Characterizing the Ovotoxicity of Cyclophosphamide Metabolites on Cultured Mouse Ovaries

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Cyclophosphamide (CPA) is reported to target dormant primordial ovarian follicles in rodents and humans. However, mechanistic studies are complicated due to the complex ovarian structure. We present here the characterization of the sensitivity of ovaries to CPA metabolites and the timing of morphological alterations induced by phosphoramide mustard (PM) in an *in vitro* system. Intact mouse ovaries (postnatal-day-4) were cultured *in vitro* and exposed to multiple breakdown products of CPA on day 0 (d0). Tissues were cultured up to d8, and then follicle counts and immunohistochemistry were performed. 4-Hydroperoxy-CPA (4-HC), a precursor of an activated form of CPA, and PM depleted primordial and primary follicles (≥ 1 μM and ≥ 3 μM, respectively, $p < 0.05$); acrolein had effects on follicle numbers only under continuous exposure (≥ 30 μM); carboxycyclophosphamide and 4-ketocyclophosphamide reduced primordial and small primary follicles only at high concentrations (100 μM). PM-induced follicle loss became significant ($p < 0.05$) by d1 or d2 following exposures to 10 μM or 3 μM PM, respectively, as determined by the numbers of pyknotic or TUNEL-positive follicles. Cellular targets were oocytes in the smallest follicles, but granulosa cells in large primary follicles. TUNEL staining was observed in both cell types, but caspase-3, a marker of apoptosis, was absent from primordial follicles. In addition, a pan-caspase inhibitor could not prevent follicle losses when administered prior to PM. Thus, brief exposures to 4-HC or PM are sufficient to induce permanent follicle loss in ovaries, and PM is likely the ultimate ovotoxicant. Furthermore, the cell death pathway is likely caspase-independent.

Key Words: cyclophosphamide; chemotherapy; phosphoramide mustard; ovarian toxicity; *in vitro*; mouse; follicle.

About 15% of adult U.S. women have had infertility visits at some point in their lives, and in North America, 5–8% of couples are diagnosed as infertile (First line physicians, 2002). Furthermore, it has been estimated that 10% of women become infertile early in life due to a premature critical loss of oocytes (eggs) (Nikolaou and Templeton, 2004). This can be caused by an accelerated rate of loss of the oocyte pool through either genetics (Nikolaou and Templeton, 2004) or detrimental exposures (smoking, occupational exposures, chemotherapy, reviewed in Hoyer and Sipes, 1996), although it has proven difficult to elucidate underlying mechanisms. Reductions in the oocyte pool reduce a woman's chances of having a baby and accelerate her progression toward menopause, which has been linked to increased risks of osteoporosis (Christiansen *et al.*, 1980), cardiovascular disease (Oparil, 1999), and mental diseases (Paganini-Hill and Henderson, 1994).

Female gametes are incorporated into structures called follicles, where the oocyte is surrounded by supporting granulosa cells. These primordial follicles are initially in a dormant state and can remain thus for decades in women. Mammalian females are born with a large number of oocytes that are slowly lost over time through atresia or ovulation (Hirshfield, 1991), eventually causing reproductive failure following exhaustion of the follicle population. Although the possible replenishment of primordial follicles during adulthood has been discussed again recently (Telfer *et al.*, 2005), it is widely believed that dormant follicle loss is permanent (Hirshfield, 1991). Furthermore, loss of dormant follicles is undetectable until menopausal symptoms are observed. Little is known about the physiology of dormant oocytes and follicles, partly due to the difficulty of separating them from the much larger growing follicles in adult ovaries.

Specific toxicant-induced alterations in ovarian follicle populations have been demonstrated for certain drugs and environmental pollutants (Hoyer and Sipes, 1996). Toxicity directed against dormant follicles can induce premature menopause, whereas exposure-induced loss of growing follicles causes only temporary infertility (Hoyer and Sipes, 1996). The frequency of chemical-induced primordial follicle loss in the human population is unknown due to our lack of any non-invasive biomarkers capable of measuring dormant follicle populations. Thus, identifying potentially dangerous exposures or mechanisms of ovarian toxicity in human populations has proven difficult.

Few experiments have explored the mechanisms underlying the susceptibility of oocytes to chemical exposures. Studies involving the occupational, ovotoxic chemical 4-vinylcyclohexene

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diepoxide (VCD) have provided evidence that oocytes may be susceptible to changes in cytoplasmic and/or mitochondrial apoptotic signaling (Hu *et al.*, 2001a,b). In support of this, inactivation of caspase 2 or 3 reduced VCD-induced primary follicle loss, whereas elimination of Bax expression appeared to protect against VCD-induced loss of both primordial and primary follicles (Takai *et al.*, 2003). While this is a model ovotoxicant that can improve our understanding of toxicant-induced dormant follicle loss, it is unlikely to be of specific concern in humans or wildlife.

A chemical exposure that is of direct human concern is cyclophosphamide (CPA), due to its use in chemotherapeutic regimens against cancer and autoimmune disorders. Multiple studies have identified primordial ovarian follicles to be a sensitive target of CPA in mice (Plowchalk and Mattison, 1991, 1992), whereas in rats, antral follicles appear more sensitive than primordial follicles (Hoyer and Devine, 2002; Jarrell *et al.*, 1991).

Metabolism and spontaneous changes involved in activation and detoxification of CPA are relatively well understood (Ludeman, 1999). Oxidative activation of CPA (a prodrug) by hepatic cytochromes P4502B1 and 3A4 to 4-hydroxycyclophosphamide (4-OH-CPA) leads to a cascade of spontaneous (nonenzymatic) reactions, ultimately resulting in the production of phosphoramide mustard (PM), which is generally accepted as the reactive alkylating agent of therapeutic consequence (Ludeman, 1999). Plowchalk and Matttison (1991) demonstrated that generation of PM is required to cause ovarian toxicity. 4-OH-CPA rapidly interconverts to aldophosphamide (AP), which can spontaneously fragment to generate PM and acrolein. Enzymatic detoxification involves AP being converted to carboxyphosphamide (CPM) or, less often, 4-OH-CPA being oxidized to 4-ketocyclophosphamide (4-KTCP).

In the present study, an *in vitro* whole ovary culture system was used to characterize the ovarian response to CPA metabolites or to a compound that breaks down to the activated form of CPA. Experiments compared the ovotoxic concentrations of each chemical, determined the timing of follicle destruction, and characterized the specific cell and follicle type(s) targeted in the ovary. Results confirmed the role of PM and/or a further breakdown product as the ovotoxic metabolite. These studies also have begun to elucidate the process by which oocytes are eliminated by PM, demonstrating a lack of caspase-3 activation in PM-targeted oocytes.

MATERIALS AND METHODS

Reagents. The different reagents used were purchased from the following sources: penicillin/streptomycin, Hank's buffered saline (without $CaCl_2$, $MgCl_2$, or $MgSO_4$), Albumax, and Ham's F-12/DMEM (1:1) medium from Invitrogen (Burlington, ON); culture plates from Sarstedt (Montréal, QC); Millicell-CM filter inserts from Millipore (Bedford, MA); the ApopTag® red *in situ* apoptosis detection kit from Chemicon International (Temecula, CA);

primary antibody against cleaved caspase-3 from Cell Signaling Technologies (Beverly, MA); biotin-conjugated secondary antibody, the blocking kit and the Vectashield mounting medium from Vector Laboratories (Burlingame, CA); streptavidin-Alexa-fluor 568 from Molecular Probes (Eugene, OR); sodium citrate and Tween 20 from Bioshop (Burlington, ON); z-VAD-fluolomethylketone (z-VAD-fmk) from Calbiochem (La Jolla, CA). PM (as the cyclohexylammonium salt) was acquired from the National Cancer Institute. CPM, 4-HC, and 4-KTCP were gifts from the laboratories of Drs. Susan M. Ludeman and O. Michael Colvin (Duke University, NC). Synthesis of these compounds has been described elsewhere (Ludeman *et al.*, 1992; Zon *et al.*, 1984). All other reagents were purchased from Sigma (St. Louis, MO).

Animals. A breeding colony consisting of 8–9 male and 30 female CD-1 mice was purchased from Charles River (Montréal, QC). Animals were housed in plastic cages, given food and water *ad libidum,* and maintained on a 12-h light/dark cycle; adults were replaced every 6 months. The colony was used to produce postnatal-day-4 (PND4) mice for use in collecting ovaries for ovarian cultures. Males and females were allowed to mate for intervals of 2 days (2d), and females were monitored for litters daily. Ovaries were collected from female pups on PND4, counting the day of birth as day 0. All experimental procedures were approved by the INRS Institutional Committee on Animal Care and Use.

Ovarian culture and exposures. Intact ovarian tissue cultures were established and cultured *in vitro* as described previously for rats (Devine *et al.*, 2004). Ovaries from PND4 CD-1 mice were placed in culture in 48-well culture plates on top of a small piece of membrane (Millicell-CM filter insert) floating on 250 μl of culture medium, one ovary per well (humidified, 37°C, 5% CO_2 in air). Culture medium was replaced every 2 days.

In vitro experiments involved exposing cultured mouse ovaries to multiple metabolites or breakdown products of CPA, because these are thought to be the chemicals that are formed *in vivo* and cause damage to the ovary (Ludeman 1999; Plowchalk and Mattison 1991). 4-HC was used as a cell-permeable precursor that spontaneously produces 4-OH-CPA in solution (Cai *et al.*, 2000; Hales, 1982; Ludeman, 1999). Preliminary *in vitro* toxicity studies were performed (from 0.01 to 300 μM) to determine the range of concentrations to be used for each chemical in these studies, based upon survival of exposed tissues. From these results, the following concentrations were used for each chemical: 0.3–30 μM for PM and 4-HC, 3–100 μM for CPM and KTCP, and 30–100 μM for acrolein. Additions of chemicals to medium were made on d0 of culture within 1 h after establishment of cultures and were not replaced when culture medium was changed. Comparisons between control and treated ovaries in each set of experiments were necessary due to the interlitter variability in the numbers of follicles per ovary. Since no effect of single exposures to acrolein was observed after 8 days (not shown), acrolein was replaced with each exchange of culture medium (called continuous exposures below). Chemicals were dissolved in culture medium or, for 4-ketocyclophosphamide (KTCP), in dimethyl sulfoxide (DMSO); the same amounts of DMSO were added in wells of control ovaries in experiments with that compound. Exposures of ovaries to these chemicals were begun within 5 min of solution preparation. Ovaries exposed to different concentrations of chemicals were cultured at opposite ends of a culture plate or in separate plates to avoid possible cross-toxicity to any volatile breakdown product of PM that has been suggested to be formed (Flowers *et al.*, 2000). When z-VAD-fmk (50 μM) was used, it was added to medium 1 h before addition of PM. The product has been reported to be effective at this concentration in cell cultures and at lower concentrations (10 μM) in ovulated oocytes (Perez *et al.*, 1997), while secondary effects were reported at 100 μM in such oocytes (Perez *et al.*, 1999).

Ovarian follicle counts and morphological analyses. Ovarian follicle populations were determined as described previously (Devine *et al.*, 2004). Briefly, standard histological processing was performed on ovaries, and every 12th section (5 μm thick) was taken for follicle counts. Healthy/nonatretic follicles were classified according to specific criteria and were only included if follicles had a distinct oocyte nucleus. Primordial follicles contained a single

layer of squamous granulosa cells, small (<20 granulosa cells) or large (≥20 granulosa cells) primary follicles had at least three cuboidal granulosa cells in a single layer, and secondary follicles had ≥2 layers of granulosa cells. Follicles with eosinophilic (pyknotic) oocytes or granulosa cells were considered as degenerating or atretic and were counted separately from healthy follicles.

Immunohistochemistry and analysis of staining. Immunohistochemistry was performed on ovaries fixed in buffered formaldehyde (3.7%) for 2 h. Paraffin-embedded tissues were sectioned at 5 μm and at every 12th section, two adjacent sections were used for cleaved caspase-3 staining and terminal UTP nick end labelling (TUNEL), respectively. Microwave antigen retrieval was done, boiling sections for 13 min in sodium citrate (1 M, pH 6.1). For active caspase-3 staining, sections were blocked with 5% BSA for 5 min, then incubated with a polyclonal rabbit antibody against cleaved caspase-3 (1:25 dilution in PBS, 1% BSA, and 0.5% Tween 20, pH 7.2) overnight at 4°C (all other steps were performed at room temperature). Sections were blocked 15 min each with streptavidin and then biotin (Vector blocking kit), followed by incubations with a goat anti-rabbit biotin-conjugated secondary antibody (1:75, 1 h) then alexa-fluor-568 conjugated to streptavidin (1:100, 1 h). Nuclei were stained with Hoechst (0.01 mg/ml) and mounted in Vectashield.

TUNEL was used to localize fragmented DNA *in situ*. TUNEL staining was performed according to kit instructions with the exception that antibody incubation was lengthened to 40 min. Nuclei were stained as above.

Sections were inspected using a Leica DMRE fluorescent microscope (Deerfield, IL). Images were digitized and merged using ImagePro Plus (version 4.0, Media Cybernetics, Silver Spring, MD). After assembling multiple images per section in Adobe Photoshop Elements (version 2.0) into images of whole sections, follicles containing at least one positively stained cell (oocyte/granulosa cell) were counted manually.

Statistics. For each set of experiments involving concentration-specific follicle counts or immunohistochemistry, data were analyzed by one-way ANOVA and, where appropriate, by the Tukey HSD post-hoc test with JMP® (Version 5.1. SAS Institute Inc, Cary, NC). For time-course experiments, follicle counts were analyzed by Student's *t*-test for each concentration and time point separately, because experiments of each concentration were performed independently. Significance was assigned at $p < 0.05$ in each case. Follicle counts were performed by two observers blinded to sample identity.

RESULTS

Postnatal-Day-4 Mouse Ovarian Development *In Vitro*

An apparent steady state was observed in cultured ovaries, with small numbers of growing follicles developing throughout the culture period up to the secondary follicle stage and others undergoing atresia or dying off. The numbers of primary and secondary follicles remained constant (Table 1), while numbers of atretic follicles increased over the course of cultures (see below). Also, primordial follicle numbers were significantly reduced by the end of culture, on d8 compared to those collected on d2 (Table 1).

Concentration-Dependent Effects of Cyclophosphamide Metabolites *In Vitro*

Phosphoramide mustard (PM) induced a significant depletion of healthy primordial and small primary follicles (3–10 μM, Fig. 1A). There were also fewer healthy large primary follicles at 10 μM, but no significant changes in small

TABLE 1
Healthy Follicle Populations in Untreated Postnatal-Day-4 CD-1 Mouse Ovaries Cultured for up to 8 Days *In Vitro*

	Follicle Stage				
Day	Primordial	Small primary	Large primary	Small secondary	Total follicles
1	357 ± 91[*,a]	67 ± 8[c]	8.8 ± 2.3[d]	0.8 ± 0.5[e]	422 ± 100[f]
2	402 ± 48[a]	75 ± 7[c]	8.9 ± 1.4[d]	0.9 ± 0.5[e]	488 ± 53[f]
4	377 ± 47[a]	92 ± 9[c]	6.5 ± 1.3[d]	0.3 ± 0.2[e]	478 ± 51[f]
6	307 ± 87[a]	96 ± 12[c]	6.4 ± 1.0[d]	0.2 ± 0.2[e]	410 ± 95[f]
8	213 ± 19[b]	109 ± 15[c]	7.2 ± 1.9[d]	3.0 ± 1.1[e]	312 ± 16[g]

*Values represent means ± SE, numbers of healthy follicles per ovary counted in every 12th section. Means within the same follicle type with different letters are significantly different, $p < 0.05$. $n = 5$–18 ovaries per condition in 3 to 6 experiments.

secondary follicles at the concentrations tested (not shown). Concentrations ≥30 μM PM caused nonspecific cell death throughout the entire ovary (not shown). Furthermore, untreated ovaries cultured adjacent to wells containing 3 μM or 10 μM PM had fewer primordial follicles (141.4 ± 12.3, $n = 5$ ovaries; and 69.3 ± 18.3, $n = 3$ ovaries, respectively) than untreated ovaries cultured in separate plates (196.5 ± 14.7, $n = 18$ ovaries; $p < 0.05$, both concentrations). Reductions in follicle populations were induced by 4-HC at ≥1 μM for primordial follicles, ≥3 μM for small primary follicles (Fig. 1B), and ≥10 μM for large primary follicles, while secondary follicle numbers were not affected (not shown). In contrast, CPM and 4-KTCP reduced primordial and small primary follicles at the highest concentration tested (100 μM, Figs. 1C and 1D), with no effects on larger follicles (not shown). Acrolein had no effect on follicle numbers at the concentrations tested (1–100 μM) following single exposures. Under continuous exposures to 30–100 μM acrolein, there were significant decreases in the numbers of primordial follicles (Fig. 1E), but there was also nonspecific toxicity in the cortical region of exposed tissues at ≥60 μM, seen as pyknotic cells (not shown).

Timing and Morphology of Phosphoramide Mustard-Induced Follicular Injury

Significant reductions in numbers of healthy primordial and small primary follicles occurred as early as 2 days or 1 day following exposures to 3 and 10 μM PM, respectively (Fig. 2, not shown for small primary follicles), while healthy large primary follicle numbers were reduced at 10 μM only, 1 day following start of exposures ($p < 0.05$, not shown). Ovaries examined at earlier time points (12–18 h) contained only a few pyknotic follicles (not shown).

In ovaries exposed to either PM or 4-HC at toxic concentrations, the cellular targets differed according to the follicular stage and were identified by changes in morphology and
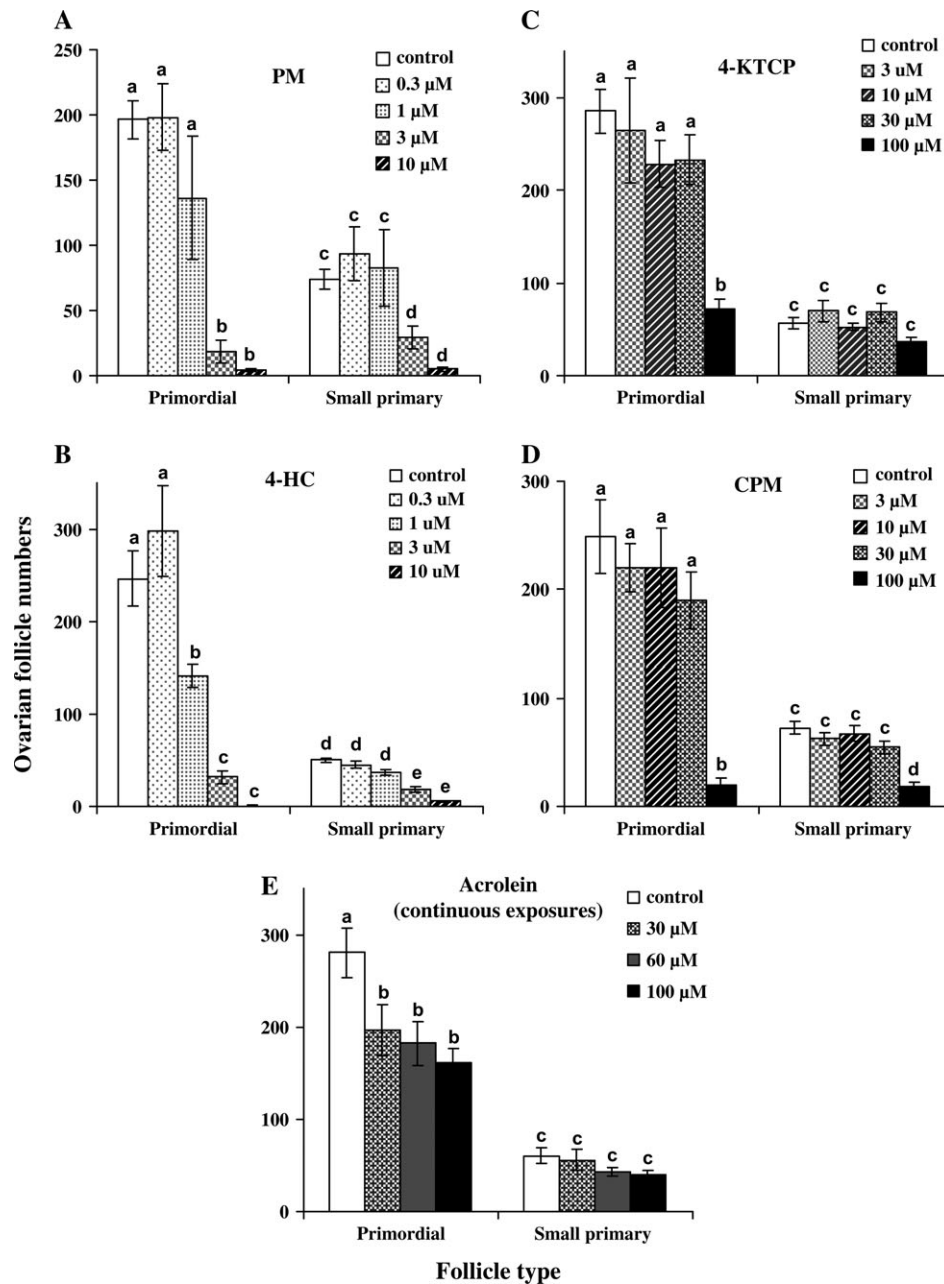
FIG. 1. Response of small ovarian follicles in intact PND4 cultured CD-1 mouse ovaries to different cyclophosphamide-related chemicals. Isolated juvenile mouse ovaries were exposed to chemicals *in vitro* for 2 days and then cultured for another 6 days. Healthy follicles were counted in cultured ovaries exposed to (A) PM, (B) 4-HC, (C) KTCP, or (D) CPM. Exposures to acrolein (E) were continued throughout the 8-day culture period. Classification and counting of healthy/nonatretic follicles were performed in every 12th section of each ovary as described in Methods. Values represent means ± SE. Means within the same follicle type with different letters are significantly different, $p < 0.05$, $n = 3$–15 ovaries per condition in three to five experiments.

histological staining. In primordial follicles, virtually all morphologic changes observed were pyknotic oocytes (Fig. 3). Primary follicles had pyknotic, eosinophilic oocytes surrounded by healthy granulosa cells, whereas some large primary follicles had granulosa cells with condensed nuclei surrounding a morphologically normal oocyte (Fig. 3). Very few small primary follicles with pyknotic granulosa cells were

observed in treated ovaries and were not included in subsequent analyses (mean number of such follicles per treated ovary ± SE: 0.19 ± 0.17, controls; 0.33 ± 0.19, 3 μM PM; 1.40 ± 0.45. 10 μM PM, $p > 0.05$, ANOVA).

When numbers of degenerating follicles were counted according to the follicle stage and cell type(s) affected, time-dependent effects were observed. There were few
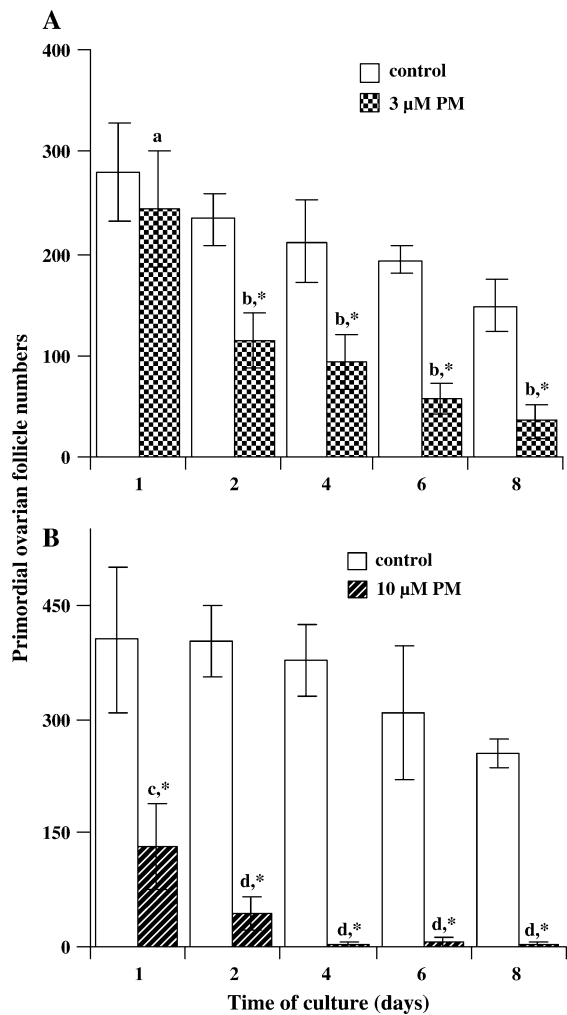
FIG. 2. Time course of the effects of PM on primordial follicle numbers in cultured ovaries. PND4 ovaries from CD-1 mice were cultured *in vitro* for 8 days after a single 2-day exposure to (A) 3 μM or (B) 10 μM PM on d0. Samples were fixed at various times and processed for histological evaluation. See Methods for classification and counting of healthy/nonatretic follicles. Values represent means ± standard error of means. Significant differences are represented by asterisks to compare control and treated ovaries at the same time points and by different letters to compare means of PM-treated ovaries to each other over time, $p < 0.05$, $n = 4$–12 ovaries per condition in three or four experiments.

FIG. 3. Morphological appearance of PND4 CD-1 mouse ovaries following *in vitro* culture for 2 days with (A) control medium or (B) medium containing 10 μM PM. Examples of healthy (hollow arrows) or degenerating (solid arrows) granulosa cells (Gc) and oocytes (O) in primordial (P), small primary (Sp), and large primary (Lp) follicles are shown. Some pyknotic primordial follicles are indicated by a narrow arrow in B. The asterisk indicates the oocyte of an atretic primary follicle. Bar = 20 μM.

degenerating primordial or small primary follicles (i.e., those with pyknotic cells) in untreated ovaries. PM-treated ovaries had significantly more primordial and small primary follicles with degenerating oocytes than untreated ovaries for up to d6, depending on the concentration and follicle type (Figs. 4B and 4C, experiments performed separately for each concentration). Large primary follicles with pyknotic granulosa cells occurred more frequently in PM-treated ovaries from d2–4 (3 μM) and d1–6 (10 μM) than in control ovaries. In parallel, there were fewer large primary follicles with atretic oocytes than in controls on d4 (3 μM) or d4–6 (10 μM, Fig. 4E). By d8,
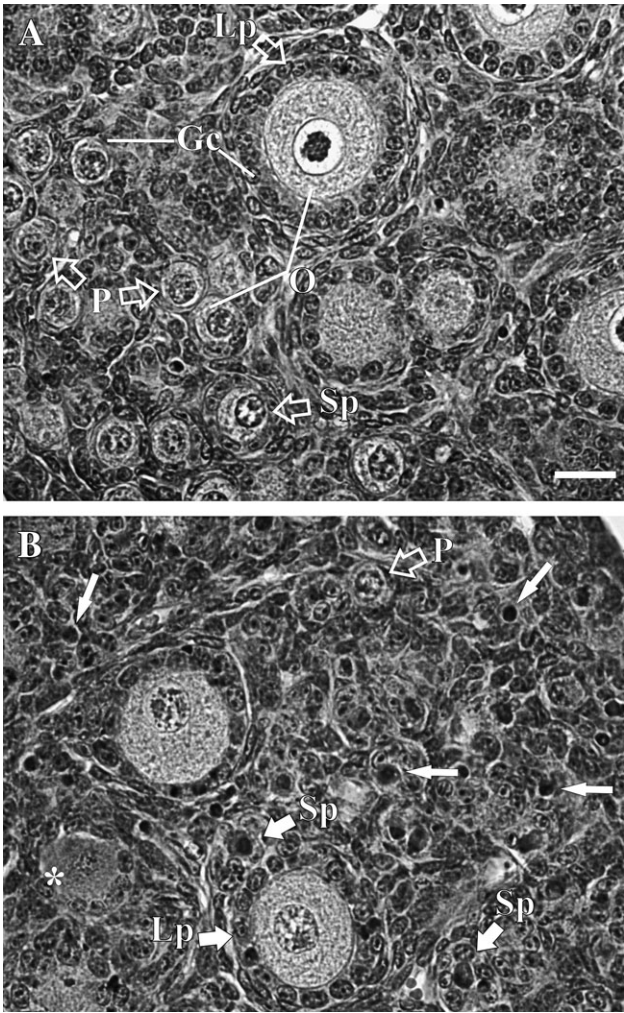
numbers of dying follicles became similar between PM-exposed and untreated ovaries. Atresia of secondary follicles was not included in this analysis due to the small numbers observed.

Cell Death Markers in Response to Culturing with Phosphoramide Mustard

Based upon results shown in Figure 4, markers of DNA degradation (TUNEL) or apoptosis (cleaved caspase-3) were examined at 24 and 48 h after start of PM exposures. In untreated ovaries, TUNEL staining was detected in small numbers of oocytes of primordial, small primary, and rarely, of large primary follicles (Supplementary Fig. 1A). Staining was
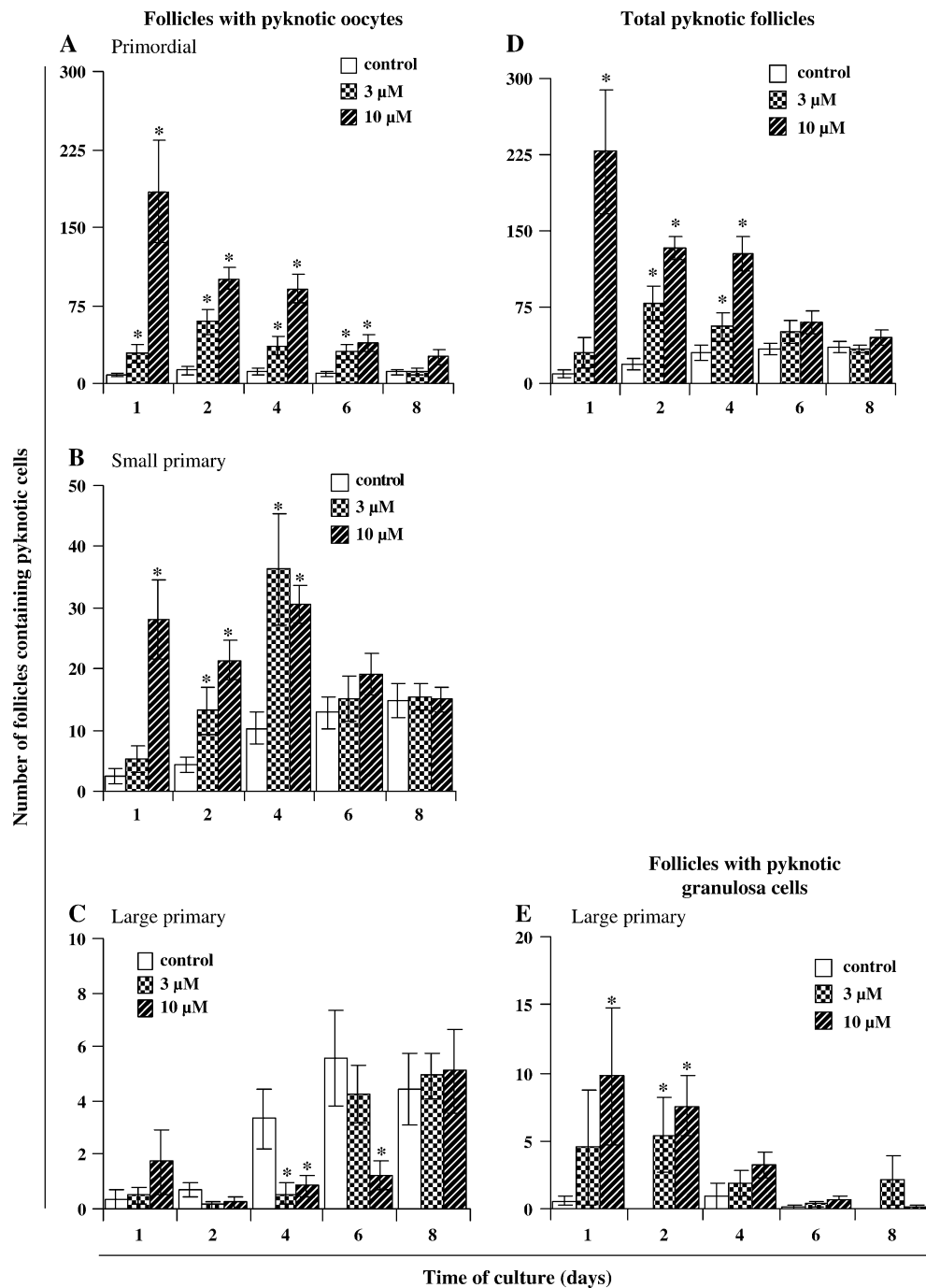
FIG. 4. Frequency of degenerating small ovarian follicles over time following exposures to PM. PND4 ovaries from CD-1 mice were cultured *in vitro* for 8 days after exposures to PM (3–10 µM) on d0. Tissue samples were collected at various times and processed for histological evaluation. Counts of follicles with (A–C) pyknotic oocytes, (D) total numbers of pyknotic follicles, and (E) pyknotic granulosa cells are shown. See Methods for classification and counting of degenerating follicles. Values represent means ± SE; the mean value for controls is from the combined data of the 3 and 10 µM experiments, since controls were not significantly different ($p = 0.49$). Asterisks represent significant differences compared to untreated ovaries cultured in parallel, $p < 0.05$, $n = 4–12$ ovaries per condition in three or four experiments.

also observed in individual granulosa cells of primary follicles. In ovaries cultured with PM, patterns of staining were similar to control ovaries, but more cells were positive. PM significantly increased the number of TUNEL-positive follicles 1 day (10 µM)

or 2 days (3 µM) after exposures (Fig. 5A). No staining was observed when terminal deoxynucleotidyl transferase (TUNEL) or the primary antibody (cleaved caspase-3) were omitted from procedures (Supplementary Figs. 1C and 1F).
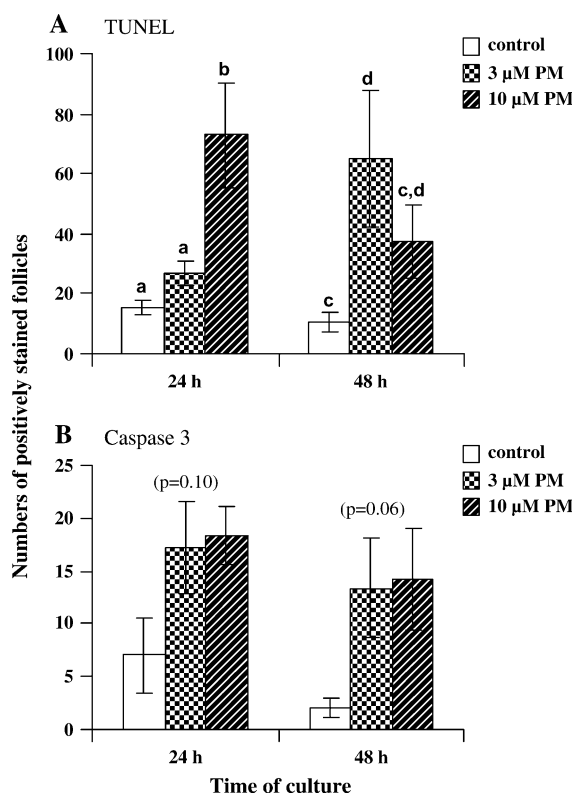
FIG. 5. Numbers of small preantral ovarian follicles stained for cell death markers in cultured ovaries exposed to PM. PND4 ovaries from CD-1 mice were cultured *in vitro* after exposures to PM (3 or 10 μM) on d0. Tissue samples were collected at various times after exposures and processed for immunohistochemistry, and then preantral follicles of any stage containing (A) TUNEL-positive or (B) cleaved caspase-3-positive cells (oocytes or granulosa cells) were counted as described in Methods. Values represent means ± standard error of means. Means within the same time point with different letters are significantly different, $p < 0.05$, $n = 5$–8 ovaries per condition in three or four experiments.

Cleaved caspase-3 staining was mostly seen in granulosa cells of primary follicles, and the numbers of stained follicles in control ovaries were very low (Fig. 5, Supplementary Fig. 1D). Few oocytes of small primary follicles were stained, and no cells in primordial follicles were clearly positive for caspases-3 staining. In contrast to TUNEL staining, the numbers of follicles containing cleaved caspase-3 positive cells in PM-treated ovaries, although higher, were not significantly different from untreated ovaries (Fig. 5B). The same observation was made for the numbers of positively-stained cells per section between untreated and 3 or 10 μM PM–treated ovaries at 24 or 48 h ($p = 0.12$ for 24 h; $p = 0.19$ for 48 h, ANOVA).

Caspase Inhibition Does Not Protect against PM-Induced Follicle Loss

To further investigate the role of caspases in PM-induced follicle death, ovaries were cultured in the presence of PM and/or z-VAD-fmk, a cell-permeable, irreversible, and specific pan-caspase inhibitor, in order to block caspase-3 and other caspases in cultured ovaries. The appearance and follicle numbers in ovaries exposed to z-VAD (50 μM) were similar to untreated ovaries during this period (Fig. 6). Co-exposure to this inhibitor and 3 or 10 μM PM did not affect either the PM-induced loss of healthy follicles (not shown) or the increase in degenerating primordial and primary follicles induced by PM (Fig. 6).

DISCUSSION

The results presented here demonstrate that effects in the ovarian *in vitro* culture system mimic those reported for CPA *in vivo* (Meirow *et al.*, 1999; Plowchalk and Mattison, 1991, 1992). We confirm that PM is the ultimate ovotoxicant of CPA (Plowchalk and Mattison, 1991), and that it can, with only a brief exposure, induce rapid loss of dormant and small preantral follicles at concentrations relevant to those found in blood of human patients (Struck *et al.*, 1987). The cellular target of PM appears to depend on the follicle type, with oocytes being affected in smallest follicles and granulosa cells being predominantly affected in larger growing follicles. A certain number of follicles survive PM exposures, and the integrity of these remaining follicles/oocytes remains unknown. Damaged but surviving oocytes could carry genomic damage, as demonstrated in other cell types (Anderson *et al.*, 1995) and male germ cells (Hales *et al.*, 2005), although concrete evidence for this in humans is lacking.

Of the five compounds characterized here, 4-HC was the most ovotoxic. The greater hydrophobicity of 4-HC compared to PM (PM is charged at physiological pH) is likely a key factor in its greater toxicity, as it allows 4-HC to more easily enter cells and generate PM intracellularly (Ludeman, 1999). These observations support the conclusion that the intracellular production of PM from 4-OH-CPA/AP results in greater cytotoxicity than that of PM outside the cell (Ludeman, 1999). Results comparing effective concentrations of 4-HC and PM were similar to reports concerning their relative mutagenicity and teratogenicity (Cai *et al.*, 2000; Hales, 1982). Although additive effects of 4-HC breakdown products (i.e., PM and acrolein) are possible, much greater acrolein exposures were needed to affect follicle numbers *in vitro* than with PM. The volatility of acrolein may be part of the reason why more of this metabolite is needed compared to others.

Our results demonstrating that a volatile compound can induce primordial follicle loss are the first to suggest this breakdown product may be at least as toxic to the ovary as PM. Flowers *et al.* (2000) presented strong evidence in cultured cells that this chemical is chloroethylaziridine (CEZ). Such effects suggest that the volatile chemical could contribute significantly to CPA-induced ovarian toxicity *in vivo*, since concentrations in those wells *in vitro* are likely much less than that of PM in wells (i.e., <10 μM). PM itself is unlikely to be
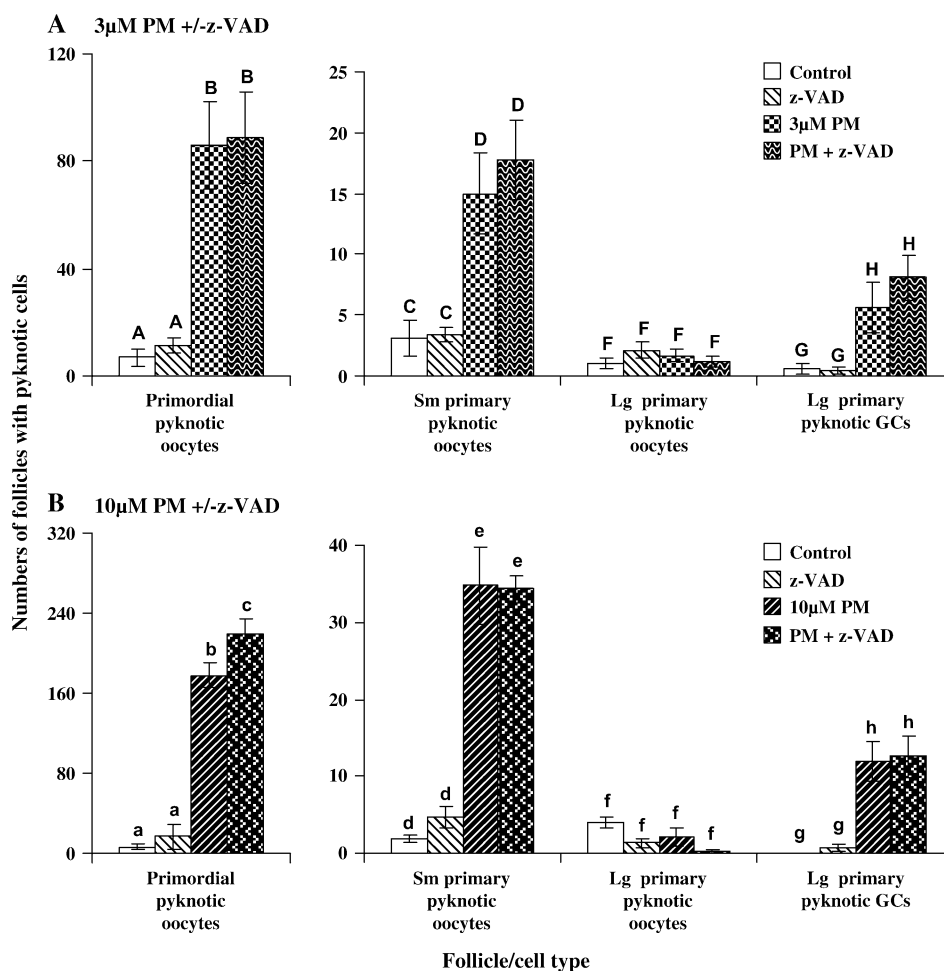
FIG. 6. Occurrence of degenerating small ovarian follicles 48 h following exposures to PM in the presence of a caspase inhibitor (z-VAD). PND4 ovaries from CD-1 mice were collected, placed in culture, exposed to z-VAD-fmk (50 μM) 1 h before PM at (A) 3 or (B) 10 μM, and cultured *in vitro* for 48 h. Values represent means ± SE. Means within the same follicle type with different letters are significantly different, $p < 0.05$, $n = 9–12$ ovaries per condition in three experiments.

the source of toxicity, because it is not volatile under these conditions (S. M. Ludeman, personal communication). The timing of follicle loss, which was similar to reports with PM-generating chemicals *in vivo* (Plowchalk and Mattison, 1992), may also indicate that a more long-lived chemical than PM is involved in this phenomenon. Although not specifically known for the culture medium used here, the half-life of PM at physiological pH has been reported to be very short (Engle *et al.*, 1982; Ludeman, 1999), and complete loss of free PM should occur within a few hours. Under identical conditions, CEZ has a much longer half-life than PM (Shulman-Roskes *et al.*, 1998), and its generation could prolong exposures of ovaries to alkylating agents.

CPM and 4-KTCP are considered nontoxic metabolites of CPA and are unable to release PM (Ludeman, 1999). CPM is also not very cell permeable, since it is charged at pH 7.4 (Ludeman, 1999; Ludeman *et al.*, 1992). Nonetheless, ovotoxicity from these chemicals was observed in our system, though at relatively high concentrations compared to PM or 4-HC.

Comparing ovarian sensitivity of ovaries to CPM and 4-KTCP with that of PM and from blood concentrations reported in the literature (1–3 μg/ml or 3–5 μM) (Baumann *et al.*, 1999; Struck *et al.*, 1987), these two metabolites are likely not the chemicals that cause ovarian toxicity *in vivo*.

It is not known what happens to ovarian follicles that are injured *in vitro*. The counts of degenerating follicles (Fig. 4) are difficult to assess due to the unknown kinetics of such follicle elimination. *In vivo*, it is believed that neighboring cells or resident macrophages remove the debris during follicular atresia (Inoue *et al.*, 2000). Following the rapid disappearance of healthy follicles and increase in pyknotic follicles on d1–2 after exposures to PM or 4-HC in our system (Figs. 2 and 4), there is a gradual decrease in follicles with pyknotic cells until d8. From this data, follicles may be killed off by exposures all at once and eliminated from the tissue slowly. Alternatively, some follicles may die more slowly than others. Further study is needed to determine these kinetics, which will be helpful in interpreting such results in the future.

The reason why primordial oocytes are targeted by DNA-damaging agents is unknown. In addition, the tight interactions between granulosa cells and oocytes make it difficult to determine which follicular cell type is affected by toxic chemicals or if effects are through disruption of the intracellular communication between cells. The alkylating potential of PM likely targets important macromolecules in follicular cells, such as DNA or critical proteins, but more research is needed to determine the specific injury that leads to follicle death. The time lag between exposure and visible effects could be due to a delay in conversion of molecular-level damage to morphological changes or in the cell death signal triggered by unsuccessful attempts to repair chemical-induced injury. Mitochondrial DNA is a possible target, because primordial oocytes were reported to have 10–100× fewer mitochondria than oocytes in other stages (Jansen and de Boer, 1998). The presence and activity of metabolizing/detoxifying enzymes, DNA repair mechanisms, or antioxidants may also vary in a cell- and follicle stage-dependent manner. For example, little is known about follicle stage-specific differences in the intracellular reducing agent glutathione, which plays a role in the inactivation of PM and CEZ (Shulman-Roskes *et al.*, 1998). Depletion of critical cellular components in target cells, such as ATP following PARP-1 activation during necrosis or DNA damage, is another possibility. Evidence for follicle stage-dependent biochemical differences is lacking due to the difficulty in isolating sufficient pure fractions of oocytes, granulosa cells, or follicles to perform such measurements. Future experiments must further localize early molecular changes in order to better understand the mechanism(s) underlying PM's ovotoxicity.

Apoptosis is clearly involved in the death of granulosa cells (Tilly *et al.*, 1991) and is shown here by positive caspase-3 and TUNEL staining in pyknotic granulosa cells of primary follicles undergoing normal or PM-induced atresia. Targeting of dividing granulosa cells by PM in growing follicles is consistent with reports that CPA targets cells with a high mitotic index (Colvin *et al.*, 1976; Jarrell *et al.*, 1991). Numbers of follicles staining for cleaved caspase-3, however, were not significantly elevated by PM, possibly due to the low numbers of large primary follicles in the ovaries used here. The failure of z-VAD to completely prevent granulosa cell death of large primary follicles may suggest that caspase-independent pathways or necrosis is triggered in injured granulosa cells if the traditional pathway is inhibited.

Results presented here suggest that primordial follicle loss in response to PM is independent of caspase-3 activation. Caspase-3 was not cleaved in PM-targeted oocytes in the numerous primordial follicles observed at the time points examined, and z-VAD-fmk could not attenuate the effects of PM. We cannot exclude that its activation does not occur earlier with the results shown here although we could not detect cleaved caspase-3 in primordial follicles after 12 h of exposure (not shown). However, the lack of caspase-3 activation is similar to results in other studies (Fenwick and Hurst, 2002) and is supported by the lack of changes in natural (Matikainen *et al.*, 2001) or VCD-induced primordial follicle loss in caspase-3-deficient mice (Takai *et al.*, 2003). Alternative pathways not involving caspases have been previously described for other cell types (Leist and Jaattela, 2001) and might be involved in oocyte elimination, as well as necrosis after PM exposures. The use of cultured ovaries from knock-out mice for caspase-3 and other caspases, in addition to more sensitive morphological and biochemical endpoints, will help further clarify this issue.

Further research is needed to determine which physiological difference(s) explain the variability in sensitivity to PM among the different cells of the various ovarian follicular stages. Examining the specific cellular targets should help us to understand the long-term consequences of such detrimental exposures on reproduction and offspring. Further mechanistic studies are needed to investigate the molecular targets of chemicals such as PM and understand the mechanism(s) by which they induce dormant follicle-specific damage. This study presents results that have established optimal conditions and chemical exposures relevant to humans for such in-depth mechanistic studies using the ovarian culture model. Understanding of underlying mechanisms will aid in designing better drugs without such side-effects as infertility/teratogenicity, or in contrast, in designing treatments that allow safe and rapid sterilization of animals.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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