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Abstract
The finite ovarian follicle reserve can be negatively impacted by chemical exposures including the anti-neoplastic agent, cyclophosphamide (CPA). CPA requires bioactivation to phosphoramide 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 PMinduced 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.

Keywords
Phosphoramide mustard, chloroethylaziridine, ovotoxicity

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Involvement of a volatile metabolite during phosphoramide mustard-induced ovotoxicity

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Abstract

The finite ovarian follicle reserve can be negatively impacted by chemical exposures including the anti-neoplastic agent, cyclophosphamide (CPA). CPA requires bioactivation to phosphoramide 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.

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Conflict of Interest Statement:
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.

Keywords

Phosphoramide mustard; chloroethylaziridine; ovotoxicity

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 phosphoramide 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 6^{th} section was mounted and stained with hematoxylin and eosin. Healthy oocyte-containing follicles were identified and counted in every 6^{th} 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 comparing 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, 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.

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Highlights

- PM depletes all stage ovarian follicles in a temporal pattern
- A volatile ovotoxic compound is liberated from PM
- The volatile metabolite depletes primordial follicles

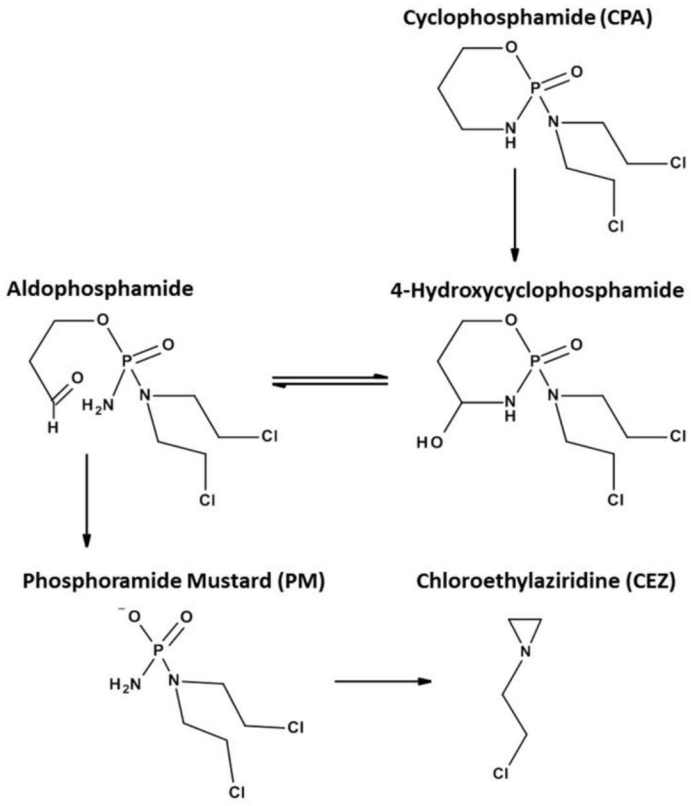

Figure 1. CPA metabolism

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.

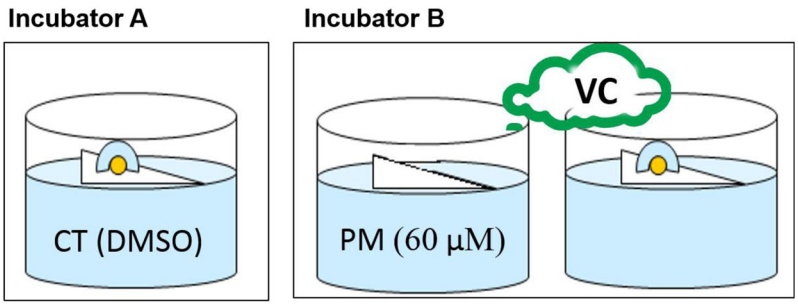

Figure 2. Experimental design to evaluate VC exposure

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.

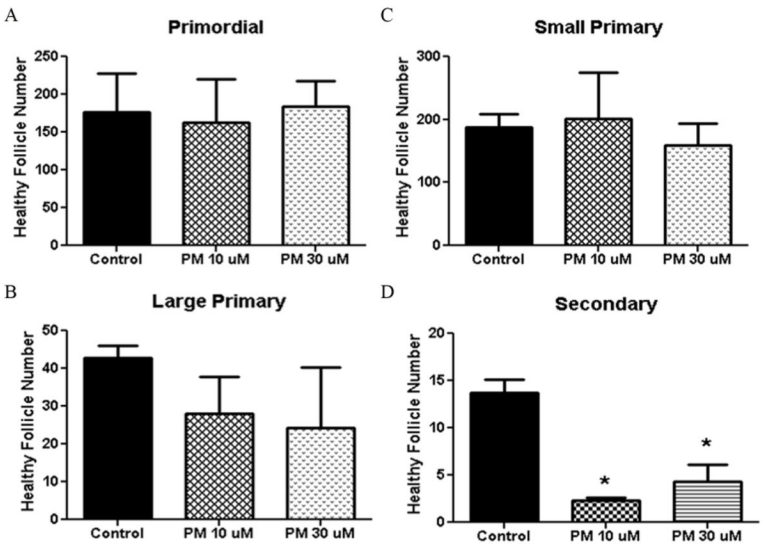

Figure 3. Effect of single PM exposure on growing ovarian follicles

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, $P < 0.05$.

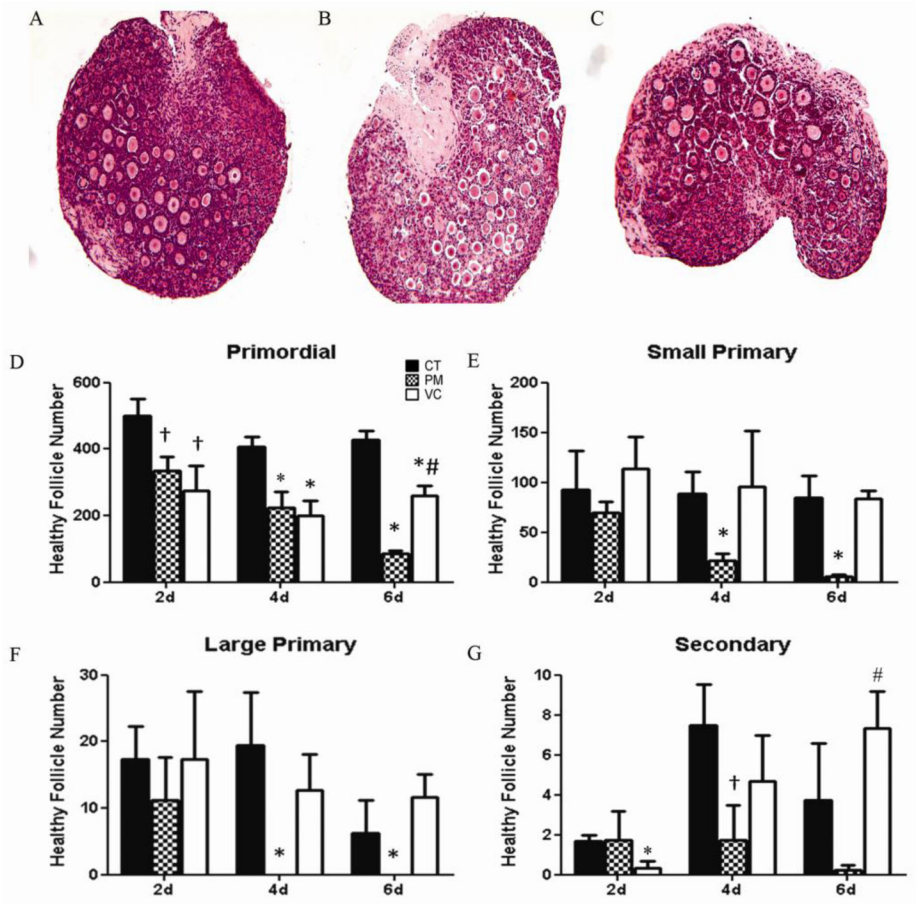

Figure 4. Temporal pattern of PM- and VC-induced ovotoxicity

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, $P < 0.05$.

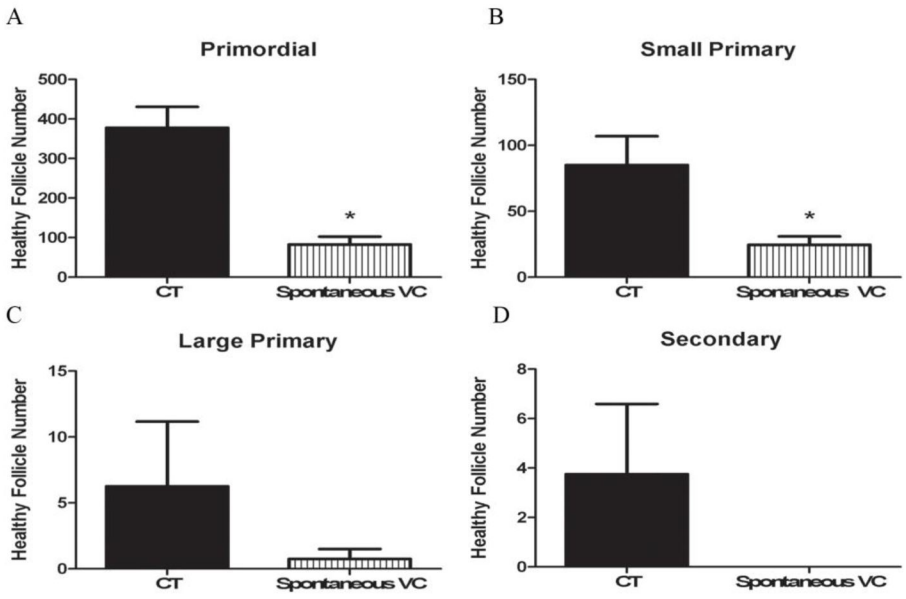

Figure 5. Ovarian-mediated biotransformation requirement for VC liberation from PM

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, $P < 0.05$.

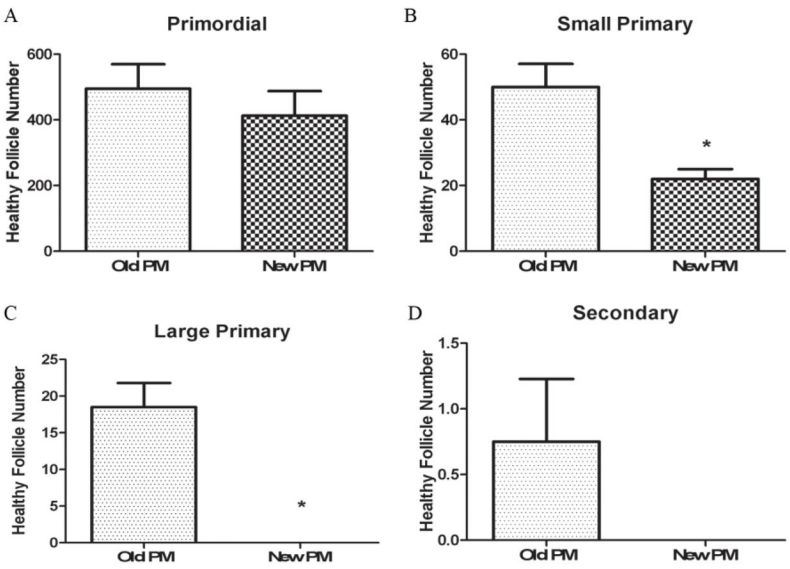

Figure 6. Impact of storage on PM degradation

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, $P < 0.05$.