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Characterization of a Rat *in Vitro* Ovarian Culture System to Study the Ovarian Toxicant 4-Vinylcyclohexene Diepoxide

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Repeated daily dosing of rats with the occupational chemical 4-vinylcyclohexene diepoxide (VCD) causes selective depletion of the smallest preantral ovarian follicles (primordial and primary). These targeted populations are difficult to study because they comprise very little of the overall mass of ovarian tissue. Additionally, they are randomly distributed throughout the ovary. Therefore, a neonatal rat ovarian culture system containing predominantly primordial and primary follicles was developed and its susceptibility to VCD was assessed. The *in vivo* sensitivity of neonatal rats to VCD dosing was first confirmed by daily injection of VCD (80 mg/kg/day ip) on postnatal days (PND) 4–19. On PND 19, depletion of primordial and small primary follicles was evident. Ovarian cultures were then established utilizing a floating organ culture system to treat ovaries from PND 4 Fischer 344 rats *in vitro*. Initial follicle populations in cultured ovaries consisted of primordial (81%) and small primary (19%) follicles, whereas larger-sized preantral follicles had developed after 15 days in culture (67% primordial, 31% small primary, and 2% large primary). Cultured rat ovaries were sensitive to follicle depletion by incubation with VCD (≥30 μM), and follicle loss occurred in a time-dependent manner (8–15 days). Evidence for apoptosis in VCD-exposed ovaries, as demonstrated *in vivo*, was obtained using immunohistochemistry. There was significantly more staining for apoptosis-associated active caspase-3 and TUNEL in ovaries incubated with VCD (30 μM, 15 days) compared with matched controls. These results demonstrate that small preantral follicles of cultured neonatal rat ovaries are sensitive to VCD exposure. The similarities between VCD's effects *in vitro* and *in vivo* demonstrate the usefulness of this system for future mechanistic studies related to ovarian follicle loss induced by VCD or other ovotoxic chemicals.

Key Words: 4-vinylcyclohexene diepoxide; ovarian toxicant; ovarian follicle; *in vitro*; ovarian culture.

Mammalian females are born with a finite number of ovarian oocyte-containing primordial follicles (Hirshfield, 1991). During each reproductive cycle, some follicles are triggered to begin developing from this dormant follicle population, and a very few (<1%) will go on to ovulate for possible fertilization (Hirshfield, 1991). Most follicles do not progress through the entire process of development but rather undergo cell death by atresia (apoptosis). The balance between follicular development and atresia maintains a continuous supply of follicles ready to provide a controlled number of fully developed ova for fertilization. This causes slow depletion of the nonrenewable pool of primordial follicles, ultimately leading to reproductive failure (menopause). Therefore, chemicals that destroy primordial and primary follicles have the potential to cause early menopause in exposed women (Hoyer and Sipes, 1996).

Quiescent primordial and earliest growing primary ovarian follicles in mice and rats are the specific targets of the occupational chemical 4-vinylcyclohexene diepoxide (VCD), a metabolite of the industrial by-product 4-vinylcyclohexene (VCH) and a solvent for other epoxides (Hoyer and Sipes, 1996; Smith *et al.*, 1990). Through time-course studies, it was determined that dosing of rats with 80 mg/kg/day ip for 15 days causes approximately 50% depletion of primordial follicles (Springer *et al.*, 1996). It has been reported that VCD enhances the number of ovarian follicles undergoing atresia relative to that in untreated animals (Hoyer *et al.*, 2001; Devine *et al.*, 2000; Borman *et al.*, 1999). VCH has only been reported to be present in the air of factories involving rubber vulcanization (0–210 μg/m^3, 8-h collections; Rappaport and Fraser, 1977; Cocheo *et al.*, 1983). Although human exposures to VCH or VCD are limited, this chemical is uniquely selective as an ovotoxic agent for primordial or primary follicles. Therefore, our findings can be used as a model for studying mechanism(s) of action of other compounds more prevalent in the environment.

Multiple mechanistic studies have been performed to assess molecular changes induced in small preantral follicles (primordial and primary) by VCD (Hu *et al.*, 2001a,b); however, the precise mechanism(s) underlying this accelerated follicle loss remains unknown. The heterogeneity of the mature ovary, as regards the diversity of follicle stages present, greatly reduces the ability to measure biochemical or molecular endpoints in the population of small follicles specifically targeted by VCD.

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Collecting isolated follicular fractions of ovarian dispersates greatly improves the selectivity of measurements in isolated small preantral follicles (25–100 μm; Flaws *et al.*, 1994b). However, the time involved in collecting the follicles may affect reversible intracellular endpoints being measured. Also, the requirement for repeated dosing of animals and the contribution of other tissues, such as liver, to the metabolism and clearance of VCD adds further complexity to the *in vivo* model. An organ culture system highly enriched in primordial and primary follicles would greatly facilitate mechanistic studies for understanding cellular and molecular mechanisms involved in ovotoxicity induced by VCD and other environmental chemicals.

Some of the earliest reports of an ovarian culture system were those of Martinovitch (1938). Intact embryonic and neonatal mouse and rat ovaries were cultured on clots of chicken embryo homogenate and blood in watch glasses to determine viability and developmental potential of ovaries in such a system. *In vitro* culture systems also have been reported for small fragments of fetal ovaries from other species (cow, Wandji *et al.*, 1996; baboon, Wandji *et al.*, 1997). Results of studies using neonatal animals have demonstrated that ovaries from younger animals survive better in culture than those from older animals (Fainstat, 1968). This is likely because ovaries from prepubertal rats are small enough to allow diffusion of nutrients and oxygen into intact organs, whereas intact ovaries from older animals do not survive when cultured for multiple days.

More recently, a culture system established for use with bulbourethral glands (Cooke *et al.*, 1987) was modified for use with ovarian tissue (Nilsson *et al.*, 2001; Parrott and Skinner, 1999). This culture system consists of ovaries from postnatal day (PND) 4 rats set on a membrane floating on 0.5 ml culture medium. This allows the ovary sufficient access to both oxygen and nutrients from the medium. Studies utilizing this system have mainly involved characterization of multiple agents that induce quiescent primordial follicles to progress to early primary follicle stages in cultured neonatal rat ovaries. These agents have included Kit Ligand (Parrott and Skinner, 1999), basic fibroblast growth factor (Nilsson *et al.*, 2001), insulin (Kezele *et al.*, 2002), and leukemia inhibitory factor (Nilsson *et al.*, 2002).

Besides facility of culture, ovaries from neonatal rats have an added benefit for the study of ovotoxicity in small preantral follicles. That is, they contain predominantly the earliest follicle stages (primordial and primary stages), which are those selectively targeted by VCD. Therefore, the present study was designed to establish and determine the potential usefulness of the neonatal rat ovarian culture system for evaluating ovotoxicity induced by environmental chemicals, such as VCD. Further, if follicle loss is induced by *in vitro* incubation with VCD, the cellular mechanism of follicular loss (i.e., necrosis versus apoptosis) will also be evaluated.

METHODS

Reagents. Penicillin/streptomycin, phosphate-buffered saline (PBS, pH 6.1), Hank's buffered saline (without $CaCl_2$, $MgCl_2$, or $MgSO_4$), Albumax, and Ham's F-12/DMEM (1:1) medium were purchased from Gibco (Grand Island, NY). Nunclon culture plates were obtained from VWR (San Francisco, CA), and Millicell-CM filter inserts from Millipore (Bedford, MA). Formaldehyde (16%) was purchased from Ted Pella (Redding, CA). Fluorescent mounting medium was purchased from Dako Corporation (Carpinteria, CA). The Apoptag Red kit was acquired from Intergen (Purchase, NY). Primary antibodies against active caspase-3 and proliferating cell nuclear antigen (PCNA) were obtained from Cell Signaling Technologies (Beverly, MA) and Novo Castra (Newcastle, UK), respectively. Biotin-conjugated goat anti-mouse and anti-rabbit antibodies were purchased from Vector Laboratories (Burlingame, CA). Streptavidin-Cy5 was purchased from Jackson Laboratories (Westgrove, PA). Streptavidin and YOYO-1 were from Molecular Probes (Eugene, OR). VCD, D-biotin, DNase-free ribonuclease, sesame oil, bovine serum albumin (BSA), transferrin, a lactate dehydrogenase kit, and all other chemicals were all acquired from Sigma Chemical Co. (St. Louis, MO).

Animals. Pregnant female Fischer 344 rats were purchased late in gestation from Harlan (Indianapolis, IN). Animals were housed in plastic cages, given food and water *ad libidum*, and maintained on a 12-h light/dark cycle. Animals were monitored for litters daily, and ovaries were collected from female pups on PND 4. All experimental procedures were approved by the University of Arizona Institutional Animal Care and Use Committee.

In vivo dosing. Sesame oil or VCD (80 mg/kg, 0.57 mmol/kg, 2.5 ml/kg) in sesame oil was administered to rats through intraperitoneal injection once daily for 15 days (Kao *et al.*, 1999). This dosing regime was chosen because previous studies have shown a 50% loss of primordial and primary follicles after 15 days of daily dosing in rats starting at PND 28 (Springer *et al.*, 1996). This route of dosing was previously chosen because it provides minimal trauma to test animals and no sophisticated exposure apparatus is required. However, ovotoxicity caused by VCD has also been demonstrated in laboratory animals by gavage and dermal exposure, routes more likely to mimic potential human exposures (Chhabra *et al.*, 1990). Animals were killed 4 h after the final injection by CO_2 inhalation and ovaries were excised and processed for histological evaluation.

Ovarian culture. Culturing of ovarian tissue *in vitro* was performed as described in Parrott and Skinner (1999) with some modifications. Culture medium (500 μl per well) was added to wells of Nunclon culture plates (four-well), and a small piece of the membrane from a Millicell-CM filter insert was placed on top of the medium. Additions to medium, such as VCD, were also added to wells at this time. Plates were preequilibrated for at least 1 h in a 37°C incubator with 5% CO_2 in air before ovaries were collected. Ovaries were cultured in Ham's F-12/DMEM (1:1) medium containing 1 mg/ml BSA, 1 mg/ml Albumax, 50 μg/ml ascorbic acid, 27.5 μg/ml transferrin, 5 U/ml penicillin, and 5 μg/ml streptomycin. Female pups were killed by CO_2 asphyxiation followed by decapitation on PND 4 after birth. Small amounts of liver and ovarian tissue were collected from some pups to be used as positive controls in lactate dehydrogenase (LDH) measurements. Ovaries were removed, excised from the oviduct, fat, and connective tissue in ice-cold Hank's buffered saline (without $CaCl_2$, $MgCl_2$, or $MgSO_4$), placed onto the floating filters, and then covered with a few drops of medium from the well using fine forceps. One or two ovaries were cultured in each well. Plates were cultured in a humidified incubator at 37°C, 5% CO_2 in air for up to 15 days. Medium was removed every 2 days and replaced with fresh medium in all wells. At the end of experiments, ovaries were removed from wells, placed in fixative, and processed for histology.

Ovarian follicle counts. To assess the number of ovarian follicles, ovaries were removed from culture or from animals and put into Bouin's fixative for 2–4 h. Tissues were dehydrated, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin. The numbers of oocyte-containing follicles at each developmental stage were counted in every 12th section for

TABLE 1
Effect of Repeated Daily Dosing of Fischer 344 rats with VCD for 15 days (PND 4–19) on Ovarian Follicle Populations

Treatment	Follicle Stage					Total follicles
	Primordial	Small primary	Large primary	Secondary	Antral	
Control	94 ± 5[a]	43 ± 5	6.0 ± 0.5	32 ± 8	29 ± 4	228 ± 13
VCD (80 mg/kg)	22 ± 5*	18 ± 4*	4.6 ± 0.8	30 ± 5	29 ± 4	118 ± 13*

[a] Values represent means ± SE, number of follicles counted in every 40th section.
* $p < 0.05$, different from control, $n = 7$ animals per group.

cultured ovaries or for those that had been taken from PND 4 rats and in every 40th section in ovaries from rats treated for 15 days *in vivo*. For cultured ovaries, results of counts from every 12th section were compared with results of counts from every 6th section or from only two of the largest sections. Results were consistent in a relative manner among the different methods of analysis (assessed by one-way ANOVA); therefore, analyses presented reflect counts from every 12th section. Only those follicles that had a distinct oocyte nucleus were included in the counts. Primordial follicles were classified as those with a single layer of squamous granulosa cells. Primary follicles were identified as those that contained at least 3 cuboidal granulosa cells in a single layer. For further distinction, primary follicles were classified as small primary if there were less than 20 granulosa cells, whereas they were designated large primary if there were more than 20 granulosa cells (Borman *et al.*, 1999). Secondary follicles were identified as those with two or more layers of granulosa cells, but no antral space in the granulosa layer. Antral follicles were identified as containing at least two layers of granulosa cells that displayed an antral space. This classification scheme was used to facilitate comparisons with *in vivo* results and with prior studies involving VCD. Follicles with eosinophilic oocytes were not included in follicle counts, because not all such structures could be definitively identified as follicles.

Measurement of LDH from medium of cultured ovaries. LDH was measured according to instructions provided in the kit. Briefly, 50 μl medium was collected from culture wells and placed in wells of 96-well plates. Homogenates of liver or ovaries were used for positive controls. Freshly collected liver (5–20 mg) or ovary (2–6 mg) samples were homogenized with a Tissue Tearor (Bio Spec, Bartlesville, OK) immediately after collection in 500 μl culture medium, and 50-μl aliquots of each were used after centrifugation at 14,000*g* for 5 min. Reagent (200 μl) was added to each homogenate or medium sample, and absorbance was measured over time at 340 nm on a Dynatech MR5000 plate reader (Dynatech, Chantilly, VA).

Immunohistochemistry. Ovaries used for immunohistochemistry were fixed in 4% formaldehyde in PBS for 2 h. Active caspase-3 and PCNA were stained in ovarian sections by the same procedure using different primary antibodies, except that there was no antigen retrieval performed on sections stained for PCNA. Paraffin-embedded tissue was sectioned at 5 μm and deparaffinized in xylene. For caspase-3 staining, antigen retrieval was carried out by boiling sections in sodium citrate buffer (1 M, pH 6.1) for 7 min. Sections were then blocked with 5% BSA for 10 min, streptavidin (10 μg/ml) for 30 min, and biotin (1 mg/ml) for 30 min. All of the following solutions were diluted with PBS containing 1% BSA. For PCNA staining, sections were incubated with a 1:25 dilution of primary monoclonal mouse antibody for 18 h at 4°C. For active caspase-3 staining, sections were incubated with a 1:25 dilution of primary rabbit polyclonal antibody for 1 h at room temperature. All other steps were performed at room temperature. After washing with PBS, sections were incubated with the appropriate biotin-conjugated secondary antibodies (goat anti-rabbit, caspase-3, or goat anti-mouse, PCNA; 1:75 dilutions) for 1 h. Following further washes, sections were incubated in Cy5-streptavidin (10 μg/ml) for 1 h. Nuclei were stained by incubating sections with ribonuclease A (DNase free, 50 μg/ml) for 1 h followed by YOYO-1 (0.55 μM) for 10 min. After three final washes with PBS, coverslips were mounted onto slides using an aqueous mounting medium.

Terminal UTP nucleotide end labeling (TUNEL) was used to measure DNA degradation by fluorescently labeling ends of DNA strands. TUNEL staining was performed according to kit instructions and was different from the other staining procedures. Briefly, following antigen retrieval, sections were incubated with terminal deoxynucleotidyl transferase and digoxigenin-labeled nucleotides for 1 h at 37°C. The reaction was stopped and sections were washed and incubated with a rhodamine-tagged anti-digoxigenin antibody for 30 min at room temperature. Nuclei were labeled with Yoyo-1 as described above, and slides were coverslipped after final washing.

Immunofluorescent staining was visualized on a Leica TCS 4D confocal microscope (Leica, Heidelberg, Germany) equipped with an argon–krypton laser. YOYO-1 staining (nuclei) was observed at 488 nm, while Cy5 staining was observed at 647 nm. Measurements of images were performed using Scion software (NIH, Bethesda, MD). An individual image was acquired on the confocal microscope (at 40× magnification) from each section at both wavelengths. For quantification, a threshold value was used to eliminate background staining, and then the overall area that was stained more intensely than that threshold was measured for each image. Also, the number of cells that stained positively (above the same threshold intensity) was determined in each image. Microscope settings and threshold values were kept constant for all images for a particular stain.

Statistics. Data were analyzed by one-way ANOVA and, when appropriate, by Fischer PLSD and Scheffe F test post-hoc tests. Significance was assigned at $p < 0.05$.

RESULTS

Treatment of Pups in Vivo

In order to ensure that a culture system utilizing PND 4 rat pup ovaries would be useful for toxicological assessments, the sensitivity of the pups' ovarian follicles to VCD was examined *in vivo*. Ovaries from rats treated with 80 mg/kg VCD each day from PND 4 to 19 had significantly fewer primordial (23% of controls) and small primary (42% of controls) follicles, although follicles at other stages of development were not significantly affected (Table 1). No other VCD-associated effects, such as necrosis or apoptosis in nontarget cells, were observed (not shown). These results demonstrate that neonatal rat ovaries are sensitive to VCD and that the same follicle populations are targeted as in adult and pubertal rats (Kao *et al.*, 1999; Flaws *et al.*, 1994a).

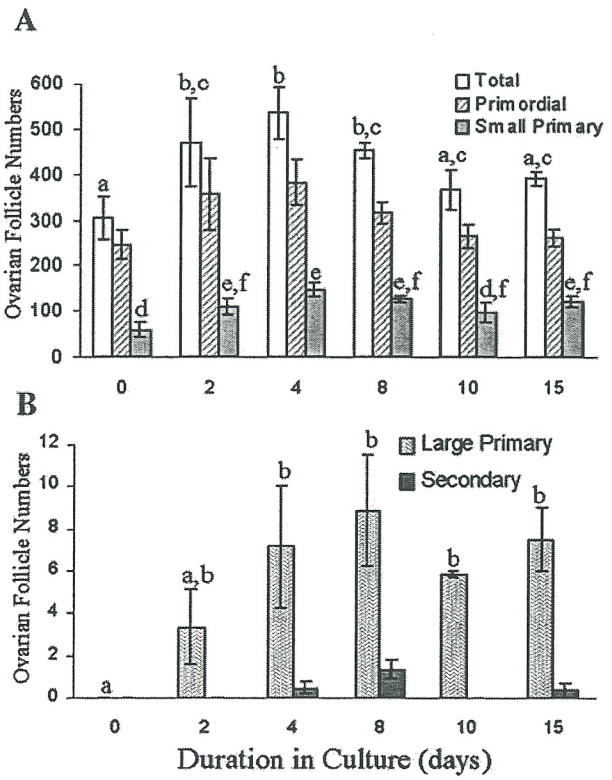

FIG. 1. Onset of development of small follicles during culture of ovaries from PND 4 Fischer 344 rats. Ovaries were collected and cultured *in vitro* for up to 15 days. Tissue samples were collected at various times and processed for histological evaluation. Ovarian follicles were classified and counted as (A) primordial, small primary follicles and (B) large primary and secondary preantral follicles as described under Methods. Values represent means ± SE; means within the same follicle type with different letters are different from each other, $p < 0.05$; $n = 4$–7 ovaries per time point in three to four experiments.

Assessment of Ovarian Tissue Viability and Follicular Development in Culture

Ovaries cultured for up to 15 days without VCD treatment remained healthy, and follicle development progressed over time. Follicle populations of ovaries collected from PND 4 rats and fixed immediately consisted solely of primordial and small primary follicles (81 ± 2 and 19 ± 2% of total follicle populations, respectively; Figs. 1 and 2A). Ovaries cultured for 4 days contained significantly more total follicles than those fixed at the time of ovary collection (Fig. 1A, $p < 0.05$). Large primary follicles were first observed in ovaries as early as 2 days of culture, whereas development of secondary follicles was seen at 4 days of culture (Fig. 1B). Following 15 days of culture, large primary and secondary follicles had developed, although only a few were observed in each ovary (67 ± 3% primordial, 31 ± 3% small primary, 2 ± 0.4% large primary, few secondary, Figs. 1A and 1B). No growing follicles containing more than two full granulosa cell layers were ever observed in these experiments.

The morphological appearance of preantral follicles from cultured ovaries (15 days; Fig. 2C) was similar to that observed *in vivo* (PND 19; Fig. 2B). In small growing follicles, granulosa cells first became cuboidal, and then oocytes began to grow noticeably in size as the granulosa cells started to proliferate (Fig. 2B). In some cultured ovaries, clusters of primordial and small primary follicles contained oocytes that stained in a more eosinophilic pattern, with the nuclei of the oocytes shrunken and dark (not shown). This morphology was not observed in ovaries collected from PND 19 rats (Fig. 2B). Some of the larger growing follicles of cultured ovaries showed signs of poor development (too few or squamous granulosa cells; Fig. 2C, arrows) or atresia (pyknotic granulosa cells and/or eosinophilic oocytes).

Effects of VCD on Ovarian Follicles in Cultured Rat Ovaries

Overall, incubation of ovaries with VCD did not induce visible signs of necrosis nor was follicle development or morphology grossly altered relative to controls (Fig. 2D). Clusters of eosinophilic oocytes were observed in some of the VCD-treated ovaries. Also present were many larger growing follicles demonstrating evidence of atresia. Additionally, in ovaries treated with VCD, some primary follicles exhibited oocytes that had a small amount of their cytoplasm split off into one or two polar body-like fragments (Fig. 2D, inset). This was observed in only 2 follicles of untreated ovaries throughout these experiments but in 1–3 follicles per ovary treated with 10 μM VCD or 5–10 follicles per ovary treated with 30 μM VCD.

To assure that cultured ovaries were not undergoing necrosis, cell damage in tissues was assessed by measurement of lactate dehydrogenase activity in the culture medium following 2, 24, and 48 h of culture. No activity was detected in medium collected from untreated cultured ovaries at the time points sampled. Furthermore, VCD exposure did not elicit measurable LDH leakage into the medium. As a positive control, LDH activity was measurable in homogenates of freshly collected tissues from 4-dayold rats (0.002 and 0.02 optical density units/min/mg tissue, for ovary and liver, respectively).

Incubation of cultured ovaries with a range of concentrations of VCD for 15 days caused a concentration-dependent loss of primordial and small primary follicles (Fig. 3). Primordial and small primary follicle numbers were significantly reduced by VCD concentrations as low as 30 μM (Fig. 3). This concentration induced a significant reduction in primordial (Fig. 4) and small primary follicle numbers (data not shown) as early as 8 days of culture. The numbers of other follicle populations were not reduced significantly by the concentrations of VCD studied, although secondary follicles were never observed in ovaries treated with 100 μM VCD (Fig. 3B).

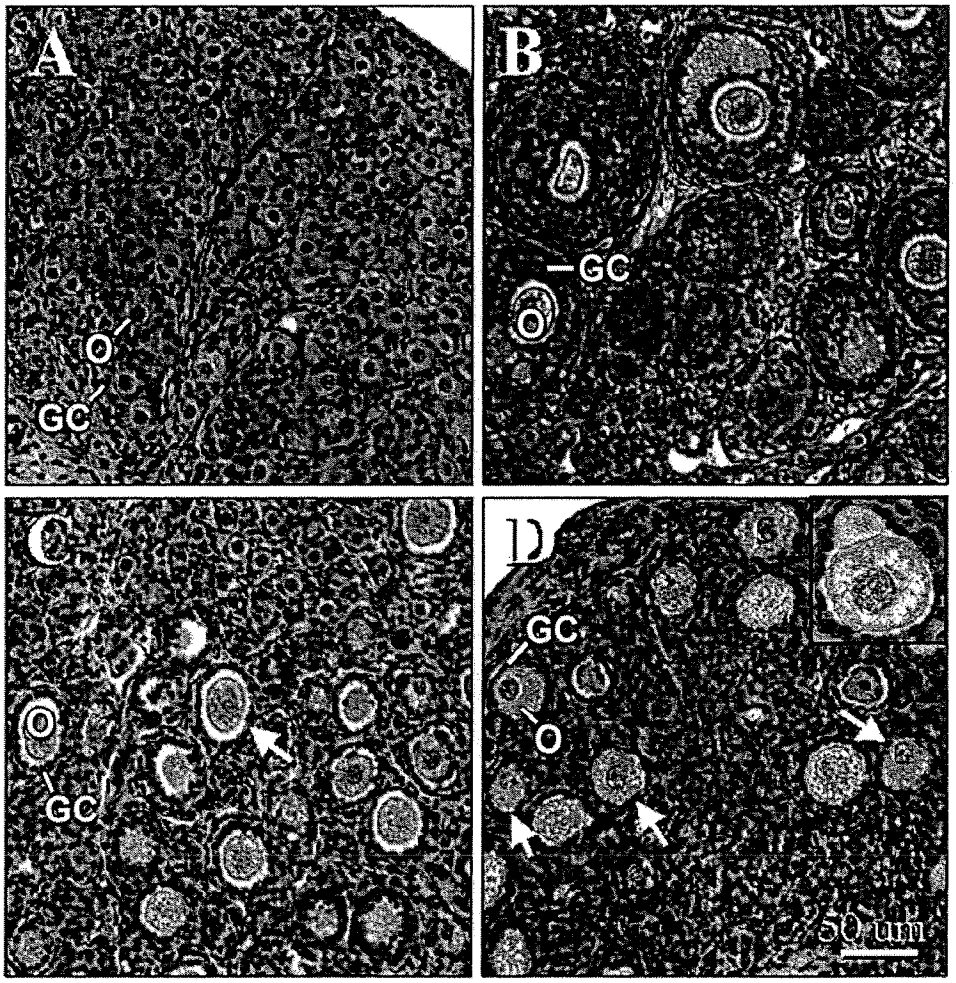

FIG. 2. Morphological appearance of PND 4 Fischer 344 rat ovaries immediately following collection or following *in vitro* culture for 15 days. (A) Freshly collected ovary from PND 4 rat, (B) freshly collected ovary from PND 19 rat, (C) PND 4 ovary cultured in control medium for 15 days (PND 4–19), or (D) PND 4 ovary cultured in medium containing 30 μM VCD for 15 days (PND 4–19). (Inset) Primary follicle with an oocyte containing a polar body-like extrusion, magnification 600×. Magnification 200×. Bar represents 50 μm. Images are of representative ovaries from three to four experiments. GC, granulosa cells; O, oocytes in primordial (A), large primary (C and D), and antral (B) follicles. Larger preantral follicles with very few or squamous granulosa cells are indicated (arrows).

Cell Proliferation and Death in Response to Culturing with VCD

In order to further characterize the ovarian culture system and the response to VCD, fresh or cultured ovaries were stained by immunohistochemistry for markers of both cell proliferation and cell death (Fig. 5). Actively proliferating granulosa cells were identified by staining for PCNA. Markers for impending cell death were identified using TUNEL, a measure of DNA degradation, and by immunohistochemical staining for active caspase-3, a final obligatory step in triggering apoptosis. No staining was observed when primary antibodies, or terminal deoxynucleotidyl transferase for TUNEL, were omitted from procedures. Ovaries fixed immediately after collection (from PND 4 rats) had very few cells staining positively for PCNA, TUNEL, or active caspase-3 (Figs. 5A–C). Following 15 days of culture, many granulosa cell nuclei in growing follicles of VCD-treated and control ovaries stained positively for PNCA (Figs. 5D and 5G). PCNA staining was also seen in oocyte nuclei in cultured ovaries (Figs. 5D and 5G, d15) but not in freshly collected ovaries (Fig. 5A). In ovaries incubated in control medium, very few cells stained positively for active caspase-3 or TUNEL (Figs. 5E and 5F, respectively). When observed, most staining for active caspase-3 and TUNEL was in individual granulosa cells of primary or secondary follicles. In ovaries that had been incubated in 30 μM VCD, the distribution of staining was similar to that in control ovaries (Figs. 5G–I). Some oocytes also stained positively for TUNEL in eosinophilic degenerating follicles of ovaries incubated in VCD, suggesting that DNA degradation is occurring in the oocyte as well (Fig. 5I).

Both the overall area and the number of cells that were

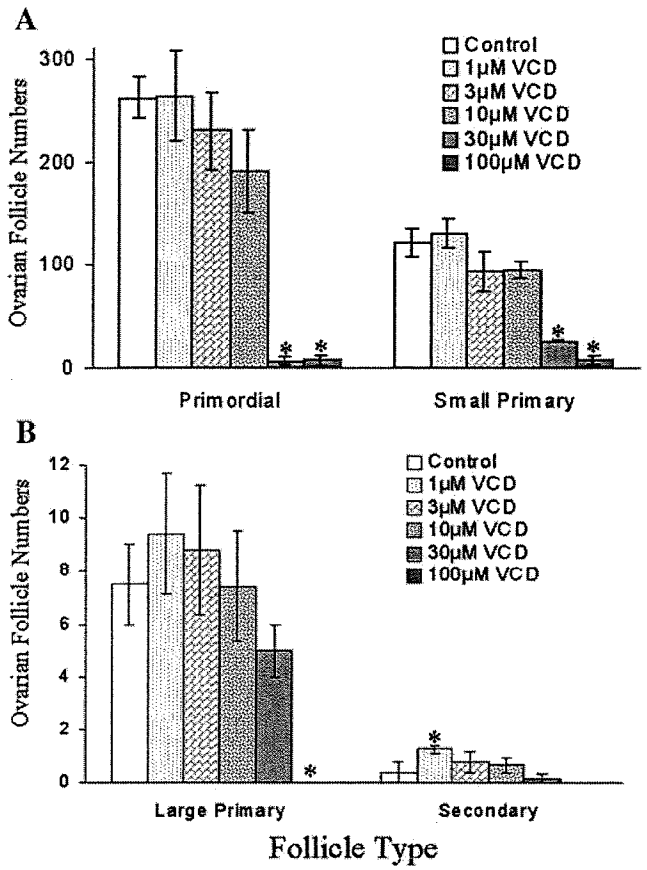

FIG. 3. Response of small ovarian follicle numbers in cultured ovaries to increasing concentrations of VCD. Ovaries from PND 4 Fischer 344 rats were collected and cultured *in vitro* for 15 days in the presence of VCD (0–100 μM). (A) Primordial and small primary and (B) large primary and small secondary ovarian follicles were classified and counted as described under Methods. Values represent means ± SE. *$p < 0.01$, different from untreated ovaries cultured in parallel; n = 5–7 ovaries per VCD concentration in three to four experiments.

positively stained above a threshold intensity value were determined in sections of ovaries. For active caspase-3 and TUNEL, positively staining cell numbers were significantly greater in 30 μM VCD-treated ovaries than in untreated ovaries (Figs. 5J and 5K, $p < 0.01$). Results were comparable when the overall area stained was assessed (data not shown). PCNA measurements were not significantly different between control and VCD-incubated ovaries ($p > 0.05$, data not shown).

DISCUSSION

The health risk for women exposed to environmental chemicals that destroy primordial and primary follicles is early menopause. A number of chemicals distributed more widely in the environment or more likely to be involved in human exposures than VCD are also known to cause this effect in laboratory animals. These include chemotherapeutic drugs (Miller and Cole, 1970), polycyclic aromatic hydrocarbons (Mattison and Thorgeirsson, 1978), and epoxides such as metabolites of butadiene (Doerr *et al.*, 1995). Therefore, the culture system will undoubtedly prove useful in the future for screening of other chemicals that are potentially ovotoxic to preantral follicles. Furthermore, having demonstrated that the ovarian organ cultures mimic the physiological response *in vivo*, more mechanistic studies can now be undertaken.

The loss of ovarian follicles *in vitro* was very similar to previous results of VCD-induced ovotoxicity in rats exposed *in vivo* (Flaws *et al.*, 1994a). Specifically, the present study demonstrates that VCD targets primordial and small primary ovarian follicles in neonatal rats both by *in vitro* and *in vivo* exposures. VCD-induced follicle loss, which was dose dependent *in vivo* (Smith *et al.*, 1990), was also concentration dependent *in vitro*. Additionally, in both systems, there is a delay between initiation of exposure and significant decreases in follicle populations. Significant loss of target follicles first occurs after 12 days of repeated dosing *in vivo* (Kao *et al.*, 1999). Likewise, follicle loss was not observed earlier than 8 days *in vitro*. Evidence for increased levels of apoptosis was found in VCD-exposed cultured ovaries, as demonstrated by increased immunohistochemical staining for both TUNEL and activated caspase-3. Similar findings of VCD-induced activation of caspase-3 (apoptosis) have also been reported following *in vivo* exposures (Hu *et al.*, 2001b). These results demonstrate that VCD can directly impact ovarian follicles, without involvement of extraovarian metabolism (e.g., in the liver) or hormonal influences (e.g., alterations in growth factor or gonadotropin levels).

VCD is known to selectively target small ovarian follicles, causing them to undergo atresia, which involves the process of

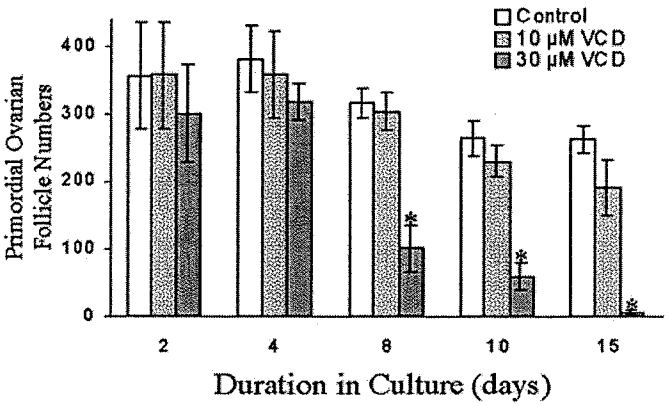

FIG. 4. Time course of the effect of VCD on primordial ovarian follicle numbers in cultured ovaries. Ovaries from PND 4 Fischer 344 rats were collected and cultured *in vitro* for up to 15 days in the presence (10 or 30 μM VCD) or absence of VCD. Ovarian follicles were classified and counted as described under Methods. Values represent means ± SE. *$p < 0.01$, different from untreated ovaries cultured in parallel; n = 4–7 ovaries per VCD concentration in three to four experiments.

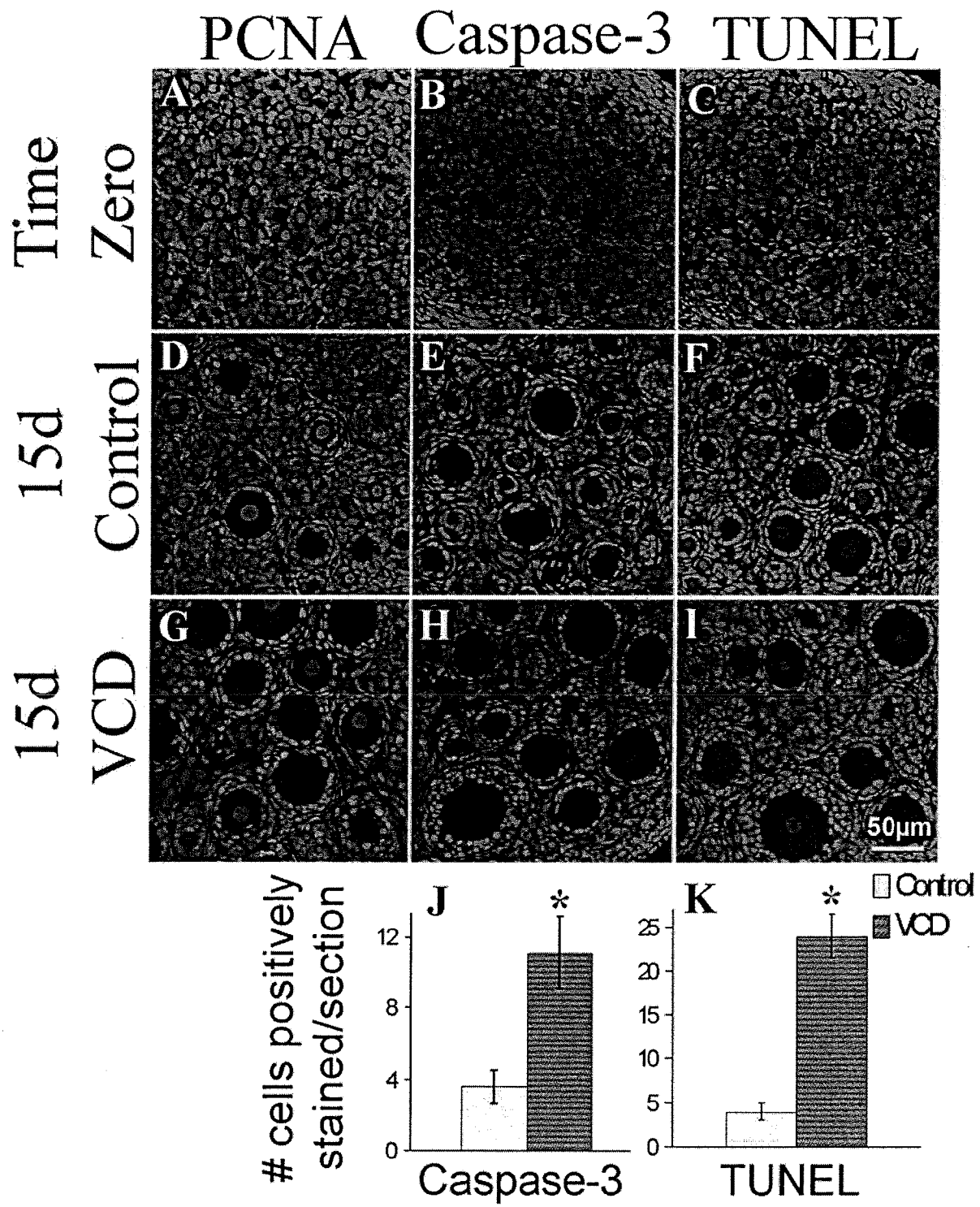

FIG. 5. Fluorescent immunohistochemical staining as visualized by confocal microscopy. Ovarian sections were incubated with antibodies directed against PCNA (A, D, and G), active caspase-3 (B, E, and H), or TUNEL (C, F, and I). Sections were from freshly collected ovaries (A–C), untreated ovaries cultured for 15 days (D–F), or ovaries cultured for 15 days in the presence of 30 μM VCD (G–I). Green (YOYO-1) represents nuclear staining in all cells; red (Cy-5) represents specific staining for the protein of interest, PCNA or active caspase-3, or for degraded DNA (TUNEL), respectively. Images are of representative ovaries from three experiments. Bar represents 50 μm. The number of cells positively stained above background for active caspase-3 (J) and TUNEL (K) were counted in each stained section of cultured ovaries as described under Methods. Values in graphs represent means ± SE; *$p < 0.01$, different from untreated ovaries cultured in parallel as determined by ANOVA.

apoptosis. The specific cellular target (oocyte versus granulosa cell) remains unknown, but some morphological evidence for effects on oocytes was observed in this study. VCD may be directly toxic to the oocyte, because eosinophilic staining and an increased incidence of polar body-like structures occurred specifically in oocytes, whereas granulosa cells usually had a normal appearance. Oocytes may have unique receptors or enzymes that are sensitive to VCD. However, it cannot be ruled out that VCD triggers changes in granulosa cells that compromise their ability to maintain the necessary support of viability for the oocyte.

The populations of follicles in cultured ovaries observed in our studies were relatively static in comparison to *in vivo* development and to that reported in other *in vitro* studies (Parrott and Skinner, 1999; Wandji *et al.*, 1996, 1997). Although a high rate of small preantral follicle loss occurs *in vivo* prior to puberty in mammals (Faddy *et al.*, 1987), there were no significant trends for loss of follicles either in our untreated cultured ovaries or in previous reports of ovarian cultures (Parrott and Skinner, 1999; Wandji *et al.*, 1996, 1997). Large primary and secondary follicles were able to develop, but larger secondary and antral follicles were absent even after 15 days of culture. This difference may be due to a lack of gonadotropins (i.e., FSH) that support secondary and antral follicle growth (Fainstat, 1968). Additionally, the rapid activation and growth of many primordial follicles described in previous culture systems (Parrott and Skinner, 1999; Wandji *et al.*, 1996, 1997) was not observed in our cultures. This activation has been reported to result from insulin (Kezele *et al.*, 2002), which was not added to the medium in the studies reported here.

Immunohistochemical staining of cultured ovarian tissue for PCNA demonstrated the quiescence of PND 4 rat ovaries and the high proliferative rate of granulosa cells in primary and secondary follicles of ovaries cultured for 15 days *in vitro*. PCNA staining in oocyte nuclei of cultured ovaries was surprising, because the oocyte is arrested in prophase of the first meiotic division (Hirshfield, 1991). However, this has also been seen in other studies (Wandji *et al.*, 1996) and in ovaries freshly collected from older rats (unpublished observations). The lack of staining in oocytes of freshly collected PND 4 rats suggests that PCNA staining in oocytes after culture is not due to nonspecific staining. As described by Wandji *et al.* (1996), PCNA may play a role in DNA repair or other nonproliferative roles.

The morphological appearance of some developing and degenerating follicles in cultured ovaries was also somewhat different from that observed *in vivo*. Some primary follicles had long, squamous granulosa cells or had gaps in the granulosa cell layer, appearing as if the oocyte is growing without sufficient granulosa cell activation and proliferation. Such follicles were present only rarely in ovaries from *in vivo* experiments (unpublished observations). Eosinophilic oocytes in atretic follicles were sometimes observed *in vitro*, whereas no such staining occurred in sections of ovaries collected from control rats or rats dosed with VCD *in vivo*. This feature of atresia *in vitro* may reflect a lack of phagocytic cells from the blood supply that would normally rapidly remove apoptotic cellular debris from atretic follicles *in vivo* (Gaytan *et al.*, 1998). These phenomena are not likely due to culture conditions because they were not unique to the center of the ovary and could be observed at any time point examined. Although there were a few morphological differences observed between cultured ovaries and those directly evaluated after collection from the animal, *in vitro* incubation did not compromise (1) follicular development, (2) overall viability of cultured tissue, (3) induction of biochemical parameters associated with physiological cell death (apoptosis), or (4) the specificity of VCD-induced follicle loss.

In summary, the results presented here demonstrate that cultured neonatal rat ovaries are susceptible to ovotoxicity by incubation with VCD. The mechanisms of this toxicity mimic those resulting from *in vivo* dosing. Thus, this ovarian culture model will prove to be very worthwhile for future studies designed to elucidate the specific mechanisms involved in ovarian toxicity at the follicular, cellular, and molecular level.

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