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ORIGINAL ARTICLE ***Reproductive endocrinology***

The magnitude of gonadotoxicity of chemotherapy drugs on ovarian follicles and granulosa cells varies depending upon the category of the drugs and the type of granulosa cells

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STUDY QUESTION: Do different chemotherapy drugs exert the same magnitude of cytotoxicity on dormant primordial follicles and the growing follicle fraction in the ovary *in vivo* and on mitotic non-luteinized and non-mitotic luteinized granulosa cells *in vitro*?

SUMMARY ANSWER: Cyclophosphamide (alkylating agent) and cisplatin (alkylating like) impacted both primordial and pre-antral/antral follicles and both mitotic and non-mitotic granulosa cells, whereas the anti-metabolite cancer drug gemcitabine was detrimental only to pre-antral/antral follicles and mitotic non-luteinized granulosa cells.

WHAT IS KNOWN ALREADY: It is already known that anti-metabolite cancer drugs are less detrimental to the ovary than alkylating and alkylating like agents, such as cyclophosphamide and cisplatin. This assumption is largely based on the results of clinical reports showing lower rates of amenorrhea in women receiving anti-metabolite agent-based regimens compared with those treated with the protocols containing an alkylating drug or a platinum compound. But a quantitative comparison of gonadotoxicity with a histomorphometric proof of evidence has not been available for many chemotherapy drugs. Therefore, we combined in this study *in vivo* and *in vitro* models of human and rat origin that allows a comparative analysis of the impact of different chemotherapy agents on the ovary and granulosa cells using real-time quantitative cell indices, histomorphometry, steroidogenesis assays, and DNA damage and cell death/viability markers. We also aimed to investigate if there is a difference between mitotic and non-mitotic granulosa cells in terms of their sensitivity to the cytotoxic actions of chemotherapy drugs with different mechanisms of action. This issue has not been addressed previously.

STUDY DESIGN, SIZE, DURATION: This translational research study involved *in vivo* analyses of ovaries in rats and *in vitro* analyses of granulosa cells of human and rat origin.

PARTICIPANTS/MATERIALS, SETTING, METHODS: For the *in vivo* assays, 54 4- to 6-week old Sprague-Dawley young female rats were randomly allocated into four groups of 13 to receive a single IP injection of: saline (control), gemcitabine (200 mg/kg), cisplatin (50 mg/kg) or

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cyclophosphamide (200 mg/kg). The animals were euthanized 72 h later. Follicle counts and serum AMH levels were compared between the groups. *In vitro* cytotoxicity studies were performed using mitotic non-luteinized rat (SIGC) and human (COV434, HGrC1) granulosa cells, and non-mitotic luteinized human (HLGC) granulosa cells. The cells were plated at a density of 5000 cells/well using DMEM-F12 culture media supplemented with 10% FBS. Chemotherapy agents were used at their therapeutic blood concentrations. The growth of mitotic granulosa cells was monitored real-time using xCelligence system. Live/dead cell and apoptosis assays were also carried out using intravital Yo-Pro-1 staining and cleaved caspase-3 expression, respectively. Estradiol (E2), progesterone (P) and anti-Mullerian hormone (AMH) levels were assayed with ELISA.

MAIN RESULTS AND THE ROLE OF CHANCE: Cyclophosphamide and cisplatin caused massive atresia of both primordials and growing follicles in the rat ovary whereas gemcitabine impacted pre-antral/antral follicles only. Cyclophosphamide and cisplatin induced apoptosis of both mitotic non-luteinized and non-mitotic luteinized granulosa cells *in vitro*. By contrast, cytotoxicity of gemcitabine was confined to mitotic non-luteinized granulosa cells.

LIMITATIONS, REASONS FOR CAUTION: This study tested only three chemotherapeutic agents. The experimental methodology described here could be applied to other drugs for detailed analysis of their ovarian cytotoxicity.

WIDER IMPLICATIONS OF THE FINDINGS: These findings indicate that *in vivo* and *in vitro* cytotoxic actions of chemotherapy drugs on the ovarian follicles and granulosa cells vary depending upon the their mechanism of action and the nature of the granulosa cells.

STUDY FUNDING/COMPETING INTEREST(S): The School of Medicine and the Graduate School of Health Sciences of Koc University, and American Hospital Women's Health Center, Comprehensive Cancer Care and Fertility Preservation Programs, Istanbul Turkey. All authors declare no conflict of interest.

Key words: gemcitabine / cisplatin / cyclophosphamide / ovary / granulosa cells / cytotoxicity

Introduction

Over the last three decades, the 5-year survival rates have significantly improved for both adult and pediatric cancer patients (Oktem and Urman, 2010a,b; Siegel *et al.*, 2014). Premature ovarian failure and other poor reproductive outcomes are now being recognized as an important sequelae of previous exposure to chemotherapy and/or radiotherapy during childhood and adulthood. Cytotoxic chemotherapy and radiotherapy regimens cause apoptosis in oocytes and in the surrounding granulosa cells in the ovary, leading to early exhaustion of the follicle stockpile and premature ovarian failure (Oktem and Oktay, 2007; Morgan *et al.*, 2012). Chemotherapy agents of the alkylating category, such as cyclophosphamide and busulfan, are much more detrimental to the ovary than the drugs of all other categories. Their effects are not cell cycle specific. They can induce genomic and organelle damage in the cells regardless of the stage of cell cycle leading to more widespread apoptosis and organ damage. By contrast, anti-metabolite cancer drugs, such as methotrexate, gemcitabine and 5-fluorouracil, are specific to the S-phase of the cell cycle (DNA synthesis), and are generally associated with milder ovarian toxicity than cyclophosphamide and cisplatin (Oktem and Oktay, 2008; Morgan *et al.*, 2012), based on the results of clinical reports showing lower rates of amenorrhea in women receiving anti-metabolite agent-based regimens compared with those treated with the protocols containing an alkylating drug or a platinum compound. Nevertheless, histomorphometric evidence with real-time quantitative cell proliferation indices, steroidogenesis and cell viability markers has not been available for many chemotherapy drugs. Thus, the available data on their gonadotoxic potential are quite vague. To answer this need, we designed an experimental methodology combining *in vivo* and *in vitro* models of human and rat ovary and granulosa cells with two specific aims: (i) to determine whether there are any differences in the magnitude of *in vivo* cytotoxicity of chemotherapy drugs on dormant primordial follicles and on growing follicles in the ovary, and (ii) to determine whether there is a difference in chemosensitivity between mitotic and non-mitotic human granulosa cells. For *in vivo* chemotherapy-induced cytotoxicity studies, we utilized the rat ovary. For *in vitro* assays, we used four different types of granulosa cells: spontaneously immortalized rat granulosa cells (SIGC), mitotic human granulosa cells (COV434 and HGrC1) and human luteal granulosa cells (HLGC). SIGC, COV434 and HGrC1 are mitotic non-luteinizing granulosa cell lines, whereas HLGC are highly specialized non-proliferating, luteinized granulosa cells obtained from follicular fluids during oocyte retrieval procedures in IVF patients. For the cytotoxicity assays, we chose three different categories of chemotherapy agents (cyclophosphamide, cisplatin and gemcitabine) whose mechanisms of action and gonadotoxic potentials are different. Cyclophosphamide and cisplatin are being widely used as the first line treatment of many solid and hematological malignancies. Both drugs were used in this study as index drugs since their cytotoxicities are well documented in human and animal studies (Oktem and Oktay, 2007; Kalich-Philosoph *et al.*, 2013; Morgan *et al.*, 2013). Gemcitabine is a pyrimidine analog chemotherapy drug mainly used in the treatment of non-small cell lung cancer, and the cancers of pancreas, bladder and breast (Mini *et al.*, 2006). It is also used in the treatment of resistant Hodgkin lymphoma and childhood osteosarcoma in combination with other chemotherapeutics (Navid *et al.*, 2008). The cytotoxic potentials of these chemotherapy drugs were assessed according to the rate of apoptosis, real-time quantitative proliferation indices, follicle reserve and steroidogenic activity of the samples before and after exposure to these drugs.

Materials and Methods

The animals

The study was approved by the Scientific Research Ethics Committees of Koc University and Cerrahpasa School of Medicine of Istanbul University. A total

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of 54 Sprague-Dawley female rats, 4–6 weeks old and weighing 100 ± 20 g, were divided into four groups, each including 13 rats. Group-1 received a single dose of 200 mg/kg gemcitabine by intraperitoneal injection (IP). Group-2 received a single dose of 200 mg/kg cyclophosphamide by IP. Group-3 received a single dose of 1.5 mg/kg cisplatin by IP. Group-4 (controls) received a single IP injection of normal saline. The doses of chemotherapy drugs were determined based on previous animal studies and corresponded to therapeutic blood levels of the drug (Mackey *et al.*, 1999; Laquente *et al.*, 2008; Morgan *et al.*, 2008, 2013). During the study, rats were housed in standard cages, specifically designed for mice or rats, with the sides and floor made of plastic and covered on the top by iron mesh. A maximum of five rats were housed in each cage. During the study, the floors of the cages were covered with dry wood shavings, which were changed every 2 days. The rats were fed with pellet-type processed food produced specifically for small experimental animals. The animals were euthanized with CO_2, their ovaries were removed and blood samples (100 μl) were obtained for AMH measurement at 72 h after the injections.

Histomorphometric assessment of the ovaries

The ovaries were processed using conventional techniques as described previously (Oktem and Oktay, 2007). In brief, they were first fixed in neutral formalin solution (10%) overnight and then paraffin-embedded and serially sectioned 7 microns apart. After hematoxylin-eosin staining, healthy and atretic follicle counts for were determined on every fifth section until the whole ovary was evaluated. Classification of primordial, primary, pre-antral and antral follicles and their corresponding healthy and atretic fractions were calculated as described previously (Oktem and Oktay, 2007). Follicle numbers were expressed as follicular density (follicle count/mm^2).

Mitotic non-luteinized human and rat granulosa cells (HGrC1, COV434 and SIGC)

HGrC1 is a human non-luteinized granulosa cell line expressing enzymes related to steroidogenesis, such as steroidogenic acute regulatory protein, aromatase and gonadotrophin receptors. Stimulation with FSH in these cells increases the mRNA levels of aromatase which consequently induces the aromatization of androstenedione to estradiol. Activin A increases the mRNA levels of the FSH receptor which is also synergistically up-regulated with FSH stimulation. Thus HGrC1 may possess the characteristics of granulosa cells in early stage follicles. HGrC1 might also be capable of the growth transition from a gonadotrophin-independent status to gonadotrophin-dependent one (Bayasula *et al.*, 2012), but they are not capable of undergoing luteinization.

COV434 was obtained from a human granulosa cell tumor. The biological characteristics of this cell line include the production of 17 beta-estradiol in response to FSH, the absence of the LH receptor, no luteinization capability, and the presence of specific molecular markers of apoptosis enabling the induction of follicular atresia (Zhang *et al.*, 2000).

Spontaneously immortalized rat granulosa cells (SIGC) have an epithelial morphology, express gonadotrophin receptors and grow in culture indefinitely. They respond to FSH stimulation with enhanced growth but without undergoing luteinization (Stein *et al.*, 1991), resembling the growth characteristics of the proliferating granulosa cells of pre-antral and early antral follicles (Oktem *et al.*, 2011, 2013).

Non-mitotic luteinized granulosa cells (HLGC)

Human luteal granulosa cells (HLGCs) were recovered from follicular fluid during oocyte retrieval procedures in 10 IVF patients. Informed consent was obtained from all participants. Follicular fluids were spun down at 500 × g for 10 min and recovered cells were plated in 24-well format culture plates at a density of 5000 cells per well using DMEM-F12 culture medium supplemented with 10% fetal bovine serum. Since they are highly specialized primary luteinized granulosa cells, they do not proliferate either spontaneously or after stimulation with a mitogenic agent. They produce large amounts of progesterone and estradiol hormones. All three types of granulosa cells were cultured with DMEM-F12 culture media supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.

Real-time and quantitative assessment of cell proliferation and viability using xCelligence

xCelligence is a cell culture system that allows real-time quantitative analysis of cell proliferation, viability, cytotoxicity and adhesion/invasion/receptor activity assays without using any compound or labeling agent. The system uses specially designed microtiter plates containing interdigitated gold microelectrodes to non-invasively monitor the viability of cultured cells using electrical impedance as the readout and generates real-time curves of cell viability and proliferation (Bird and Kirstein, 2009). Once the cells were attached to the bottom of the E-96 well culture plate (4–6 h after plating), they cells were monitored until the log phase where they were treated with the chemotherapy drugs at the indicated concentrations. The proliferation and the viability of the cells were monitored in a real-time and quantitative manner. Gemcitabine was used at 6, 12, 25, 50 and 100 μg/ml concentrations, which correspond to minimal and peak blood levels of the drug (Felici *et al.*, 2009). Cisplatin was used at 20, 40 and 100 μg/ml concentrations (Bonetti *et al.*, 1996). Since cyclophosphamide requires a 4-hydroxylation reaction in the liver to be converted to active compound, it cannot be used *in vitro*. Instead 4-hydroperoxy cyclophosphamide, the active *in vitro* metabolite of the drug was used (4-HC) at 25, 50 and 100 μg/ml concentrations (Teicher *et al.*, 1996). The viability and proliferation of the cells were monitored for every 30 min for up to 140 h. The results were expressed by normalized cell index (CI) which are derived from the ratio of CIs before and after the addition of the compounds. The normalization of CI arbitrarily sets the CI to 1 at the indicated time points. Recording of CI and normalization CI was performed using the RTCA Software 1.2.

Live cell imaging with YO-PRO-1 staining for the assessment of cell viability

Apoptotic cells become permeant to the green-fluorescent carbocyanine nucleic acid stain YO-PRO®-1 (absorbance 491 nm, emission 509 nm), whereas live cells are impermeable to it. YO-PRO-1 (1 μM) was added to the culture medium and incubated for 30 min. Then live/dead cell imaging of the cells were undertaken under appropriate channels using an IF microscope (Olympus IX71, Japan). Lots of 500 cells were counted at four different high magnification areas (×20) and the percentage of the cells expressing Yo-PRO-1 was calculated.

Detection of apoptosis with cleaved caspase-3 expression by immunofluorescence

Chemotherapy-treated cells and controls were fixed with 10% neutral formalin for 20 min, and then treated with blocking buffer (1X PBS/5% normal goat serum/0.3% Triton™ X-100) for 1 h. After rinsing with PBS, they were incubated with cleaved caspase-3 antibody (Rabbit monoclonal [E83-77], Abcam, MA, USA) in antibody dilution buffer (1X PBS/1% BSA/0.3% Triton™ X-100) at 1:50 dilution overnight at 4°C. After rinsing with PBS, the cells were incubated with fluorochrome-conjugated secondary antibody (Alexa 486, Molecular Probes, USA) diluted in antibody dilution buffer for 1 h. This step was followed by rinsing the coverslips slides and adding Hoechst 33342 for DNA staining. The images were taken under appropriate channels using an IF microscope (Olympus IX71, Japan). The percentage of

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the caspase-3 positive cells was calculated after counting 500 cells at four different high magnification areas (×20).

Hormone assays

AMH levels were determined using ACTIVE Müllerian Inhibiting Substance/Anti-Müllerian Hormone (MIS/AMH) (Diagnostic Systems Laboratories, Inc., USA) ELISA kit. The analytical sensitivity of the kit was 0.006 ng/ml. Intra-assay precision was given by the manufacturer as the coefficient of variation of the three independent assays, each running eight samples. According to this, the coefficients of variation were as follows: 4.6, 2.4 and 3.3% for the mean ± SD AMH levels of 0.144 ± 0.006, 0.843 ± 0.02 and 4.408 ± 0.147 ng/ml, respectively.

The levels of estradiol and progesterone were determined using the electrochemiluminescence immunoassay 'ECLIA', an immunoassay for the *in vitro* quantitative determination of estradiol and progesterone levels (Elecsys and cobas e immunoassay analyzers, Roche Diagnostics, USA). Lower detection limits for estradiol and progesterone were 18.4 pmol/l (5.00 pg/ml) and 0.095 nmol/l (0.030 ng/ml), respectively.

Chemicals

DMEM-F12 culture media, FBS, YO-PRO-1 and Alexa probes were purchased from Life Technologies (Thermo Fisher Scientific Inc., MA, USA). 4-HC was purchased from Niomech (Bielefeld, Germany). Gemcitabine and cisplatin were from Eli Lilly and Company (IN, USA).

xCelligence system® is a product of Roche Diagnostics (Mannheim, Germany). Anti-cleaved caspase-3 antibody (mAb#9664) was purchased from Cell Signaling Technology Inc. (MA, USA). SIGC cell line was a gift from Dr. Joshua Johnson (Yale University, New Haven, CT, USA). COV434 cell line was purchased from Sigma (St. Louis, MA, USA). HGrC1 was a gift from Dr. Ikara Iwase (Nogoya University, Japan).

Statistical analysis

Follicle counts, AMH levels and cell index readouts of xcelligence system were expressed as the mean ± SEM. Statistical analyses were done using SPSS for windows 20.0 statistical package program. Data were first compared among the groups using ANOVA or Kruskal–Wallis test where appropriate and the data were subjected to *post hoc* analysis if multiple comparison test results returned $P < 0.05$. The percentages of viable and apoptotic cells were compared between the groups using Fisher' exact test. A *P*-value of <0.05 was considered significant.

Results

In vivo gonadotoxicity assays

The numbers of primordial (0.2 ± 0.04 versus 3.43 ± 0.4, $P < 0.01$) and pre-antral/antral follicles (0.6 ± 0.2 versus 4.78 ± 0.07, $P < 0.01$) were significantly decreased in the ovaries of the animals treated with a single dose of cyclophosphamide 200 mg/kg compared with control animals. Similar gonadotoxic effects on the follicles were observed after cisplatin treatment, which caused atresia of both primordials (1.4 ± 0.4 versus 3.43 ± 0.4, $P < 0.01$) and pre-antral/antral follicles (1.71 ± 0.1 versus 4.78 ± 0.07, $P < 0.01$) in comparison to control animals. However, gemcitabine impacted only pre-antral/antral follicles (2.18 ± 0.3 versus 4.78 ± 0.07, $P < 0.05$) while the primordial follicles were preserved (4.4 ± 0.2 versus 3.43 ± 0.4, $P > 0.05$) compared with controls. The degree of cytotoxicity of gemcitabine on the pre-antral/antral follicles was less pronounced than that of cyclophosphamide and cisplatin (Fig. 1A). Serum AMH levels of the animals treated with these drugs were reduced in parallel with the decreasing number of pre-antral/antral follicles but to a lesser extent after gemcitabine (0.9 ± 0.3 versus 1.25 ± 0.4 ng/ml, $P < 0.05$) than cisplatin (0.3 ± 0.08 versus 1.25 ± 0.4 ng/ml, $P < 0.01$) and cyclophosphamide (0.1 ± 0.08 versus 1.25 ± 0.4 ng/ml, $P < 0.01$) (Fig. 1B).

Histological analysis of the control ovaries showed healthy follicles at primordial, transitional and pre-antral stages. The ovarian stroma was uniformly stained and many interstitial cells were identified. By contrast, the samples treated with cyclophosphamide or cisplatin were characterized by a less cellular, more fibrotic stroma, with fewer interstitial cells and a marked disarray of the cells and extracellular matrix. Atretic follicles were visible within the surrounding stroma. The damage was less obvious in the gemcitabine treated samples (Fig. 2).

In vitro cytotoxicity assay

First, we analyzed the growth characteristics of the mitotic granulosa cells plated at different densities in E-96 well plates of the xcelligence system to monitor the progression of the cell cycle and to determine the time periods required for transition from lag to log phase (Supplementary Fig. S1). Then the cells were treated with the chemotherapy agents at the indicated concentrations when they reached log phase. All three drugs caused a dose-dependent growth arrest and apoptosis in the mitotic granulosa (SIGC, HGrC1 and COV434) cells. The real-time growth curves of the cells treated with these drugs were characterized by a marked downward shift for several hours particularly after exposure to cyclophosphamide and cisplatin, indicative of a rapid onset of apoptosis (Fig. 3 and Supplementary Fig. S2). To further validate these findings and confirm the apoptotic death after exposure to the chemotherapy drugs, we carried out live/dead cell analysis with Yo-PRO-1 staining, cleaved caspase-3 expression and nuclear fragmentation by immunofluorescence on the same populations of the cells (Figs 3 and 4, Supplementary Fig. S3). Overall, gemcitabine induced apoptosis in 48% of the cells, whereas the rate increased to 76 and 84% when the cells were treated with cisplatin and cyclophosphamide, respectively (Fig. 4). There was a reciprocal decrease in viability and increase in apoptosis with incrementally increased doses of the drugs.

Next, we treated non-mitotic granulosa cells (HLGCs) with the same drugs at the same concentrations. Since HLGCs are adherent but non-mitotic, they are not suitable for assays in the xcelligence system. The cytotoxic effects of the chemotherapy drugs on the mitotic granulosa cells became evident hours after administration of the drugs on the xCelligence system. We therefore incubated HLGCs with the drugs for at least 24 h and compared the steroidogenic activity and the viability/apoptosis between control and chemotherapy-treated cells. While all three drugs were able to induce the apoptosis marker, cleaved caspase 3, in the mitotic granulosa (HGrC1) cells, the non-mitotic HLGC produced cleaved caspase 3 in response to cyclophosphamide and cisplatin but not in response gemcitabine (Fig. 5). Cyclophosphamide (100 μM) and cisplatin (40 μg/ml) exerted a significant degree of toxicity on the cells, as shown by a dramatic increase in apoptosis along with a concurrent decline in the steroidogenic activity of the cells. While only 4% of the control cells were apoptotic, 75 and 88% of the cells underwent apoptosis after exposure to cisplatin and cyclophosphamide, respectively (Fig. 6). As a result of the apoptosis, these cells also produced significantly lower amounts of E2 and progesterone *in vitro* than the control cells (Fig. 6). However, the cells exposed to gemcitabine

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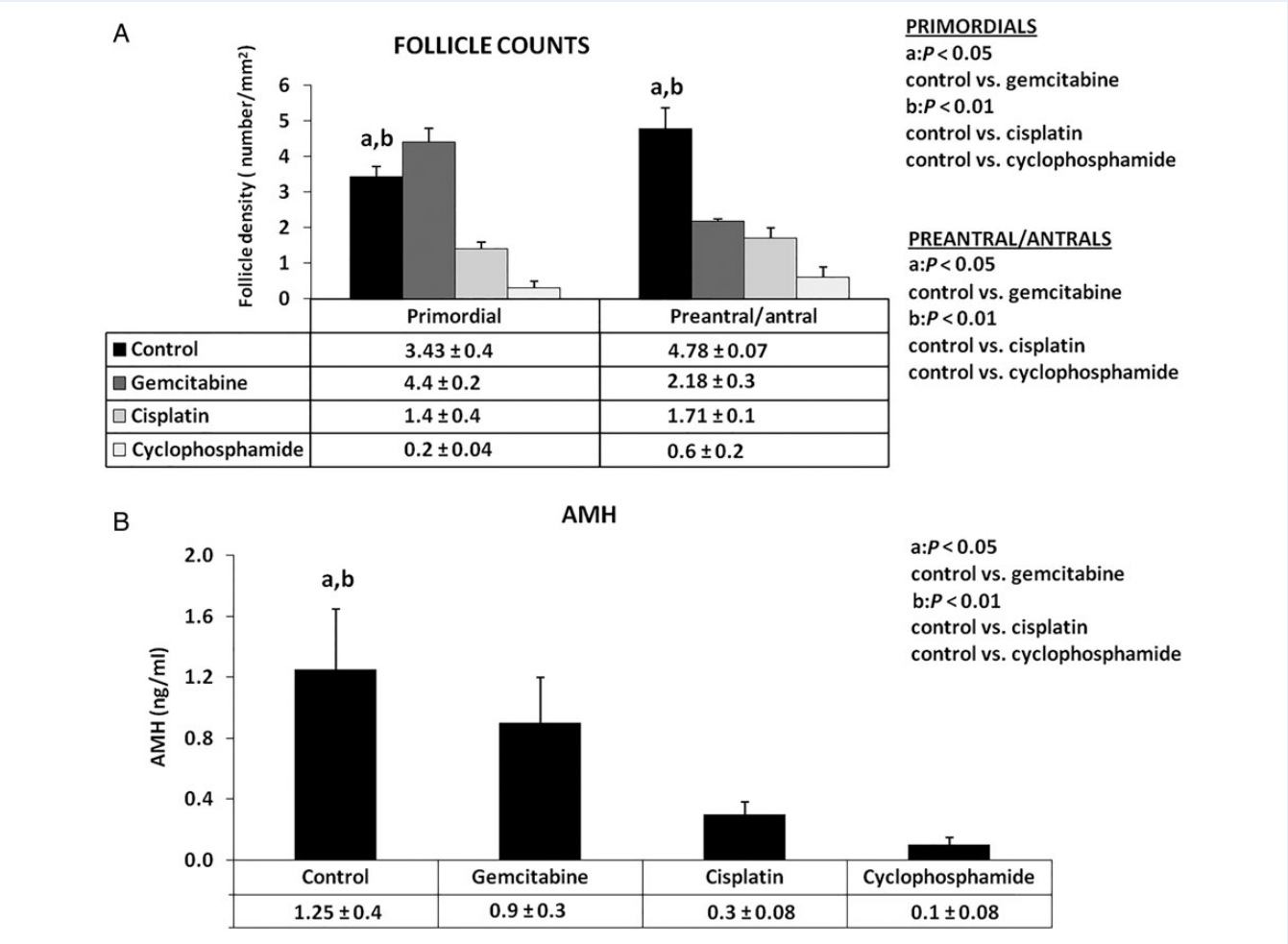

Figure 1 Comparison of *in vivo* gonadotoxic effects of chemotherapy drugs in the rat ovary using follicle counts and serum AMH levels 72 h after intraperitoneal injection of a single dose of gemcitabine (200 mg/kg), cisplatin (1.5 mg/kg) and cyclophosphamide (200 mg/kg). Cyclophosphamide and cisplatin impacted both primordial and pre-antral/antral follicles in the ovary whereas gemcitabine impacted pre-antral/antral follicles only, while the primordial follicles were preserved (**A**). Serum AMH levels were reduced in the animas treated with these drugs, indicative of their detrimental effects on the growing follicle fraction (**B**). The extent of follicle loss and the reductions in serum AMH levels were less pronounced after gemcitabine treatment.

(50 μg/ml) did not show any evidence of cellular dysfunction or death. The rate of apoptosis was comparable between the control and gemcitabine treated cells (4 versus 6%, $P > 0.05$). In addition, the mean levels of E2 (1122 ± 36 versus 1381 ± 45 pg/ml, $P > 0.05$) and progesterone (578 + 21 versus 665 + 26 pg/ml, $P > 0.05$) produced by gemcitabine treated cells were not any different from controls (Fig. 6). At 50 μg/ml dose, gemcitabine was capable of inducing apoptosis in the mitotic granulosa cells, but the non-mitotic HLGC apparently did not undergo apoptosis after being exposed to the drug at this concentration. We therefore repeated the experiment by increasing the concentration of the drug up to 100 μg/ml, almost four times its therapeutic blood concentration. Even at that dose the cells continued to produce E2 and progesterone at levels comparable to the control cells (Supplementary Fig. S4).

Discussion

Several important findings were obtained in this study about the cytotoxic effects of chemotherapy drugs on the ovarian follicles and granulosa cells. First, we provided histomorphometry-based evidence that the *in vivo* gonadotoxic effects of chemotherapy drugs on the follicles might vary depending upon their mechanism of action. Second, we showed that the chemosensitivity of mitotic granulosa cells is different from that of non-mitotic granulosa cells.

Cyclophosphamide as an index drug of the alkylating category with the greatest gonadotoxic potential impacts both dormant primordial follicles and the fraction of growing follicles (pre-antral/antral) *in vivo* (Oktem and Oktay, 2007). Many alkylating drugs have a complicated metabolism. For instance, cyclophosphamide is inert *in vitro* because the drug requires a 4-hydroxylation reaction in the liver for activation and generates two different major metabolites thereafter, phosphoramide mustard and acrolein. The former is responsible for the gonadotoxic effects of the drug while the latter is more associated with the urinary side effects of the drug (Plowchalk and Mattison, 1991). Additionally, there are other cytotoxic by-products being formed, which may have potentiated the cytotoxic effects of the drug.

Cisplatin as an alkylating like agent produced similar cytotoxic effects to cyclophosphamide. By contrast, the anti-metabolite cancer drug,

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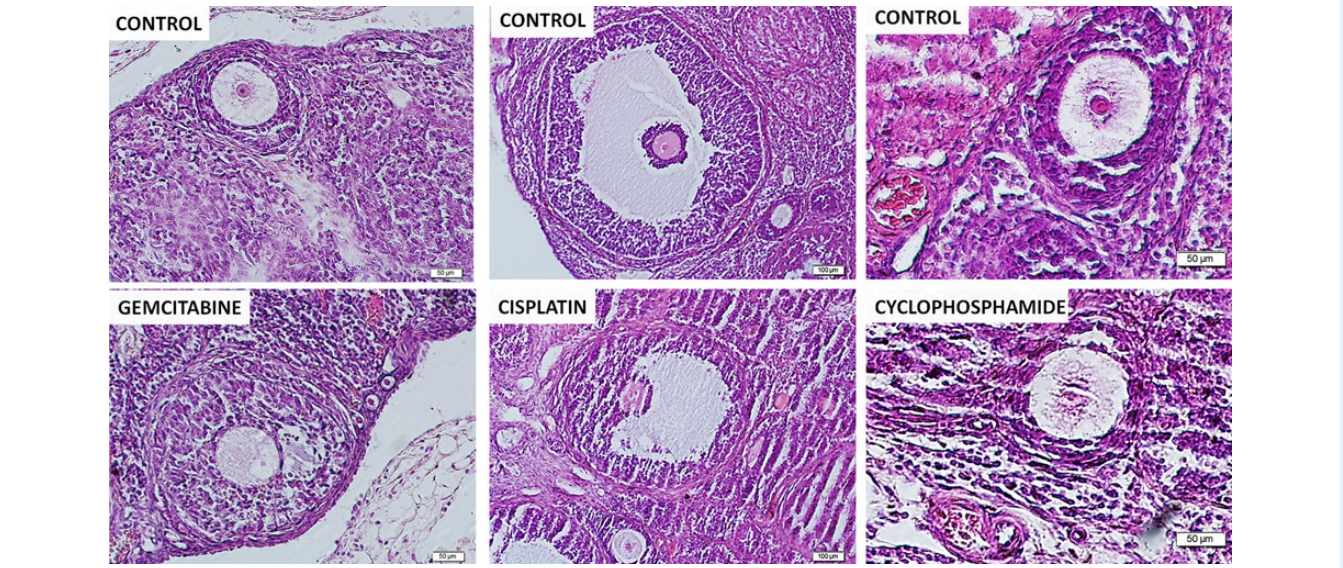

Figure 2 Histological sections of the ovaries exposed to chemotherapy drugs after hematoxylin-eosin staining (H&E). Healthy follicles at primordial, transitional and pre-antral stages were easily identified in the control ovaries. Ovarian stroma was stained uniformly with H&E and many interstitial cells easily identified. By contrast, the samples treated with cyclophosphamide or cisplatin showed a less cellular, more fibrotic stroma, with a marked disarray of the cells and extracellular matrix. Interstitial cells were more sparse. Atretic follicles were visible within the surrounding stroma. The damage was less prominent in gemcitabine treated samples.

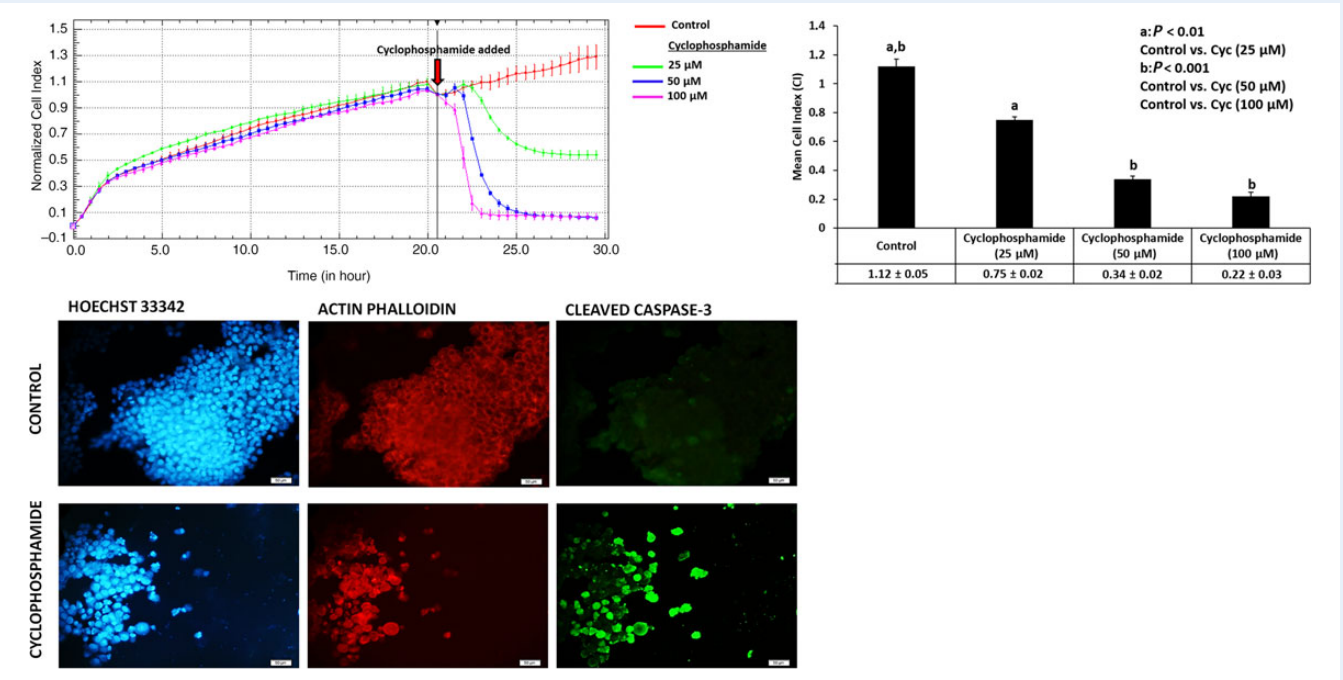

Figure 3 Real-time growth curves of human mitotic granulosa cells (HGrC1) treated with cyclophosphamide at 25, 50 and 100 µM concentrations. Cyclophosphamide halted proliferation and induced apoptosis of mitotic granulosa cells. Note the marked downward shift in the curve of the cells exposed to drug, indicative of rapid onset of apoptosis in the hours post exposure. The bar graphs show the mean cell indices of control and chemotherapy-treated cells, confirming the dose-dependent inhibition of cell proliferation after treatment with cyclophosphamide. The presence of apoptosis was also confirmed using immunofluorescence expression of apoptosis marker cleaved caspase-3 on the same population of the cells.

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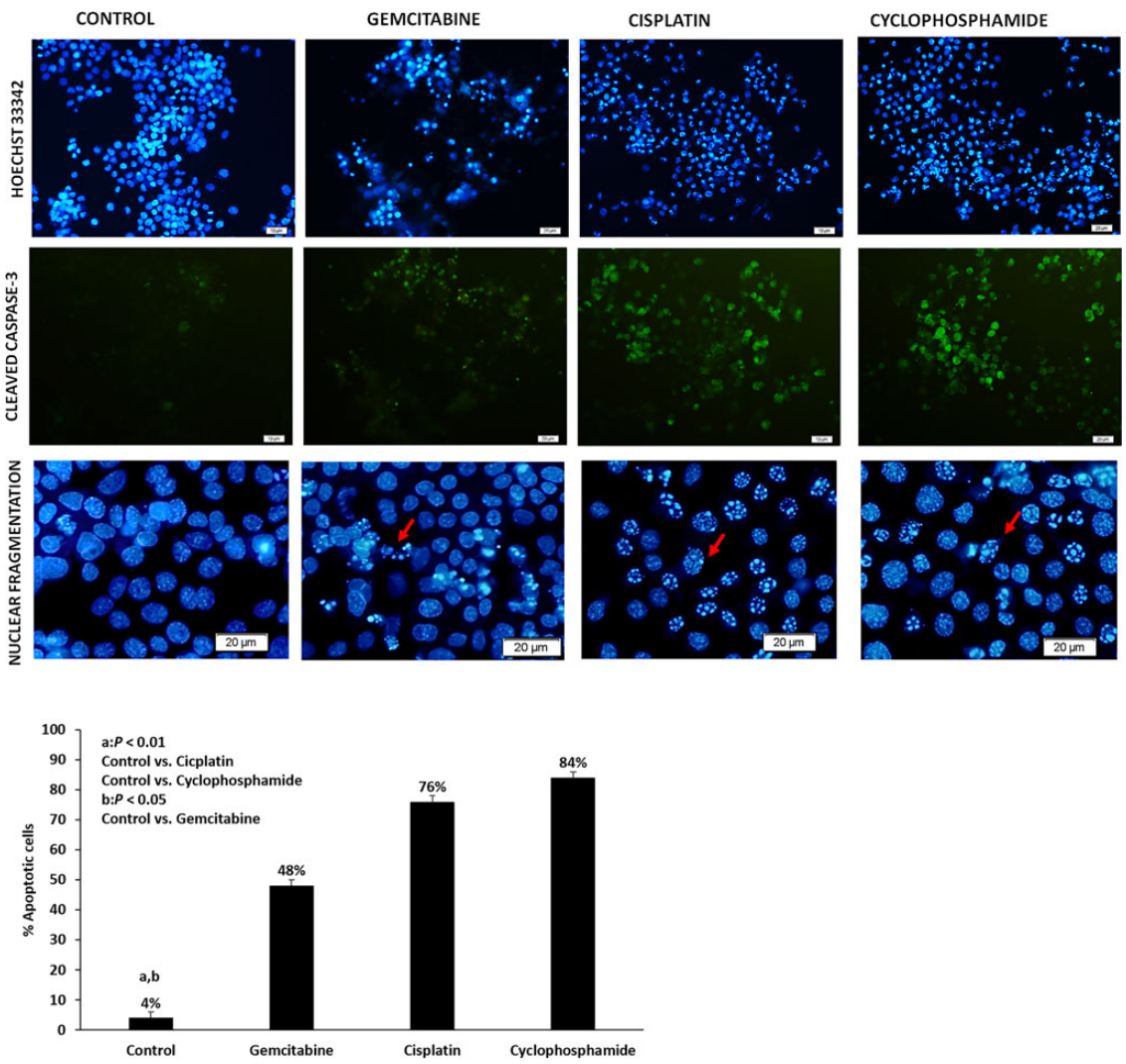

Figure 4 Immunofluorescence images of human mitotic granulosa cells (HGrC1) 24 h after exposure to cyclophosphamide (100 μM), cisplatin (40 μg/ml) and gemcitabine (50 μg/ml). Detection of apoptosis was by cleaved caspase-3 expression and nuclear fragmentation. A greater proportion of the cells treated with cyclophosphamide and cisplatin underwent apoptosis compared with those exposed to gemcitabine, as shown in the bar graphs in the lower panel.

gemcitabine, selectively targeted pre-antral/antral follicles. Primordial follicles were spared. The degree of follicle loss and reductions in serum AMH levels in the animals treated with gemcitabine were less significant than with the other drugs tested. A plausible explanation for the selective destruction of growing follicles by gemcitabine is that the higher mitotic rate and metabolic demand of the rapidly expanding granulosa cell layer in the growing pre-antral and antral follicles render them more vulnerable to the mitosis specific anti-proliferative effects of gemcitabine. However, when more toxic chemotherapy drugs such as cyclophosphamide and cisplatin are administered, both quiescent primordials and growing follicles are affected leading to a more widespread damage in the ovary and a higher risk of ovarian failure.

It is not possible to explore the *in vivo* effects of chemotherapy drugs on human ovary using histomorphometry studies. Such an experiments can only be done after xenografting ovarian tissue from younger donors into immunodeficient mice, which may not be available at many research centers (Oktem and Oktay, 2007). Further, the distribution of the follicles is not uniform and approximately only 10% belongs to growing follicles at primary stage onward in an adult human ovary (Gougeon, 2010; Oktem and Urman, 2010a,b). By contrast, pre-antral and antral follicles constitute a greater proportion of follicle stockpile in the rat ovary than the human ovary. Therefore the rodent ovary appears to be a viable option for conducting such experiments, while recognizing at the same time the possibility that the sensitivity of human follicles to chemotherapy agents might be different from that of rodents.

As another important finding, we demonstrated in this study that mitotic human granulosa cells are more sensitive to the cytotoxic actions of chemotherapy drugs than non-mitotic granulosa cells. Gemcitabine caused dose-dependent growth arrest and apoptosis of proliferating human (COV434 and HGrC1) and rat (SIGC) granulosa cells *in vitro*,

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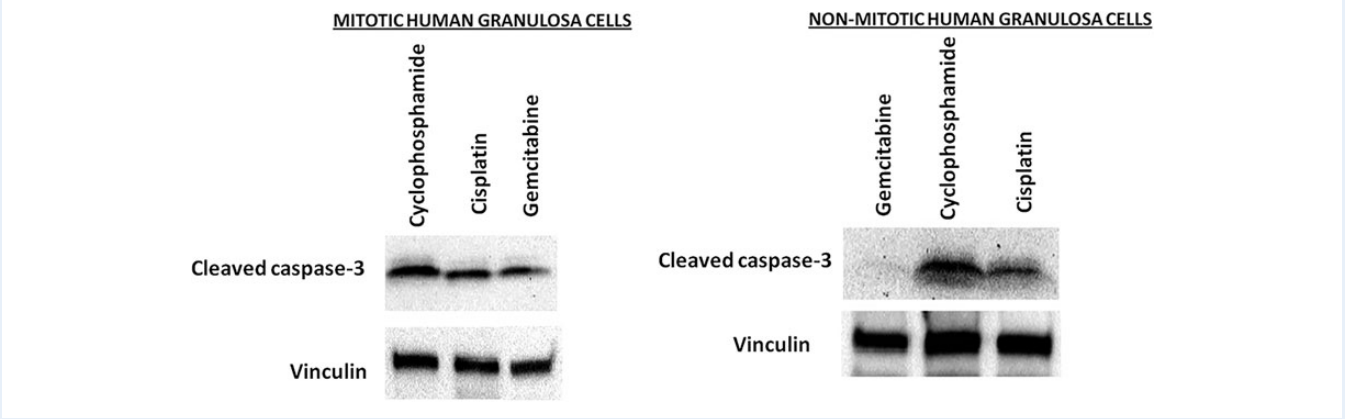

Figure 5 Detection of apoptosis in the mitotic (HGrC1) and non-mitotic luteinized (HLGC) granulosa cells exposed to the chemotherapy drugs by western blot. Cyclophosphamide (100 μM), and cisplatin (40 μg/ml) induced apoptosis of both mitotic and non-mitotic granulosa cells as evidenced by the expression of apoptosis marker cleaved caspase-3. By contrast, gemcitabine (50 μg/ml) was capable of inducing apoptosis only in the mitotic granulosa cells.

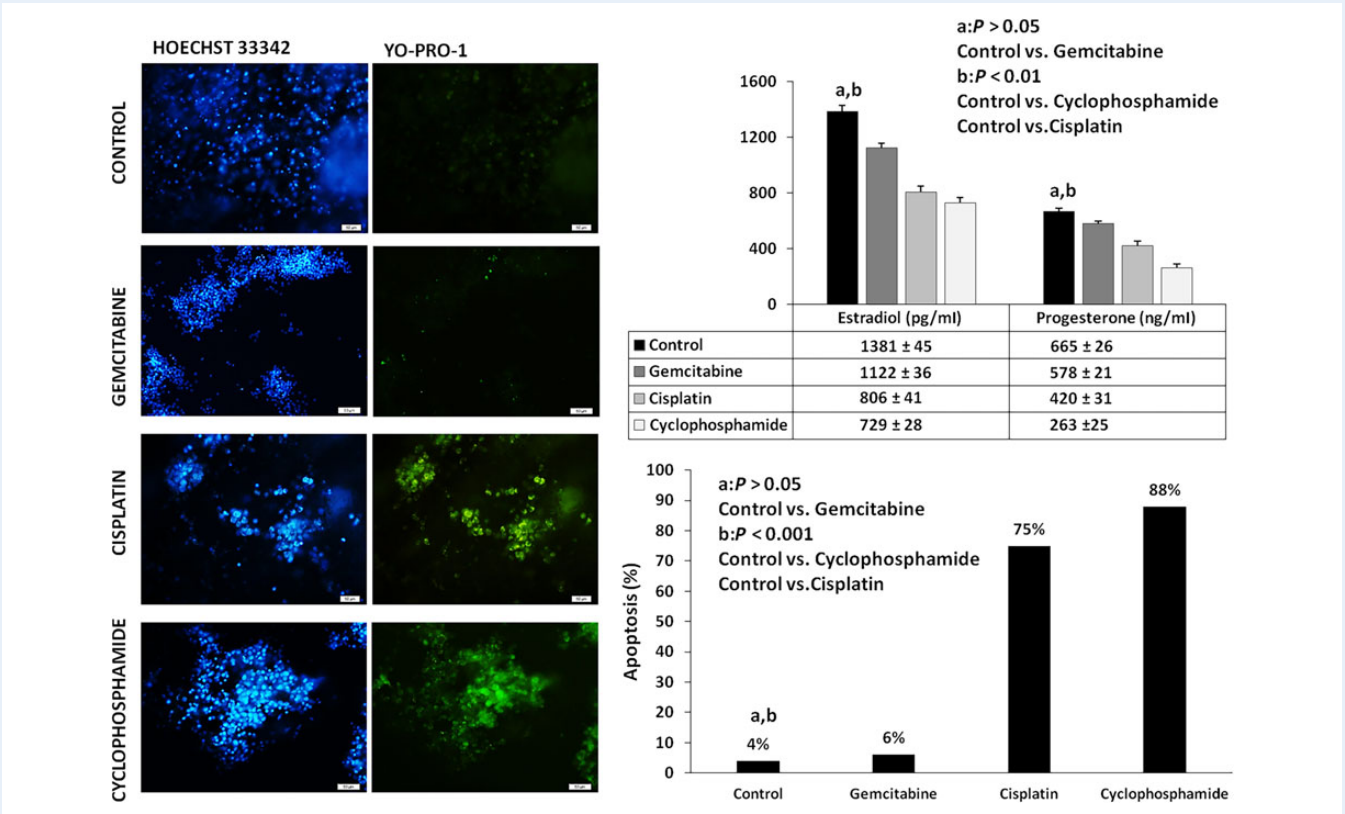

Figure 6 Comparison of cytotoxic effects of the chemotherapy drugs on human luteinized granulosa cells (HLGC) using the rate of apoptosis and steroidogenic activity. Exposure of these cells to cyclophosphamide (100 μM) and cisplatin (40 μg/ml) resulted in a dramatic increase in apoptosis and reduction in the steroidogenic activity of the cells. The rate of apoptosis and E2 and P production of the cells treated with gemcitabine (50 μg/ml) were comparable to that of the control cells.

whereas non-mitotic human luteinized granulosa cells (HLGC) were resistant to the cytotoxic effects of the same doses of the drug. It is likely that the non-mitotic nature of these cells confers them resistance to the mitosis-specific anti-neoplastic actions of gemcitabine. Cisplatin and cyclophosphamide are more cytotoxic than gemcitabine and their actions are not cell cycle specific, which means that they can non-selectively destroy entire classes of follicles. At this point it should be remembered that ovarian toxicity of chemotherapy drugs is not limited

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to the granulosa cells, as the damage in the oocytes and surrounding stroma and vascular structures are also involved in the chemotherapy-induced follicle depletion and premature ovarian aging and failure (Roness *et al.*, 2014).

Our results also showed that the xCelligence system appears to be a reliable method to analyze growth kinetics and monitor the proliferation and viability of the cells in a real-time and quantitative manner without using any labeling agent. This method can be particularly useful when the cytotoxic potential of a drug is investigated in human granulosa cells. The mean cell indices attained after exposure to different chemotherapy agents or different doses of a certain chemotherapy drug can be compared quantitatively, and a threshold cytotoxic dose can be determined. Furthermore, the same population of the cells plated in the xcelligence plate and their supernatants can be used for other experiments such as western blot (WB), immunofluorescence (IF) and PCR. Unfortunately primary and non-dividing cells such as HLGCs are not suitable for this method as they either undergo replicative senescence after a few rounds of mitosis or do not proliferate *in vitro* at all.

In conclusion, the experimental methodology described here is straightforward, easy to conduct, and can provide more detailed information about the gonadotoxic effects of chemotherapy drugs given that there is an ever-growing list of chemotherapeutics and targeted molecules being tried for different human cancers at different phase studies and that their ovotoxic potentials are unknown.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

A.Y., G.B., F.S., N.A., F.U.,Y.K., I.K., B.E., P.Y. and C.O.: conducted the experiments. M.A., C.T., S.A., Y.G., B.B. and B.U.: helped in the design of the work and the interpretation of results. U.I.: prepared the ovarian tissue sections for histomorphologic examination. A.I.: provided the cell line HGrC1 and helped in the interpretation of results. O.O.: conceived the work, designed experimental methodologies, conducted statistical analyses, interpreted the results, drafted the work and finally approved the manuscript.

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Conflict of interest

None declared.

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