GGCATCTGAAGCCTTTTGAG</td>
<td>GAGCCACATAGGCAGAGAGC</td>
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<td>GSTm</td>
<td>TTCAAGCTGGGCTGGAC</td>
<td>CAGGATGGCATTGCTCTG</td>
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<td>Cyp2e1</td>
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<td>CAATCACACCACAAGG</td>
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<td>Gapdh</td>
<td>GGATGGAATTGTGAGGGAGA</td>
<td>GTGGACCTCATGGCCTACAT</td>
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**Table 1.** RT-PCR primer sequences.
Figure 1. Impact of single DMBA exposure on follicle morphology and number.
PND4 rat ovaries were cultured for 4 d in control media and thereafter exposed once to 1% DMSO (vehicle control) or 12.5 nM DMBA or 75 nM DMBA. Following four additional days of culture, follicles were classified and counted: (A) Primordial Follicles; (B) Small Primary Follicles; (C) Large Primary Follicles; (D) Secondary Follicles. Values represent mean ± SE total follicles counted/ovary, n=5. * = P < 0.1.
Figure 2. Effect of single DMBA exposure on follicle morphology and number.
PND4 rat ovaries were cultured for 4 d in control media and thereafter exposed once to (A) 1% DMSO (vehicle control) or (B) 12.5 nM DMBA or (C) 75 nM DMBA. D depicts DMBA-induced shrunken oocytes indicated by the open arrow. Following eight additional days of culture, follicles were classified and counted: (E) Primordial Follicles; (F) Small Primary Follicles; (G) Large Primary Follicles; (H) Secondary Follicles. Values (E-H) represent mean ± SE total follicles counted/ovary, n=5; * = different from control in each follicle type, P < 0.05.
Figure 3. Effect of DMBA on ovarian expression of chemical biotransformation genes. PND4 rat ovaries were cultured for 4 d in control media and thereafter exposed to a single 1% DMSO (vehicle control) or DMBA (12.5 nM or 75 nM). Following 1, 2 or 4 additional days of culture, mRNA was isolated and (A) Cyp2e1, (B) Gstp, (C) Gstm, and (D) Ephx1 levels evaluated by quantitative RT-PCR. Values represent fold-change ± SEM relative to a control value of 1, normalized to Gapdh. * = different from control, $P < 0.05$; # = $P < 0.1$. 
Figure 4. Effect of DMBA on ovarian expression of reactive oxygen species metabolism genes.
PND4 rat ovaries were cultured for 4 d in control media and thereafter exposed to a single 1% DMSO (vehicle control) or DMBA (12.5 nM or 75 nM). Following 1, 2 or 4 additional days of culture, mRNA was isolated and (A) Sod1 or (B) Sod2 levels evaluated by quantitative RT-PCR. Values represent fold-change ± SEM relative to a control value of 1, normalized to Gapdh. * = different from control, $P < 0.05$; # = $P < 0.1$. ## = $P = 0.1$. 
Figure 5. Effect of DMBA on ovarian expression of autophagy genes.
PND4 rat ovaries were cultured for 4 d in control media and thereafter exposed to a single 1% DMSO (vehicle control) or DMBA (12.5 nM or 75 nM). Following 1, 2 or 4 additional days of culture, mRNA was isolated and (A) Atg7 or (B) Beclin1 levels evaluated by quantitative RT-PCR. Values represent fold-change ± SEM relative to a control value of 1, normalized to Gapdh. * = different from control, $P < 0.05$; # = $P < 0.1$. 

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Figure 6. Effect of DMBA on ovarian expression of autophagy genes.
PND4 rat ovaries were cultured for 4 d in control media and thereafter exposed to a single 1% DMSO (vehicle control) or DMBA (12.5 nM or 75 nM). Following 1, 2 or 4 additional days of culture, mRNA was isolated and (A) Kitlg or (B) cKit or (C) Akt1 levels evaluated by quantitative RT-PCR. Values represent fold-change ± SEM relative to a control value of 1, normalized to Gapdh. * = different from control, $P < 0.05$; $\#$ = $P < 0.1$. 
Figure 7. Localization and effect of DMBA on ovarian EPHX1, BECN1, cKIT or pAKT\textsuperscript{Thr308} protein.
PND4 rat ovaries were cultured for 4 d in control media and thereafter exposed to a single (A) 1% DMSO (vehicle control) (B) 12.5 nM DMBA or (C) 75 nM DMBA. Following four additional days of culture, ovaries were fixed in formalin, and immunofluorescence staining was performed using a primary antibody directed against (A-C) EPHX1, (E-G) BECN1, (I-K) cKIT or pAKT\textsuperscript{Thr308}. EPHX1 is represented in green and the Hoechst nuclear stain is in blue. BECN1, cKIT and pAKT\textsuperscript{Thr308} are represented in red and the Hoechst nuclear stain is in green. Quantification of (D) EPHX1, (H) BECN1, (L) cKIT or (P) pAKT\textsuperscript{Thr308} was performed and * = different from control, $P < 0.05$; # = $P < 0.1$. 
References


Chapter 6. DISSERTATION CONCLUSION

As described in this dissertation, the ovarian follicle reserve is essential to sustain fertility throughout the lifespan of a woman. When this vital pool is compromised by ovotoxicants such as PM or DMBA, the effects of these chemicals are apparent both at the molecular level as described herein, but also systemically by affecting a woman’s ability to conceive, her overall health and potentially even her quality of life, since many negative health associations are increased post-menopause. Although infertility affects millions of women around the world, there is much to learn about ovarian physiology and toxicology, thus, the goal of this dissertation was to help close some of these knowledge gaps by gaining an understanding of how ovotoxic compounds contribute to infertility and characterizing the ovarian protective response to such exposures.

Volatile Ovotoxic Compound

Despite PM’s recognition as the cancer-fighting and follicle-killing metabolite of the chemotherapy drug CPA (Plowchalk and Mattison, 1991; Desmeules and Devine, 2006), little is known about the mechanisms that are activated upon ovarian exposure to PM. In our endeavor to understand the mechanism(s) of PM-induced follicle depletion, we discovered that PM partitions to a volatile and ovotoxic compound, which we assume to be CEZ. Although the generation of CEZ is known to occur in vitro and has been demonstrated in other tissues, we were first to characterize its ovotoxicity. We found that CEZ and PM each induced follicle loss after 4 days in the neonatal rat ovary and that CEZ appears to primarily target the primordial follicle pool (Madden et al., 2014). The generation of CEZ was
confirmed to be a spontaneous reaction, independent of ovarian metabolism (Madden et al., 2014).

Upon discovery of the generation of CEZ occurring in our culture system, separate incubators and separate incubation times per experiment were employed to eliminate contamination and improve exposure consistencies across treatments. To reduce risk of our own exposure, the incubator containing PM or CEZ treatments was maintained in a fume hood. Although literature strongly supports CEZ as the volatile compound generated in our system, attempts were made to capture and confirm that CEZ was, in fact, our volatile compound of interest. Despite our efforts, we were unable to confirm this, as many questions still remain at to how much of PM is converted to CEZ in our system, when is this conversion occurring, how long does it persist, and without a standard CEZ to compare to, this was an uphill battle.

In agreement with previous reports, we too concluded that, “the chemistry of PM is complex and very condition-dependent” (Shulman-Roskes et al., 1998). Regardless of its challenges, the potential impact of the generation of CEZ and its ovotoxicity is substantial. Potentially, CEZ, not PM, is the active metabolite of CPA, which would help explain some of the confusion in the field as to how PM elicits its toxic effects while having a very short half-life and poor membrane permeability (Shulman-Roskes et al., 1998). Additionally, CEZ generation was originally observed in rat exhalants who had received CPA (Rauen and Norpoth, 1968), thus anyone receiving CPA may be expiring CEZ from their lungs and exposing those in close proximity such as doctors, nurses and family members to this ovotoxicant. Taken together, our data and the reports of others suggest that CEZ is a
chemical requiring further investigation to understand its role in ovotoxicity as well as chemotherapy.

**Phosphoramide Mustard (PM)**

With the appreciation that our PM treatments also include the effects of CEZ, we moved forward to investigate the mechanism underlying PM-induced infertility using the neonatal rat ovary culture system previously described. This system is advantageous for these studies since hepatic biotransformation contributions are removed, allowing direct ovarian exposure to be studied.

**Metabolism:** PM exposure altered the mRNA and protein levels of the key xenobiotic metabolism genes EPHX1, GSTM, and GSTP, thus suggesting their involvement in the ovarian response to PM. By competitively inhibiting EPHX1, we found that this enzyme likely contributes to the detoxification of PM and/or CEZ. Manipulation of GSH levels demonstrated that increasing GSH levels in the ovary reduces PM-induced follicle loss and decreasing the GSH levels has no impact on PM ovotoxicity. In this experiment, the addition or depletion of GSH alone reduced follicle number and promoted further questions, not answered by current literature, regarding the GSH levels present in neonatal rat ovary, particularly in the small preantral follicles. Additionally, our work has identified novel genes to be altered during PM exposure through use of a PCR array. This work therefore has opened up many avenues for further exploration.
Autophagy: PM-induced follicle loss has been previously reported (Plowchalk and Mattison, 1991; Desmeules and Devine, 2006) and was confirmed in our neonatal rat ovary culture system (Madden et al., 2014), but the PCD mechanism driving PM follicle depletion is unknown. PM-treated ovaries do not have an increased in atretic (unhealthy) follicles to mirror loss of the healthy population, suggesting rapid clearance of these follicles is occurring (Madden, unpublished), thus autophagy was hypothesized to be a PCD mechanism activated by PM.

Increased mRNA levels of PUMA, p21 and Becn1, and decrease in Caspase-1, provided encouragement that autophagy could be occurring following PM exposure, which was furthered by also finding an increase in BECN1 protein level. Our hypothesis was confirmed via visualization by TEM of the formation of autophagosomes and within these structures seeing the digestion of organelles such as mitochondria. To gain an understanding for the functional role of PM-induced autophagy, LY294002 was used to inhibit autophagy via inhibition of the PI3K pathway and rapamycin was used to activate autophagy by inhibiting mTOR. LY294002 had no impact on the ovotoxicity of PM, while rapamycin prevented PM-induced follicle loss. While it appears that PM-induced activation of autophagy is occurring, however, we appreciate that the pathways manipulated are complex and this area is a noteworthy one for further investigation to understand the mechanism preventing PM follicle depletion.

7,12-dimethylbenz[a]anthracene (DMBA)

In contrast to PM, DMBA ovotoxicity has been focus of many studies and although its bioactivation is well characterized, its detoxification and mechanism of follicle destruction
remain unclear. Therefore, the focus of our study was to investigate the effect of DMBA on the larger preantral follicles by allowing PND4 rat ovaries to develop over 4 d in culture before receiving a single exposure to 12.5 nM or 75 nM DMBA, which are both considered as low-dose exposures relative to prior studies, and may mimic those received through passive cigarette smoke exposure. Our results indicated that these acute low-dose DMBA exposures still invoked an ovarian response, resulting in follicle loss of large primary (12.5 nM and 75 nM) and secondary (12.5 nM) follicles as well as altered mRNA levels of numerous genes including those involved in xenobiotic metabolism, autophagy, the PI3K pathway and the oxidative stress response. Of note, while most of the studies outlined in this dissertation and in the literature examine the effects of repetitive ovotoxicant exposures, this DMBA study sheds light on the value of low-level and acute exposure studies.

**Future Areas of Interest**

Each of the PM studies described in this dissertation are the initial steps towards understanding how CPA-induced infertility occurs. Additional research is required to confirm CEZ as the volatile, ovotoxic compound, which in itself will require investigation in order to gain an understanding for the kinetics of this reaction. Our results suggest that EPHX1 likely plays a key role in the metabolism of PM or, as we hypothesize, in the detoxification of CEZ. The EPHX1 inhibitory experiment could be expanded to include treatments similar to that of the GSH experiment, so that the ovaries exposed to the volatile compound are also treated with the EPHX1 inhibitor. Another interesting avenue would be to investigate the effect of CPA and its metabolite using an EPHX1 knockout mouse in
comparison to wild type. This experiment would provide the opportunity to investigate pharmokinetic, biological and morphological differences between treatment and genotypes.

Another future area of interest includes the investigation of the level of GSH in the primordial and small primary follicle, which the neonatal rat ovary will provide a great model to understand this because at 4 days of age, the rat ovary only consists of these small follicles. There is no information in the literature regarding these levels, thus, considering the importance of GSH conjugation in many physiological functions, understanding the level of GSH in these early follicle types could have a large impact in reproductive toxicology as it is largely assumed that GSH conjugation is occurring to detoxify ovotoxicants and protect this value pool of follicles. Furthermore, generation of GSH conjugated PM compounds would also further our knowledge on the ovotoxicity (increased or decreased) that GSH conjugation imparts.

An avenue for further exploration that has been generated by these studies is to understand the action of autophagy following ovotoxicant exposures like PM and DMBA. For PM, in particular, follicle loss was prevented by using a clinically available compound, rapamycin to activate autophagy via inhibition of mTOR, while inhibition of the upstream PI3K pathway with LY294002 did not impact PM follicle depletion. Performing these same studies during DMBA exposure would aid in understanding if autophagy is a common phenomenon induced by ovotoxicants. Additional molecular studies using rapamycin and LY294002 will need to be done to understand the impact of these pathway manipulations on gene expression up- and downstream of the target pathway.

Lastly, while the PND4 rat ovary culture method used is a great model to understand the direct impact of chemicals on the ovary as well as enable us to manipulate pathways,
validation of these results \textit{in vivo} will be a critical step to fully understand CPA-induced fertility.

\textbf{Dissertation Summary}

Ovotoxicants, such as DMBA and PM, elicit an ovarian response that includes activation of xenobiotic metabolism, autophagy and altered PI3K signaling in order to maintain a balance between pro-survival and pro-death all with the main goal of ensuring quality oocytes for the production of the next generation. For PM, EPHX1 and mTOR are good candidate proteins for further investigation as both were shown in our model to have a determining role in the ovotoxicity of PM. In addition, the potential involvement of CEZ in the ovotoxic, and presumably antineoplastic, effects of CPA is of great interest and concern. Unfortunately, much is left to be learned in this field before fertility preservation techniques are able to provide the promise of fertility following chemotherapy or other harmful exposures.
References


## APPENDIX

<table>
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<tr>
<th>Gene Name</th>
<th>Symbol</th>
<th>P-value</th>
<th>Fold Change</th>
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Table 1. Effect of PM exposure on ovarian expression of glucose metabolism genes. PND4 rat ovaries were treated with 1% DMSO (vehicle control), PM (30 μM), or PM (60 μM) for 2 d. Following RNA isolation, mRNA levels were quantified with an RT^2 Profiler PCR array. Values represent fold-change ± SEM relative to a control value of 1, normalized to Hprt1 and Rplp1. * = different from control, P < 0.05; † = P < 0.1.
GLUTATHIONE S-TRANSFERASE CLASS MU REGULATION OF APOPTOSIS SIGNAL-RELATED KINASE 1 PROTEIN DURING VCD-INDUCED OVOTOXICITY IN NEONATAL RAT OVIES

A paper published by *Toxicology and Applied Pharmacology*

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**Contribution Statement:**
I contributed to the mRNA results, assisted in interpretation and contributed to writing the manuscript; Bhattacharya, P., did a significant portion of the experiments and writing of the manuscript; Sen, N., performed the experiments for Figure 2. Hoyer, P.B., was the post-doctoral mentor for Keating, A.F. thus provided some of the animals and edited the manuscript; Keating, A.F. designed experiments, assisted in data interpretation and figure construction, edited the manuscript and served as corresponding author.

**Abstract**

4-vinylcyclohexene diepoxide (VCD) destroys ovarian primordial and small primary follicles via apoptosis. In mice, VCD exposure induces ovarian mRNA expression of glutathione S-transferase (GST) family members, including isoform mu (*Gstm*). Extravascular GSTM negatively regulates pro-apoptotic apoptosis signal-related kinase 1 (ASK1) through protein complex formation, which dissociates during stress, thereby initiating ASK1-
induced apoptosis. The present study investigated the ovarian response of *Gstm* mRNA and protein to VCD. Induction of *Ask1* mRNA at VCD-induced follicle loss onset was determined. Ovarian GSTM:ASK1 protein complex formation was investigated and VCD exposure effects thereon evaluated. Phosphatidylinositol-3 kinase (PI3K) regulation of GSTM protein was also studied. Postnatal day (PND) 4 rat ovaries were cultured in control media ±: 1) VCD (30 μM) for 2-8d; 2) VCD (30 μM) for 2d, followed by incubation in control media for 4d (acute VCD exposure); or 3) LY294002 (20 μM) for 6d. VCD exposure did not alter *Gstm* mRNA expression, however, GSTM protein increased (*P* < 0.05) after 6d of both the acute and chronic treatments. *Ask1* mRNA increased (0.33-fold; *P* < 0.05) relative to control after 6d of VCD exposure. Ovarian GSTM:ASK1 protein complex formation was confirmed and, relative to control, the amount of GSTM bound to ASK1 increased 33% (*P* < 0.05) by chronic but with no effect of acute VCD exposure. PI3K inhibition increased (*P* < 0.05) GSTM protein by 40% and 71% on d4 and d6, respectively. These findings support involvement of GSTM in the ovarian response to VCD exposure, through regulation of pro-apoptotic ASK1.

**Introduction**

The ovary is the female gonad composed of oocyte-containing follicles at various stages of development. Females are born with a finite number of the most immature follicular stage, termed primordial follicles, which, once destroyed, cannot be regenerated (Hirshfield, 1991). Exposure to environmental factors that cause follicular damage and depletion can impair fertility and induce ovarian failure (menopause; Mattison *et al.*, 1980). An occupational chemical known to selectively destroy primordial and small primary
follicles in the ovaries of mice and rats is 4-vinylcyclohexene diepoxide (VCD, Kao et al., 1999; Smith et al., 1990). VCD is the active metabolite of 4-vinylcyclohexene (VCH), an industrial diluent for epoxides (IARC, 1976). Repeated daily dosing of rats with VCD (80 mg/kg/day, intraperitoneal) for 15 days results in approximately 50% depletion of primordial and small primary follicles relative to controls, with no significant effect on larger follicles or corpora lutea (Smith et al., 1990; Flaws et al., 1994; Springer et al., 1996a,b). VCD-induced primordial and small primary follicle loss begins on day 6 of in vitro exposure in postnatal day (PND) 4 cultured Fisher 344 (F344) rat ovaries (Keating et al., 2009). Mechanistic studies have revealed that VCD induces follicle loss by accelerating the natural process of atresia (apoptosis) in both a time- and dose-dependent manner (Springer et al., 1996a; Hu et al., 2001a; Devine et al., 2002).

VCH may be metabolized (bioactivated) in the ovary to VCD by the action of the cytochrome P450 (CYP) family member CYP 2E1 (Cannady et al., 2003; Rajapaksa et al., 2007). Cyp 2e1-null mice had less follicle loss compared to their wild type control littermates (Rajapaksa et al., 2007), suggesting the involvement of CYP 2E1 in VCH bioactivation. Conversely, VCD can be detoxified in the ovary to a less ovotoxic tetrol metabolite through the action of microsomal epoxide hydrolase (mEH) (Flaws et al., 1994; Cannady et al., 2002; Keating et al., 2008; Bhattacharya et al., 2012). Studies have shown that VCD induces mEH mRNA and protein expression both in vivo and in vitro prior to an observed follicle loss in mice and rats (Cannady et al., 2002; Keating et al., 2008; Bhattacharya et al., 2012). Additionally when mEH is inhibited in vitro using cyclohexene oxide (CHO) in the presence of VCD, more follicle loss results relative to those ovaries
treated only with VCD, thereby supporting an ovarian detoxification role for mEH during VCD exposure (Bhattacharya et al., 2012).

Glutathione (GSH) is a ubiquitous antioxidant that protects cells against oxidative stress and electrophilic compounds (DeLeve and Kaplowitz, 1991). GSH functions in two major ways: 1) through conjugation to electrophilic compounds resulting in their more rapid excretion from the body; or 2) directly reducing and neutralizing reactive oxygen intermediates in a coupled reaction involving GSH peroxidase (DeLeve and Kaplowitz, 1991). The glutathione S-transferase (GST) enzyme family catalyze conjugation of GSH to xenobiotics (Jakoby et al., 1978), and the ovary is capable of synthesizing GSH (Luderer et al., 2001). The mRNA encoding the GST isoforms pi (Gstp) and mu (Gstm) are increased in response to VCD exposure in cultured PND4 mouse ovaries (Keating et al., 2008). Additionally, VCD increases Gstp mRNA and protein in cultured PND4 rat ovaries prior to (d4) and at times of (d6, d8) VCD-induced follicle loss (Keating et al., 2010). These observations support that the GST isoforms mu and pi are involved in the ovarian response to VCD exposure, and may potentially catalyze VCD:GSH conjugation.

In addition to GSH conjugation, GST enzymes are involved in cell signaling pathway regulation (Cho et al., 2001; Dorion et al., 2002; Keating et al., 2010). GSTP forms a protein complex with c-JUN N-terminal kinase (JNK; Adler et al., 1999) and is likely to negatively regulate its action in the rat ovary (Keating et al., 2010). The amount of JNK protein bound to GSTP increased after 6 days of VCD exposure (Keating et al., 2010) with a concomitant decrease in unbound JNK and phosphorylated c-JUN (p-c-JUN) protein levels. Furthermore, a decrease in p-c-JUN preceded the increase in JNK bound to GSTP, indicating that JNK activity was impaired through the GSTP interaction (Keating et al., 2010).
Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase kinase (MAPKKK) which is capable of activating the pro-apoptotic JNK and p38 MAPK signaling pathways (Ichijo et al., 1997). In extra-ovarian tissues, GSTM physically interacts with ASK1 and functions to repress ASK1-mediated apoptosis (Cho et al., 2001). The GSTM:ASK1 protein complex dissociates in response to stress and thereby triggers activation of apoptosis by ASK1 (Dorion et al., 2002). It is therefore known that GSTM modulates stress-activity signals by suppressing ASK1. Interestingly, VCD-induced apoptosis involves the action of JNK and p-c-JUN (Hu et al., 2002). Thus, ovarian GSTM is potentially involved in the ovarian response to VCD exposure.

Phosphatidylinositol 3-kinase (PI3K) is a lipid kinase involved in various cellular functions including cell growth and survival, proliferation, and differentiation (Vivanco and Sawyers, 2007). In the ovary, the PI3K signaling pathway plays an important role in primordial follicles by maintaining oocyte viability and directing their recruitment into the growing follicular pool (Castrillon et al., 2003; Reddy et al., 2005; Liu et al., 2006). Impairment of PI3K signaling occurs during VCD-induced ovotoxicity (Keating et al., 2011), while PI3K inhibition prevented VCD-induced primordial follicle loss in cultured PND4 rat ovaries (Keating et al., 2009). Recently, it has been shown that the xenobiotic biotransformation enzymes mEH and Gstp are regulated by PI3K signaling in cultured PND4 rat ovaries and Gstm mRNA was increased by PI3K inhibition (Bhattacharya et al., 2012; Bhattacharya and Keating, 2012). Taken together, these findings also point to regulation of Gstm by PI3K signaling.

To determine if GSTM is associated with the ovarian response to VCD as well as in regulation of pro-apoptotic ASK1, several approaches were taken: the first was to study the
temporal pattern of *Gstm* mRNA and protein expression in response to VCD. The second was to determine any effect of VCD on *Ask1* mRNA expression at the time of the onset of VCD-induced follicle loss. The third approach was to establish the presence of a GSTM:ASK1 protein complex in the rat ovary and to study the effect of VCD exposure (both acute and chronic) upon this complex. Finally, because *Gstm* mRNA expression is increased in cultured PND4 rat ovaries during inhibition of the PI3K pathway (Bhattacharya and Keating, 2012) regulation of GSTM by PI3K was evaluated.

**Materials and Methods**

**Reagents:**

4-vinylcyclohexene diepoxide (VCD, mixture of isomers, >99% purity), ascorbic acid, transferrin, bovine serum albumin (BSA), 2-β-mercaptoethanol, 30% acrylamide/0.8% bis-acrylamide, ammonium persulphate, glycerol, N’N’N’N’-Tetramethyl-ethylenediamine (TEMED), Tris base, Tris HCL, sodium chloride, Tween-20 were purchased from Sigma-Aldrich Inc. (St Louis, MO). Dulbecco’s Modified Eagle Medium: nutrient mixture F-12 (Ham) 1x (DMEM/Ham’s F12), Albumax, penicillin (5000U/ml), Hanks’ Balanced Salt Solution (without CaCl₂, MgCl₂ or MgSO₄), *Gstm* and β-actin primers, and superscript III one-step RT-PCR system were obtained from Invitrogen Co. (Carlsbad, CA). *Gapdh* and *Ask1* primers were obtained from the DNA facility of the Office of Biotechnology at Iowa State University. Millicell-CM filter inserts and anti-GSTM antibodies were purchased from Millipore (Bedford, MA). The anti-ASK1 primary antibody was obtained from Cell Signaling Technology (Danvers, MA). 48-well cell culture plates were purchased from Corning Inc. (Corning, NY). RNeasy Mini kit, QIA shredder kit, RNeasy Mini Elute kit, and
Quantitect™ SYBR Green PCR kit were purchased from Qiagen Inc. (Valencia, CA). RNAlater was obtained from Ambion Inc. (Austin, TX). 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002; CAS#154447-36-6) was purchased from A.G. Scientific, Inc. (San Diego, CA). Pierce BCA protein Assay Kit was purchased from Thermo Scientific (Rockford, IL). The goat anti-rabbit secondary antibody was obtained from Thermo Scientific, (Lafayette, CO). Ponceau S was from Fisher Scientific (Asheville, NC). ECL plus chemical luminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK).

**Animals:**

Rats were housed two per cage in plastic cages and maintained in a controlled environment (22 ± 2°C; 12h light/12h dark cycles). The animals were provided a standard diet with *ad libitum* access to food and water, and allowed to give birth. All animal experimental procedures were approved by the Iowa State University’s Institutional Animal Care and Use Committee.

**In vitro ovarian cultures:**

Ovaries from PND4 Fisher 344 (F344) rats were cultured as described by Parrott and Skinner (1999). Briefly, pups were euthanized by CO₂ inhalation followed by decapitation. Ovaries were removed, trimmed of oviduct and other excess tissue and placed onto Millicell-CM membrane floating on 250 µl of DMEM/Ham’s F12 medium containing 1 mg/ml BSA, 1 mg/ml Albumax, 50 µg/ml ascorbic acid, 5 U/ml penicillin and 27.5 µg / ml transferrin per well in a 48 well plate previously equilibrated to 37°C. One ovary was placed in the control
treatment, while the contralateral ovary was exposed to the experimental treatment. A drop of medium was placed on top of each ovary to prevent it from drying. Ovaries were treated with vehicle control medium (1% DMSO), VCD (30 µM) or LY294002 (20 µM) and maintained at 37°C and 5% CO₂ for 2-8 d. For chronic VCD exposure, ovaries were continuously exposed to VCD (30 µM) on alternate days and samples were collected accordingly. For the acute VCD exposure, ovaries were exposed for 2d to VCD (30 µM) beginning at the onset of culture and were subsequently incubated in control medium for a further 4d for a total of 6d of culture. The concentration (30 µM) of in vitro VCD exposure was previously determined to cause about 50% loss of both primordial and small primary follicles after 6d (Devine et al., 2002; Keating et al., 2009). The concentration of LY294002 (20 µM) was previously shown to be effective in the rat PND4 ovary culture system (Keating et al., 2009). For cultures lasting for more than 2d, media was changed every alternate day and treatment added.

**RNA isolation and polymerase chain reaction (PCR):**

Following 2, 4, 6 or 8 d of in vitro culture, ovaries treated with control or VCD (30 µM) were stored in RNA later at -80°C. Total RNA was isolated from ovaries (n=3; 10 ovaries per pool) using an RNeasy Mini kit (Qiagen) following the manufacturer’s instructions. RNA was eluted in 14ul of RNase-free water and the concentration was determined using a NanoDrop (λ=260/280 nm; ND 1000; Nanodrop Technologies Inc, Wilmington, DE). Total RNA (500 ng) was reverse transcribed to cDNA using Superscript III One-Step RT-PCR System (Invitrogen). Gstm and β-actin (Actb) were amplified on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). An Eppendorf mastercycler (Hauppauge, NY) was used to
amplify Gapdh and Ask1. A Quantitect™ SYBR Green PCR kit (Qiagen Inc. Valencia, CA) was used for amplification. The primers used for Gstm and Actb were those described in (Keating et al., 2008). The Gapdh primers were from Bhattacharya and Keating (2012). Ask1 primers were: forward - 5’-AGAAAAGGACCAAGAAATTAAGCAC-3’; reverse-5’-AACCGACTTATAGTGTCTTCGTCAG-3’. There was no difference in Actb or Gapdh mRNA expression level between vehicle control and VCD treated ovaries.

**Protein isolation:**

Pools (n = 3 pools; 10 ovaries per pool for Western blotting; 20 ovaries per pool for immunoprecipitation) of whole ovarian protein suspensions were obtained by homogenization in tissue lysis buffer containing protease and phosphatase inhibitors (Thompson et al., 2005). SDS-free lysis buffer was used to isolate protein for immunoprecipitation. After homogenization, samples were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min. Protein quantification was performed using a standard BCA protocol on a 96-well assay plate. Emission absorbance was detected with a λ= 540nm excitation on a Synergy™ HT Multi-Detection 1 Microplate Reader using KC4™ software (Bio-Tek® Instruments Inc., Winooski, VT). Protein concentrations were calculated from a BSA protein standard curve.

**Protein immunoprecipitation:**

Protein immunoprecipitation was performed as previously described (Bhattacharya and Keating, 2012). Briefly, ovarian protein (30 µg) was incubated overnight at 4°C with 15 µl of anti-ASK1 antibody. Protein G agarose beads were washed, added to the protein-antibody
mixture and incubated at 4°C for 3 h with shaking, followed by centrifugation at 10,000 rpm for 10 mins. The supernatant containing the unbound protein was saved for Western blotting. After washing with lysis buffer beads were incubated with Laemmli sample buffer (40 μl) at 95°C for 10 min. Thereafter, the beads were centrifuged and the bound protein (supernatant) fraction (40 μl) was collected.

**Western blot analysis:**

10% SDS-PAGE gels were used to separate proteins (n=3; 10 μg total protein or 20 μl unbound or bound immunoprecipitation fractions) followed by transfer onto nitrocellulose membranes as previously described (Thompson et al., 2005). Membranes were blocked for 1 h with shaking at 4°C in 5% milk in Tris-buffered saline with Tween-20 (TTBS). Primary antibody diluted in 5% milk in TTBS was added to the membranes and incubated overnight at 4°C with rocking. The GSTM and ASK1 antibody dilutions used were 1:200 and 1:1000. Membranes were washed three times (10 min) in TTBS and incubated in appropriate HRP-conjugated secondary antibody (1:2000) for 1 hr at room temperature. Membranes were washed three times (10 min) in TTBS followed by a single wash for 10 min in Tris Buffered Saline (TBS). Membranes were incubated in ECL plus chemiluminescence detection substrate for 5 min and exposed to X-ray film. Equal total protein loading was confirmed by Ponceau S staining of membranes. Densitometry of the appropriate bands was quantified using ImageJ software (NCBI). Each band was normalized to an appropriate internal control – Ponceau S for total protein measurements and to ASK1 for immunoprecipitation. The treatment values were compared to control values using Prism 5.04 software to determine any difference between experimental treatments.
**Statistical analysis:**

All statistical analyses were performed using Prism 5.04 software (GraphPad Software). One ovary from each animal was placed in control media while the contralateral ovary was placed in the experimental treatment, thus data were analyzed by paired t-tests comparing the treated ovaries to controls. Statistical significance was defined as $P < 0.05$. For Western blotting, statistical analysis was performed on the raw data comparing experimental treatment to the respective control at each time-point. For each experiment, the n-value equals three groups that consisted of 8-10 ovaries per group, and data are presented as mean value +/- standard error.

**Results**

*Temporal effect of chronic VCD exposure on Gstm mRNA expression in PND4 F344 rat ovaries*

To investigate the effect of VCD on ovarian *Gstm* mRNA, real-time PCR was performed over the time-course of VCD exposure; 2d, 4d, 6d or 8d. Relative to control ovaries, no change in *Gstm* mRNA expression was observed at any time point (Figure 1).

*Temporal effect of chronic VCD exposure on GSTM protein in F344 rat ovaries*

To determine any impact of VCD exposure on GSTM protein level, PND4 rat ovaries were cultured in control media or media containing VCD (30 μM) for 4d, 6d or 8d. There was no effect of VCD on GSTM protein level on d4. Relative to control-treated ovaries,
VCD increased ($P < 0.05$) GSTM protein level on d6 by 13% and on d8 by 9% (Figure 2A, B).

*Induction of Ask1 mRNA in F344 rat ovaries by VCD at the time of follicle loss onset*

To study if VCD induces *Ask1* mRNA, PND4 rat ovaries were cultured in control media or media containing VCD (30 μM) for 6 d. *Ask1* expression was measured on d6 since this time point coincides with the beginning of VCD-induced follicle loss. Relative to control-treated ovaries, *Ask1* mRNA was increased (0.33-fold; $P < 0.05$) by VCD exposure (Figure 3).

*Effect of chronic VCD exposure on GSTM:ASK1 protein complex formation in F344 rat ovaries*

To evaluate the presence of a GSTM:ASK1 protein complex in the ovary as well as to investigate the effect of VCD exposure on this complex during VCD exposure, PND4 F344 rat ovaries were collected at the time points prior to (d4) and at the time of (d6) VCD-induced follicle loss. A GSTM:ASK1 protein complex was detected in ovarian tissue (Figure 4A). There was no effect of VCD on the amount of GSTM bound to ASK1 on d4 but this was increased by 33% ($P < 0.05$) on d6 (Figure 4B). There was no impact of VCD exposure or ASK1 interaction on GSTM protein that was unbound to ASK1 at either time point (Figure 4C, D).
Acute VCD exposure effects on GSTM protein level in F344 rat ovaries

Whether a single (acute) exposure of PND4 rat ovaries for 2d to VCD was sufficient to induced increased GSTM protein levels was determined. Protein was collected 6d after the onset of culture. Relative to control-treated ovaries, GSTM protein was increased \( (P < 0.05) \) on d6 by 6\% (Figure 5A, B).

Determination of VCD acute exposure effect on the GSTM:ASK1 protein complex in F344 rat ovaries

To determine if acute VCD exposure could cause dissociation of the GSTM:ASK1 protein complex, ovaries were exposed to VCD for 2d and ovaries collected 6d after the onset of culture. There was no effect of VCD exposure on the amount of GSTM protein bound to ASK1 (Figure 6A, B). Also, no impact on the amount of GSTM in the unbound protein fraction was observed (Figure 6C, D).

Effect of PI3K inhibition on ovarian GSTM protein level

An increase in \( \text{Gstm} \) mRNA was previously demonstrated after 2d and 4d of PI3K inhibition (Bhattacharya and Keating, 2012). In order to investigate whether altered \( \text{Gstm} \) mRNA was correlated with GSTM protein after PI3K inhibition, LY294002 treated ovaries were collected on 4d and 6d. Inhibition of PI3K increased \( (P < 0.05) \) GSTM protein levels by 40\% and 71\% on d4 and d6, respectively (Figure 7A, B).
Effect of PI3K inhibition on ovarian Ask1 mRNA level

Any impact of PI3K inhibition on Ask1 mRNA expression was determined in ovaries that had been treated for 6d with LY294002. There was no effect of LY294002 treatment on Ask1 mRNA, relative to control treated ovaries (Figure 8).

Discussion

Ovarian GSTP is increased in response to VCD exposure and inhibits JNK activity through a protein:protein interaction, potentially contributing to the ovarian xenobiotic protective response (Keating et al., 2010). In the PND4 mouse ovary, it has been shown that VCD (30 μM) exposure increases Gstm mRNA expression prior to observed VCD-induced follicle loss (Keating et al., 2008). However, further investigation into a functional role for GSTM during VCD-induced ovotoxicity has been lacking. Therefore, this study investigated the ovarian response of Gstm to VCD exposure in cultured PND4 rat ovaries. Although there was no change at the mRNA level at any time point studied, GSTM protein level was increased at both 6d and 8d, and was highest at the time point at which follicle loss is first observed (d6). These results support an involvement of GSTM in the ovarian protective response to VCD exposure in rats.

Several studies have reported that GSTM inhibits the action of pro-apoptotic ASK1 (Cho et al., 2001; Dorion et al., 2002). This negative regulation of ASK1 by GSTM is through formation of an ASK1:GSTM protein complex. The complex is disrupted following external environmental stress stimulus such as heat shock (Dorion et al., 2002). Such stress releases GSTM from ASK1, and the unbound ASK1 initiates the c-JUN and p38 MAPK
signaling pathways in favor of apoptosis (Dorion et al., 2002; Ichijo et al., 1997). Thus, ASK1 is involved in activating apoptotic signaling pathways.

It is known that VCD-induced primordial and small primary follicle loss is via apoptosis (Hu et al., 2001; Devine et al., 2002; Springer et al., 1996a). Moreover, VCD-induced apoptosis involves activation of JNK resulting in phosphorylation of c-JUN in small pre-antral follicles which are the selective target of VCD (Hooser et al., 1994; Hu et al., 2002). Because ASK1 activates JNK signaling, a role for ASK1 during VCD-induced ovotoxicity was hypothesized. Additionally, GSTM is increased in response to VCD exposure, and can form a complex with ASK1 in extra-ovarian tissues as an anti-apoptotic mechanism. Thus, it was logical to investigate whether this was part of an ovarian protective response to VCD exposure. Our data demonstrates induction of Ask1 mRNA at the time of VCD-induced follicle depletion, thus supporting pro-apoptotic Ask1 involvement during VCD-induced ovotoxicity. It was further shown that ovarian GSTM protein is bound to ASK1 and that this complex is increased in response to VCD exposure at the time of follicle loss. This is similar to the response of a GSTP:JNK complex during both VCD (Keating et al., 2010) and DMBA exposures (Bhattacharya and Keating, 2012). There was no disruption of the GSTM:ASK1 protein complex by VCD in terms of changes in the unbound form of GSTM. Since the ovaries were exposed to VCD every alternate day, we surmised that this chronic exposure may be the reason for lack of a clear disruption in the protein complex, since GSTM may be continuously activated, and thus constantly binding ASK1. For this reason an experiment was performed to determine if a single acute VCD exposure would unveil more radical changes in the GSTM:ASK1 complex. GSTM protein was confirmed to increase in this paradigm, albeit marginally, however no major impact on GSTM:ASK1
protein binding was observed. It may however be the case that more distinct changes may have taken place immediately after VCD exposure (d2 or d4). Collectively, from both the chronic and acute VCD exposures, it is evident that GSTM binds pro-apoptotic ASK1. GSTM-induced inactivation of ASK1 may also lead to reduced JNK activity as was evident during VCD-exposure (Keating et al., 2010) since ASK1 is an upstream regulator of JNK (Tobiume et al., 2001). Therefore, it is hypothesized that the complexing of GSTM with ASK1 is part of an ovarian protective response to VCD. That is, in response to VCD-induced apoptosis, GSTM may be involved in an effort to prevent toxicity through binding and suppressing pro-apoptotic action of ASK1 in cultured rat ovaries. Whether GSTM plays an additional role in GSH conjugation cannot be confirmed by these studies.

Inhibition of PI3K prevents VCD-induced primordial follicle loss (Keating et al., 2009) and VCD has been shown to inhibit phosphorylation of the PI3K members c-KIT, pAKT and to decrease total FOXO3 protein (Keating et al., 2011). Inhibition of PI3K signaling increases both mEH (Bhattacharya et al., 2012) and Gstp mRNA and protein expression (Bhattacharya and Keating, 2012), indicating that this pathway regulates ovarian xenobiotic metabolism gene expression. Also, inhibition of PI3K increases Gstm mRNA levels in the rat ovary (Bhattacharya and Keating, 2012). Because a role for GSTM in VCD-induced toxicity was supported by the data herein, an investigation of a regulatory role for PI3K in GSTM translation was made. GSTM protein was increased by PI3K inhibition on d4 and d6 of culture. Although we did not observe any impact of PI3K inhibition on Ask1 mRNA induction, it is possible that increased GSTM binding to ASK1 could result during PI3K inhibition however, this study cannot confirm that this is the case. Thus, these data further add to an understanding of the involvement of PI3K in xenobiotic biotransformation.
enzyme regulation (Bhattacharya et al., 2012; Bhattacharya and Keating et al., 2012). *Gstm* mRNA was increased in mouse ovaries exposed to VCD, while protein levels were not affected (Keating et al., 2008), thus there appears to be some species-specific differences in *Gstm* regulation. Whether this regulation is through control of *Gstm* transcription or translation remains to be determined, but could potentially involve the action of microRNA’s, as is the case for *Gstp* (Zhang et al., 2012; Patron et al., 2012).

VCD exposure decreases PI3K signaling (Keating et al., 2011) and it is hypothesized that this leads to impaired follicle viability, and accelerate activation of primordial follicles into the growing follicular pool, both of which would lead to premature ovarian failure (Keating et al., 2009). During the same time frame VCD also increases ovarian expression of *mEH* (Bhattacharya et al., 2012), *Gstp* (Keating et al., 2010) and *Gstm*, likely as a result of PI3K inhibition (summarized in Figure 9). PI3K regulates ovarian arylhydrocarbon receptor (*Ahr*) and nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*) mRNA and protein expression (Bhattacharya et al., 2012), both transcription factors that regulate xenobiotic metabolism genes, including *Gstm*, thus they may be involved in VCD-induced *Gstm* activation. Why the increased expression of the xenobiotic metabolism enzymes is insufficient to protect against VCD-induced follicular destruction remains unclear, but may result either because the enzymes are overwhelmed by continuous VCD exposure, or because VCD-induced impairment of oocyte viability becomes impossible to counteract.

In summary, GSTM appears to be involved in the ovarian response to VCD; potentially contributing to both VCD detoxification and prevention of apoptosis. Future studies are aimed at further characterizing *Gstm* involvement during VCD-induced ovotoxicity.
Conflict of Interest Statement

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Figure 1. Temporal effect of chronic VCD exposure on Gstm mRNA expression.
F344 PND4 rat ovaries were cultured in control (CT) media or media containing 30 µM VCD for 2-8 d. Following incubation, total RNA was isolated, reverse transcribed to cDNA and analyzed for Gstm and Actb mRNA expression by RT-PCR as described in materials and methods. Values are expressed as fold-change relative to control ± SE; n=3 (10 ovaries per pool).
Figure 2. Temporal effect of chronic VCD exposure on GSTM protein level.
F344 PND4 rat ovaries were cultured in control (CT) media or media containing 30 µM VCD for 4-8 d. Following incubation, total protein was isolated and Western blotting was performed to detect GSTM protein as described in materials and methods. (A) Representative GSTM Western blot on d6. (B) Quantification of GSTM protein levels on d4, d6, d8; Values are expressed as a percentage of control; n=3 (10 ovaries per pool). * P < 0.05; different from control.
Figure 3. Effect of chronic VCD exposure on *Ask1* mRNA expression.
F344 PND4 rat ovaries were cultured with control (CT) media or media containing 30 µM VCD for 6d. Following incubation, total RNA was isolated, reverse transcribed to cDNA and analyzed for *Ask1* mRNA expression by RT-PCR as described in materials and methods. Values are expressed as fold-change relative to control ± SE; n=3 (10 ovaries per pool). * P < 0.05; different from control.
Figure 4. Effect of chronic VCD exposure on the GSTM:ASK1 protein complex.
Ovaries from F344 PND4 rats were cultured in control media (CT) or media containing VCD (30 µM) for 4 or 6 d. Ovaries were exposed to VCD on alternate days from the beginning of culture. Following incubation, total protein was isolated and immunoprecipitation was carried out as described in materials and methods. (A) ASK1 immunoprecipitation followed by detection of GSTM protein on d4 or d6; control = c, VCD = v. (B) Quantification of the amount of GSTM protein bound to ASK1. (C) Representative Western blot for unbound GSTM protein on d6. (D) Quantification of the amount of unbound GSTM protein levels on d4 and d6. Values are expressed as a percentage of control; n=3 (20 ovaries per pool). * P < 0.05; different from control.
Figure 5. Effect of acute VCD exposure on GSTM protein level.
Ovaries from F344 PND4 rats were cultured in control (CT) media or media containing VCD (30 µM) for 6d with exposure to VCD at the onset of culture for 2d. Following incubation, total protein was isolated and Western blotting was performed to detect GSTM protein as described in materials and methods. (A) Representative Western blot on d6; control = c, VCD = v. (B) Quantification of GSTM protein levels, values are expressed as a percentage of control; n=3 (10 ovaries per pool). * \( P < 0.05 \); different from control.
Figure 6. Effect of acute VCD exposure on GSTM:ASK1 protein complex formation. Ovaries from F344 PND4 rats were cultured in control (CT) media or media containing VCD (30 µM) for 6d with exposure to VCD at the onset of culture for 2d. Following incubation, total protein was isolated and immunoprecipitation was carried out as described in materials and methods. (A) ASK1 immunoprecipitation, followed by detection of GSTM protein by Western blotting on d6; control = c, VCD = v. (B) Quantification of the amount of GSTM protein bound to ASK1, values are expressed as a percentage of control; n=3 (10 ovaries per pool). * P < 0.05; different from control. (C) Western blotting was performed on the unbound protein fraction to detect GSTM protein; control = c, VCD = v. (D) Quantification of the amount of unbound GSTM protein levels, values are expressed as a percentage of control; n=3 (20 ovaries per pool). * P < 0.05; different from control.
Figure 7. Effect of PI3K inhibition on ovarian GSTM protein level.
F344 PND4 rat ovaries were cultured in media containing control or LY294002 (20 µM) for 4d or 6d. Total protein was isolated and Western blotting was performed to detect GSTM protein levels. (A) Representative Western blot on d6; control = c, LY294002 = LY. (B) Quantification of Western blotting for GSTM protein, values are expressed as a percentage of control; n=3 (20 ovaries per pool). * P < 0.05; different from control.
Figure 8. Effect of PI3K inhibition on *Ask1* mRNA expression.

F344 PND4 rat ovaries were cultured with control (CT) media or media containing 20 µM LY294002 for 6d. Following incubation, total RNA was isolated, reverse transcribed to cDNA and analyzed for *Ask1* mRNA expression by RT-PCR as described in materials and methods. Values are expressed as fold-change relative to control ± SE; n=3 (10 ovaries per pool).
Figure 9. Proposed mechanistic model.
VCD exposure inhibits PI3K signaling, leading to increased GSTM protein, resulting in increased GSTM:ASK1 protein interaction.
References


