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SHORT COMMUNICATION

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Possible Direct Cytoxicity Effects of Cyclophosphamide on Cultured Human Follicles: An Electron Microscopy Study*

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***Purpose*:** To evaluate the direct effect of cyclophosphamide on cultured human ovarian follicles.
***Methods*:** Human ovarian cortical slices from premenopausal women were incubated with medium containing cyclophosphamide (0.0005–0.5 mg/mL) for 2–48 h and assessed by transmission electron microscopy. Noncultured specimens and samples cultured without cyclophosphamide were used as controls.
***Results*:** There were significantly more damaged granulosa cell nuclei after incubation with 0.5 mg/mL cyclophosphamide for at least 4 h. There were also more changes in the basement membrane after incubation with cyclophosphamide at concentrations of 0.05 and 0.5 mg/mL.
***Conclusions*:** Although the cyclophosphamide dose that caused damage to the granulosa cell nuclei was above the pharmacological level, our results suggest that cyclophosphamide, and not only its active metabolite phospharamide mustard, might have a destructive effect on human follicles, as it remains in the circulation longer. This effect could be mediated by damage to the granulosa cells and perhaps the basement membrane.

KEY WORDS: Cyclophosphamide; granulosa cells; oocytes; ovarian failure; primordial follicles.

INTRODUCTION

More and more young women with cancer are surviving, thanks to improvements in treatment. However, many suffer from ovarian failure and infertility due to chemotherapy (1). The mechanism underlying this damaging side effect is not yet fully understood as chemotherapeutic drugs affect dividing cells, whereas most human follicles are primordial and do not undergo mitotic division. It is possible that apoptosis, i.e. physiologically programmed cell death, which is associated with various ovarian atresia events (2), is responsible for the follicular damage (1). The morphological characteristics of apoptosis can be identified by transmission electron microscopy (TEM), as follows: deletion of single cells; membrane blebbing; cell shrinkage; phagocytosis by adjacent normal cells and macrophages; and compaction of chromatin into uniformly dense masses (2).

The main chemotherapeutic drugs that induce ovarian damage are alkylating agents such as cyclophosphamide (Cp) (3). Cp acts on malignant cells only after it undergoes hepatic transformation to form an active metabolite, phospharamide mustard. This agent irreversibly binds to a nucleophilic site, especially to DNA. The DNA strands then break and cannot be further synthesized, leading to cell death. However, whereas phospharamide mustard has a half-life of only 40–50 min, Cp has a half-life of up to 8 h and can be detected in the circulation even 24 h after administration. Therefore, despite its lack of direct toxicity to cancer cells (3), Cp may be toxic to noncancerous cells. The aim of the present study was to determine if Cp has a direct effect on cultured human ovarian follicles.

METHODS

Ovarian Material

Ovarian tissue was obtained from the right ovaries of six premenopausal patients aged 13–39 years (the youngest was premenarcheal). The patients had lymphoma and leukemia, and the tissue was retrieved prior to anticancer treatment at laparoscopy for cryopreservation of ovarian tissue for putative fertility preservation (1). The hospital's ethics committee approved the study. All participants or guardians provided written informed consent.

Cryopreservation and Thawing of Ovarian Tissue

All the ovarian cortical specimens were cut to a size of 2–5 mm and cryopreserved and thawed with a 1,2- propanediol (PROH) (Sigma) and sucrose (Sigma) solution (4). In the present study the

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freezing and thawing medium contained synthetic serum (Irvine Scientific, Santa Ana, CA). Prior to cryopreservation, the samples were transferred through increasing concentration gradients of the freezing solution for several minutes and then frozen slowly in a programmable freezer (Kryo 10; series 10/20, Planer Biomed, Sunbury on Thames, U.K.). Ovarian pieces were thawed by transferring them in decreasing concentration gradients of PROH and sucrose for several minutes. After thawing, the specimens were transferred to an Earle's balanced salt solution (EBSS) (Biological Industries, Beit Haemek, Israel).

Results from our laboratory demonstrate very little follicular damage after freezing–thawing (4). Therefore, by using such tissue, we were able to evaluate the follicular content prior to culture and to minimize the possibility of culturing specimens with low follicular density. Immediately after thawing one-two uniform-sized samples measuring 0.5–1.0 mm were fixed (uncultured controls) in 3% glutaraldehyde (Sigma). Only this sample size allowed for proper fixation and preparation for TEM (4).

Culturing Methods

Millicell CM inserts (Millipore, Bedford, MA) fitted into 24-well plates (Nunclon, Delta, Roskilde, Denmark) were precoated with extracellular matrix gel (Sigma) (4). Thin ovarian slices were cut (0.5–1.0 mm, the same size as the uncultured controls) and placed in the inserts and covered with medium. The basic culture medium was supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biological Industries, Beit Haemek, Israel) and 0.5 U/mL purified human follicle stimulating hormone (FSH) (Metrodin, Teva Pharmaceutical Industries, Petah Tikva, Israel) (4). Cp was then added to the culture medium in concentrations of 0.05 mg/mL and 0.5 mg/mL (L and H, respectively, in Tables I and II). As every cultured slice weighed approximately 1 g, and pharmacological doses given to patients are 1.5–60 mg/mL/kg (3), 0.05 mg/mL (L) was in the pharmacological range. Furthermore, the concentration of Cp might be lower in the circulation and near the ovary, so lower doses (0.005 and 0.0005 mg/mL) were initially used as well. Other samples were cultured without Cp (cultured controls). The plates were incubated for 2, 4, 24, and 48 h in a standard incubator. At termination of every incubation, pH was measured and the ovarian samples were removed from the inserts and immediately fixed in 3% glutaraldehyde (Sigma). The spent medium samples were collected for subsequent osmolarity measurement.

Preparation for Transmission Electron Microscopy

Our TEM preparation method has been described previously for intact ovarian specimens (4). The blocks were sectioned serially, with intervals of at least 50 μm between follicles to avoid resectioning the same follicle. Semithin (0.5–0.75 μm) sections for light microscopy (LM) were stained with toluidine blue (BDH Chemicals Ltd, Poole, U.K.) and ultrathin (1-Å) sections for TEM were stained with uranil acetate (BDH) and lead citrate (BDH). The follicles were examined with a JEOL (JEM 1010) TEM.

The follicles were first counted in all LM sections. Various general parameters were observed and measured using the TEM bar-scale: type; diameter F; diameter of the granulosa cell (GC) layer G; and G/F ratio, as a measure of the thickening of the GC layer (1). Changes in the basement membrane (BM) were also noted (5). A thin BM was considered normal, and changes were defined as follows: thin but interrupted (Ti); thickened (Th); and totally destroyed (D). In addition, the GC layer was observed for percent of normal nuclei, and the GC layer and the oocytes were observed for the number of vacuoles (4–6) and lipid droplets (4,6). The latter were given a range of 1–3 (4); with 1 indicating very few vacuoles/lipid droplets and 3 indicating many, according to a scaling method used by our group previously (4). Two authors (Ahud Raz, Ronit Abir) evaluated these follicular parameters independently, twice each. The condition of the oocyte nuclei was not taken into account, as in many cases the follicles were not at the midsection. Morphological characteristics of apoptosis were noted (2). The follicles were photographed by a computerized system (Gatan BioScan Camera, Digital Micrographs) attached to the electron microscope.

Initial observations (7–8 follicles/group) revealed no changes in the follicles cultured with 0.005 and 0.0005 mg/mL or in the follicles cultured for only 2 h at all Cp concentrations; these blocks were, therefore, not sectioned further.

Statistical Analysis

Data were statistically analyzed by unpaired Student's t test and chi-square test.

Table I. General Characteristics of Follicles and Basement Membrane

Group	UC	0/4 h	0/24 h	0/48 h	L/4 h	L/24 h	H/4 h	H/24 h	H/48 h
Follicles Number	44	26	35	14	35	22	27	32	12
Follicles Class	pf = 30[a], 1st = 10, 2nd = 4	pf = 13[a], 1st = 12, D = 1	pf = 11, 1st = 17, 2nd = 6[c], D = 1	pf = 1, 1st = 9, D = 3	pf = 22[b], 1st = 12, 2nd = 1	pf = 6, 1st = 10, 2nd = 4, D = 2	pf = 15[a], 1st = 8, D = 4	pf = 12, 1st = 14, D = 2	pf = 2, 1st = 8, D = 2
Basement Membrane	Ti = 4, Th = 3, D = 1	Ti = 8	Ti = 5, Th = 3	Ti = 5, Th = 3, D = 2	Ti = 18[d], Th = 4, D = 1	Ti = 10[e], Th = 1, D = 1	Ti = 12[f], Th = 3, D = 3	Ti = 14[g], Th = 3, D = 2	Ti = 2, D = 3[h]

Note. Values are means ± SD; UC, uncultured control; 0, cultured control; L, 0.05 mg/mL; H, 0.5mg/mL; pf, primordial; 1st, primary; 2nd, secondary follicles; D, destroyed; Ti, thin interrupted; Th, thickened.
[a] Significantly higher than 0/4 h; 0/24 h, 0/48 h, H/48 h ($P < 0.02$ to $P < 0.0001$).
[b] Significantly higher than L/24 h and 0/48 h ($P < 0.0001$).
[c] Significantly more 1st and 2nd than uc; 0/4 h, L/4 h, H/4 h ($P < 0.01$ to $P < 0.0001$).
[d] Significantly higher than uc; 0/4 h, L/4 h, H/4 h and 0/24 h ($P < 0.01$ to $P < 0.0001$).
[e] Significantly higher than uc and 0/24 h ($P < 0.03$ and $P < 0.006$ respectively).
[f] Significantly higher than 0/24 h ($P < 0.007$).
[g] Significantly higher than uncuc; 0/4 h; 0/24 h and 0/48 h, H/48 h ($P < 0.05$ to $P < 0.0001$).
[h] Significantly higher than 0/4 h and 0/24 h ($P < 0.05$ and $P < 0.02$ respectively).

RESULTS

There were no significant differences in pH (7.14–7.4) or osmolarity (283–296 mOsmol) of the spent media samples in any of the groups. Owing to a technical problem with the incubator, the blocks of ovarian tissue of one patient at 48 h were excluded from the analyses, leading to a total lower number of follicles in all 48 h groups. Moreover, only three follicles were identified in the group cultured with 0.05 mg/mL for 48 h (0.05 mg/mL–48 h) (results not shown). No intracellular changes could be seen under LM.

Table I and Fig. 1 show the most significant of the TEM observations, as follows: 1) A significantly higher number of primordial follicles in the control and study samples and samples cultured for 4 h, as well as a higher number of primary and secondary follicles in control–24 h (0/24 h in Tables I and II). 2) Significantly more follicles with thin–interrupted (Ti) BM at 0.05 mg/mL–4 h (L/4 h in Tables I and II), 0.5 mg/mL–4 h (H/4 h in Tables I and II), 0.05 mg/mL–24 h (L/24 h) and 0.5 mg/mL–24 h (H/24 h), with more follicles with destroyed (D) BM at 0.5 mg/mL–48 h (H/48 h). 3) A decrease in normal GC nuclei in follicles cultured with 0.5 mg/mL (40.4 ± 34.4 to 51.8 ± 40.1). 4) More vacuoles in GCs of uncultured controls (UC) and at 0.5 mg/mL–4 h (H/4 h); 0.5 mg/mL–24 h (H/24 h) and 0.5 mg/mL–48 h

Table II. General Characteristics of Granulosa Cells and Oocytes

Group	UC	0/4 h	0/24 h	0/48 h	L/4 h	L/24 h	H/4 h	H/24 h	H/48 h
% Normal GC Nuclei	97.0 ± 0.8^a	83.7 ± 32.6^a	91.2 ± 24.3^a	83.0 ± 31.6^a	79.5 ± 27.6^a	80.4 ± 27.0^a	40.4 ± 34.4	50.3 ± 39.2	51.8 ± 40.1
Vacuoles GC	1.9 ± 1.2^b	1.1 ± 0.9	2.0 ± 0.6^c	2.4 ± 0.7^d	1.8 ± 0.8	2.3 ± 0.6^e	2.3 ± 0.8^c	2.4 ± 0.8^d	2.5 ± 0.9^d
Lipids GC	0.5 ± 0.8	1.2 ± 0.7^e	1.3 ± 0.7^e	2.0 ± 0.8^f	1.3 ± 0.8^c	2.3 ± 0.7^g	1.2 ± 0.6	1.8 ± 1.0^f	2.1 ± 1.1^f
Vacuoles Oocyte	0.4 ± 0.5	0.6 ± 0.8	0.9 ± 0.6	1.9 ± 1.0^h	0.5 ± 0.5	1.3 ± 1.0^d	0.9 ± 1.1	2.1 ± 1.1^i	1.0 ± 1.2

Note. Values are means ± SD; UC, uncultured control; 0, cultured control; L, 0.05 mg/mL; H, 0.5mg/mL; GC, granulosa cells.
[a] Significantly higher than h/4 h; H/24 h, and H/48 h ($P < 0.05$ to $P < 0.0001$).
[b] Significantly higher than 0/4 h ($P < 0.05$).
[c] Significantly higher than 0/4 h, L/4 h ($P < 0.005$ to $P < 0.0003$).
[d] Significantly higher than UC; 0/4 h, L/4 h ($P < 0.03$ to $P < 0.0001$).
[e] Significantly higher than UC ($P < 0.002$ to $P < 0.006$).
[f] Significantly higher than UC; 0/4 h, L/4 h, H/4 h; 0/24 h ($P < 0.05$ to $P < 0.0001$).
[g] Significantly higher than UC; 0/4 h, L/4 h, H/4 h; 0/24 h, H/24 h ($P < 0.08$ to $P < 0.0001$).
[h] Significantly higher than UC; 0/4 h, L/4 h, H/4 h and 0/24 h ($P < 0.01$ to $P < 0.0001$).
[i] Significantly higher than UC; 0/4 h, L/4 h, H/4 h; 0/24 h, L/24 h and 0/48 h ($P < 0.01$ to $P < 0.0001$).

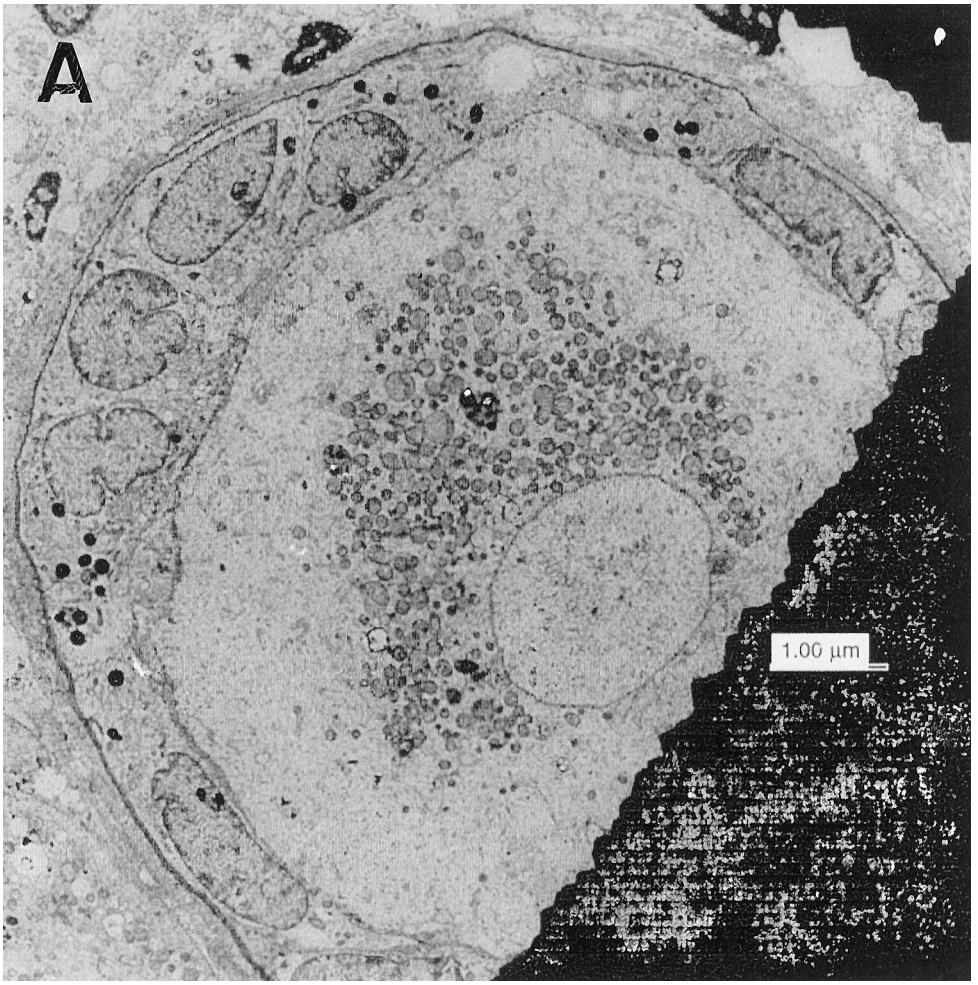

Fig. 1. TEM photographs of human follicles. (A) Electron microscope section of an uncultured primordial follicle from a 39-year-old patient. Note the normal oocyte nucleus, its surrounding organelles, normal GC, and the thin but normal BM (original magnification X2500). (B) Electron microscope section of a follicle from a 21-year-old patient cultured at 0.5 mg/mL–24 h. Note the destroyed oocyte, leakage (L) of nuclear material into the oocyte, abnormal GC nuclei (arrow), and thin but normal BM (original magnification X3000). (C) Electron microscope section of a follicle from the same patient as in Fig. 1(A) cultured at 0.5 mg/mL–48 h. Note that this is not a midsection, the numerous large lipid droplets (L), vacuoles (V), the normal GC nucleus (arrow), and the totally destroyed BM (D) (original magnification X8000).

(H/48 h). 5) An increase in lipid droplets in the GCs in control–4 h (0/4 h) and 0.05 mg/mL–4 h (L/4 h), control–24 h (0/24 h) and 0.05 mg/mL–24 h (L/24 h), control–48 h (0/48 h) and 0.5 mg/mL–48 h (H/48 h). 6) Regarding oocytes, more vacuoles appeared at 0.05 mg/mL–24 h (L/24 h), 0.5 mg/mL–24 h (H/24 h), and control–48 h (0/48 h).

There was no correlation between age and follicular damage. Moreover, although the ovarian samples from the youngest premenarcheal patient had more follicles, they were not less damaged than the samples of the other patients. We could not identify any morphological signs of apoptosis in any of the specimens examined. There were no significant differences in follicular diameter, diameter of GC, or *G*/*F* ratio between any of the groups (results not shown). There were also no significant changes in oocyte lipid droplets in any of the groups.

DISCUSSION

The present study showed for the first time that the incubation of human follicles for at least 4 h with Cp caused damage to the GC nuclei (0.5 mg/mL) and the BM (0.05 and 0.5 mg/mL). In addition more

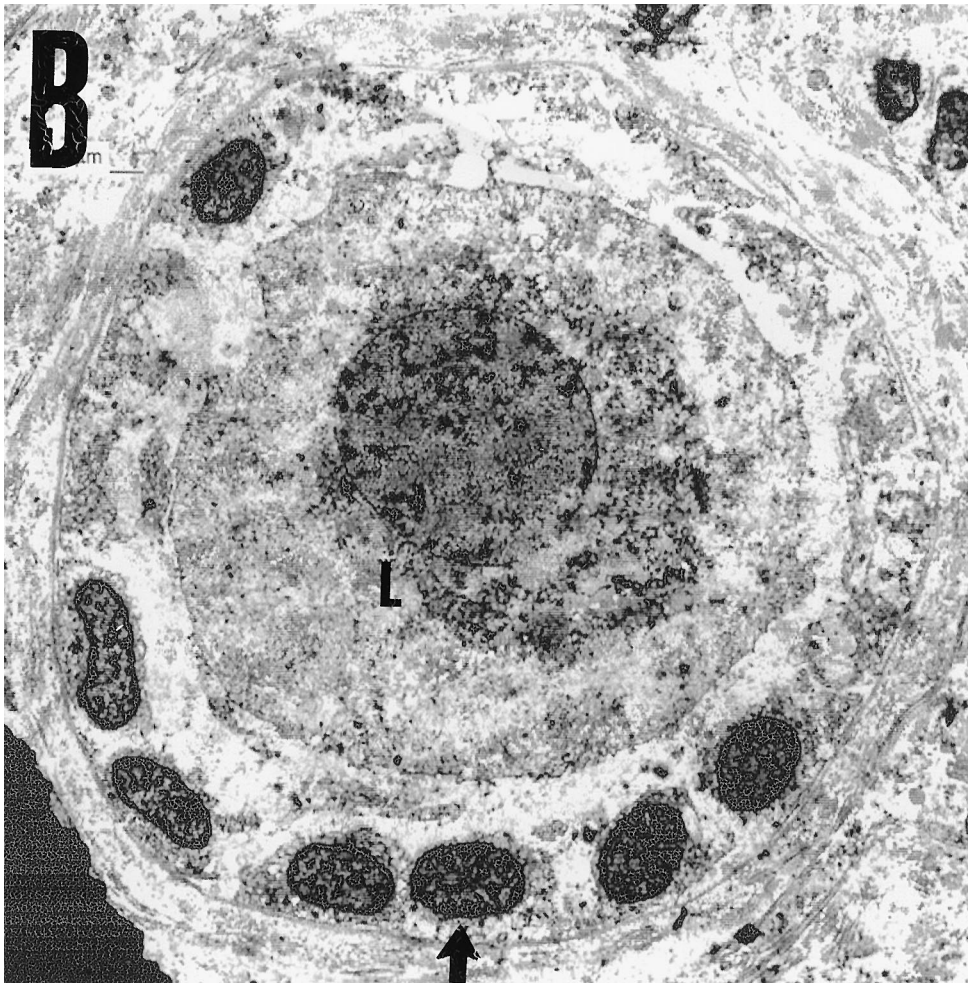

Fig. 1. (*Continued*)

primordial follicles were identified initially, whereas the proportion of primary and secondary follicles increased at 24 h.

Although 0.5 mg/mL is above the pharmacological dose (3), high doses of Cp are often administered continuously for up to 4 days in patients with cancer, leading to high circulating levels. Moreover, our previous studies showed that cultured human follicles required higher FSH dose than the physiological level for growth (4). Thus, it is possible that in vitro conditions do not reflect the actual in vivo concentrations. Indeed, Chen *et al.* (7) found that incubation of cumulus-enclosed germinal-vesicle-stage porcine oocytes with Cp (0.001–1.0 mg/mL) for up to 48 h induced a dose–response inhibition of nuclear maturation, especially from 24 h with 0.1 mg/mL. This concentration (0.1 mg/mL) was also above pharmacological levels, and our optimal concentration (0.5 mg/mL) was within the range used by Chen *et al.* (7). Fein *et al*. (8) cultured rat blastocysts, with 0.1 mg/mL Cp for 2 and 48 h. There was a clear toxic effect of Cp on the cultured blastocysts and when they were transferred to psuedopregnant rats, large numbers of resorptions and retarded embryos were observed.

Meirow (1) performed LM studies and a terminal deoxynucleotidyl transferase (TUNEL) assay to evaluate apoptosis in human primordial follicles cultured with cisplatin for up to 36 h. Swelling of the GCs as well as their nuclei was noted. In the present study there was no swelling of the GC layer after incubation with Cp, as reflected by consistencies in the diameters of the GC layer and the *G*/*F* ratios. Meirow (1) reported that cisplatin induced apoptosis in GC; but we failed to identify any morphological signs of apoptosis. Our results, however, are in line with Tannock and

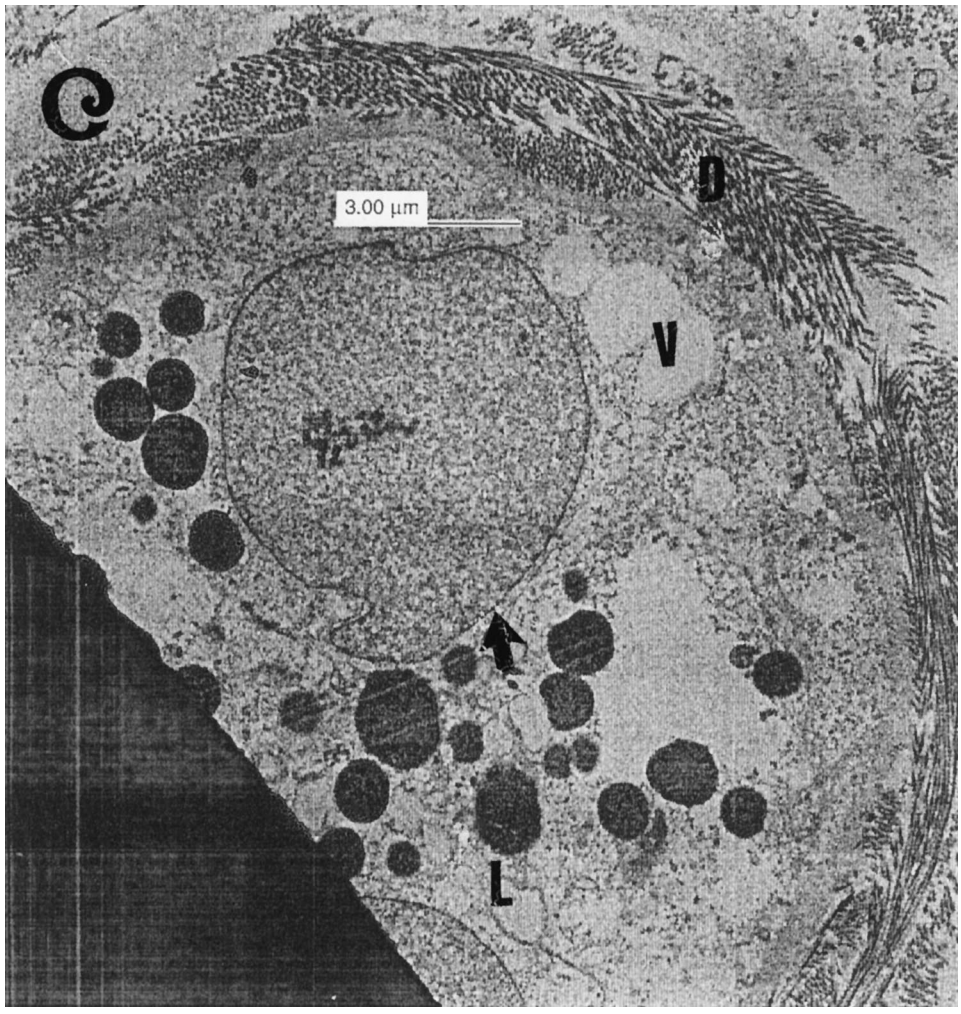

Fig. 1. (*Continued*)

Lee (9), who used a TUNEL assay and a DNA ladder visualization method to show that apoptosis was not responsible for the cell death of cultured cancerous cell lines after exposure to various chemotherapeutic drugs, including cisplatin. Both our study and Meirow's (1) showed that chemotherapeutic drugs cause damage to the GC layer even after only a few hours of incubation. It is, therefore, possible that in the present study, the follicles were damaged by direct cytotoxicity of Cp to the granulosa layer, as follicles cannot remain viable without interactions between the GCs and the oocytes (4,10).

An increase in lipid droplets and vacuoles in both the GCs and the oocytes (4), as well as irregular changes in the BM (5), suggest a possible deterioration in follicular health. One study reported an increase in lipid droplets in isolated human unilaminar follicles (4), and another showed radiation-induced accumulation of lipid droplets in oocytes of mouse unilaminar follicles (6). Familliari *et al*. (5), in a TEM study of four ovarian biopsy samples from women after chemotherapy, noted vacuoles in the follicular GC layer and in the oocyte cytoplasm, in addition to an abnormally thick BM. Accordingly, in the present study, there was an increase in vacuoles in the GC and oocytes after culture, even in some of the control groups. It is noteworthy that a significantly higher number of vacuoles in the GCs of follicles from uncultured controls (frozen–thawed) was also observed, suggesting that cryopreservation may cause more damage than reported previously (4). Our study is also in line with that of Familliari *et al*. (5) regarding chemotherapy-induced irregular changes in the BM. However, the thickened BM

observed here might be attributable to a transition to the primary–secondary stages (11) and not to the effect of Cp. The BM changes in our study included an increase in interrupted BM and totally destroyed BM.

Our study also showed more primordial follicles in uncultured control samples and samples at 4 h, and more primary and secondary follicles in control–24 h samples. This finding might be interpreted as a rapid activation of primordial follicles during 24 h of incubation, owing either to the removal of the follicles from in vivo inhibitors (4), or perhaps to FCS induction. At the same time, the quality of the follicles deteriorated during culture independent of the addition of Cp, as evidenced by the increase in lipid droplets in GCs from 4 h onwards in all samples, including controls. Researchers using this culture system for growing follicles should take this factor into account.

Age is directly correlated with the effect of chemotherapy on human ovaries (1,5,11) and researchers believe there is less damage to ovaries of premenarcheal girls (11). Marcello *et al*. (11) used TEM to study the ovaries of 10 girls with leukemia, 3 of whom were postmenarcheal. There was a clear reduction in the number of follicles in all the girls; however, signs of follicular damage were noted only in the postmenarcheal girls; namely stromal fibrosis and capillary changes. Our study included only one relatively old (13 years) premenarcheal girl; although her ovaries contained more follicles, the ovarian damage was not different from that found in older patients.

In the present study, the relevant hepatic enzymes (3) were not added to the culture medium to convert Cp into its metabolites. Therefore, the findings suggest that Cp, and not only its metabolite, plays a role in follicular destruction in culture. Phospharamide mustard may be even more toxic to human follicles than its precursor, and it should be added to cultures of human follicles in future studies. To the best of our knowledge the product is currently unavailable commercially.

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