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ORIGINAL ARTICLE *Reproductive endocrinology*

GnRH agonist leuprolide acetate does not confer any protection against ovarian damage induced by chemotherapy and radiation *in vitro*

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STUDY QUESTION: Is there any *in vitro* evidence for or against ovarian protection by co-administration of a GnRH agonist with chemotherapy in human?

SUMMARY ANSWER: The co-administration of GnRH agonist leuprolide acetate with cytotoxic chemotherapy agents does not preserve ovarian reserve *in vitro*.

WHAT IS KNOWN ALREADY: Randomized controlled trials of the co-administration of gonadotrophin-releasing hormone (GnRH) agonists with adjuvant chemotherapy to preserve ovarian function have shown contradictory results. This fact, together with the lack of a proven molecular mechanism of action for ovarian protection with GnRH agonist (GnRHa) places this approach as a fertility preservation strategy under scrutiny. We therefore aimed in this study to provide *in vitro* evidence for or against the role of GnRHa in the prevention of chemotherapy-induced damage in human ovary.

STUDY DESIGN, SETTINGS, SIZE AND DURATION: This translational research study of *ex vivo* and *in vitro* models of human ovary and granulosa cells was conducted in a university hospital between 2013 and 2015.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Ovarian cortical pieces ($n = 15$, age 14–37) and mitotic non-luteinized (COV434 and HGrC1) and non-mitotic luteinized human granulosa cells (HLGC) expressing GnRH receptor were used for the experiments. The samples were treated with cyclophosphamide, cisplatin, paclitaxel, 5-FU, or TAC combination regimen (docetaxel, adriamycin and cyclophosphamide) with and without GnRHa leuprolide acetate for 24 h. DNA damage, apoptosis, follicle reserve, hormone markers of ovarian function and reserve (estradiol (E2), progesterone (P) and anti-mullerian hormone (AMH)) and the expression of anti-apoptotic genes (bcl-2, bcl-xL, bcl-2L2, Mcl-1, BIRC-2 and XIAP) were compared among control, chemotherapy and chemotherapy + GnRHa groups.

MAIN RESULTS AND THE ROLE OF CHANCE: The greatest magnitude of cytotoxicity was observed in the samples treated with cyclophosphamide, cisplatin and TAC regimen. Exposure to these drugs resulted in DNA damage, apoptosis and massive follicle loss along with a concurrent decline in the steroidogenic activity of the samples. GnRHa co-administered with chemotherapy agents stimulated its receptors

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and raised intracellular cAMP levels. But it neither activated anti-apoptotic pathways nor prevented follicle loss, DNA damage and apoptosis induced by these drugs.

LIMITATIONS, REASONS FOR CAUTION: Our findings do not conclusively rule out the possibility that GnRHa may offer protection, if any, through some other mechanisms *in vivo*.

WIDER IMPLICATIONS OF THE FINDINGS: GnRH agonist treatment with chemotherapy does not prevent or ameliorate ovarian damage and follicle loss *in vitro*. These data can be useful when consulting a young patient who may wish to receive GnRH treatment with chemotherapy to protect her ovaries from chemotherapy-induced damage.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by the School of Medicine and the Graduate School of Health Sciences of Koc University, and the American Hospital Women's Health Center, Comprehensive Cancer Care and Fertility Preservation Programs, Istanbul, Turkey. The authors declare no competing interests.

Key words: chemotherapy / ovarian failure / GnRH agonist / apoptosis / follicle reserve / granulosa cells / steroidogenic activity

Introduction

Premature ovarian failure and other poor reproductive outcomes are important sequelae of previous exposure to chemotherapy and/or radiotherapy in children and adults with cancer. Cytotoxic chemotherapy regimens and radiotherapy induce apoptotic death of the oocytes and surrounding granulosa cells in the ovary leading to early exhaustion of the follicle stockpile, infertility and premature ovarian failure (Oktem and Oktay, 2007; Morgan *et al.*, 2012). Young females diagnosed with breast cancer, lymphomas/leukemias or non-malignant diseases requiring the use of cytotoxic chemotherapy regimens (i.e. alkylating regimens) are at the greatest risk of premature ovarian failure and infertility following adjuvant chemotherapy (Partridge *et al.*, 2004; Oktem and Urman, 2010a,b). The administration of gonadotrophin-releasing hormone agonists during chemotherapy has been proposed as a potential fertility preservation strategy to preserve ovarian reserve after emergence of the promising findings from anecdotal reports, primate models and non-randomized trials in human (Turner *et al.*, 2013). However, randomized controlled trials (RCTs) have shown inconsistent results in female patients with cancer (for review, see Turner *et al.*, 2013), giving rise to a debate among the physicians and scientists in the fields of oncology and reproductive medicine over the actual role of GnRHa in the prevention of chemotherapy-induced ovarian failure. Very recently, another RCT stirred the debate further by showing in breast cancer patients that the administration of GnRH agonist goserelin during adjuvant chemotherapy protected against ovarian failure and reduced the risk of early menopause (Moore *et al.*, 2015). The professional societies of oncology and reproductive medicine/fertility preservation currently emphasize the lack of a proven molecular mechanism for gonadal protection with GnRHa during chemotherapy, and underscore the need for research in this under-studied issue (Kim *et al.*, 2012; Turner *et al.*, 2013). Several mechanisms of action have been proposed to explain GnRH-induced protection. These include reduced activation of primordial follicles, decreased ovarian perfusion due to hypoestrogenic state c, decreased ovarian apoptosis or enhanced anti-apoptotic pathways activated by GnRH during chemotherapy.

However none of these theories has been validated so far (Oktem and Oktay, 2007; Morgan *et al.*, 2012; Turner *et al.*, 2013). Therefore we designed a translational research study to investigate if GnRHa administration during chemotherapy preserves the ovarian reserve during chemotherapy, possibly through the activation of its cognate receptors and up-regulation of anti-apoptotic genes in the ovary.

Materials and Methods

The experimental design of the study is depicted in Fig. 1A.

Patients

Ovarian cortical tissues were obtained from 15 patients (mean age ± SD: 27.8 ± 2.7, range: 14–37) undergoing laparoscopic surgery for the removal of the benign ovarian cysts between the years 2014–2015. One patient (age 14) had mature cystic teratoma. The remaining patients had ovarian endometrioma. All patients underwent operations at late follicular phase of the cycle (the mean ± SD of the cycle day: 8.2 ± 2.4). Human luteinized granulosa cells (HLGCs) were recovered from follicular fluid during the oocyte retrieval procedure in 20 IVF patients (mean age ± SD: 32.6 ± 3.5). The etiologies for infertility were as follows: unexplained ($n = 12$), diminished ovarian reserve ($n = 8$). Informed consent was obtained from all patients and the study was approved by the institutional review board of Koc University.

Preparation and culture of ovarian cortical tissues

Ovarian cortices embedded in the cyst wall were removed under sterile conditions, minced into pieces of equal size (0.5 × 0.5 cm) and cultured for 24 h in 24-well format culture plate using 1 ml of DMEM-F12+10% FBS. Chemotherapy agents ± GnRHa were added to culture media at the indicated concentrations.

Chemicals, chemotherapy drugs and GnRH agonist leuprolide acetate

Chemotherapy drugs and GnRHa were administered at their therapeutic blood concentrations. 4-hydroperoxy cyclophosphamide (4-HC), the active *in vitro* metabolite of the drug, was used at 50 and 100 µg/ml concentration (Teicher *et al.*, 1996) (Niomech, Bielefeld, Germany). 5-FU and cisplatin were used at 50 and 40 µg/ml, respectively (Bonetti *et al.*, 1996; Casale *et al.*, 2004) (Eli Lilly and Company, IN, USA). Paclitaxel was used at 2 µg/ml (Bristol-Myers Squibb Company, NY, USA) (Rowinsky *et al.*, 1999). Docetaxel was from Rhone-Poulenc Rorer/Sanofi company (France). Adriamycin was obtained from Sandoz, Novartis Inc. (Germany). TAC was administered at the following concentrations; cyclophosphamide 100 µg/ml, adriamycin and docetaxel at 10 ng/ml (Martin, 2006; Eiermann *et al.*, 2011). GnRH agonist leuprolide acetate (Abbott Pharmaceutical Products, USA), was given at three different concentrations (12.5, 25 and 50 ng/ml), which correspond to the serum and ovarian follicular fluid concentrations of the drug (Dodson *et al.*, 1988). DMEM-F12 culture media, fetal bovine serum (FBS), YO-PRO-1, Alexa probes were purchased from Life Technologies (Thermo

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Fisher Scientific Inc., MA, 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). Anti-phospho-Histone H2A.X antibody (Ser139, clone JBW301) was from Millipore (MA, USA). COV434 cell line was purchased from Sigma (St. Louis, MA, USA). HGrC1 was a gift from Dr. Ikara Iwase (Nagoya University, Japan).

Irradiation of ovarian cortical samples

A single fraction dose of 2 Gy (at a dose-rate of 600 MU/min) mimicking conventional daily fractionation in clinical use was prescribed for one piece of tissue in culture medium per Petri dish per beam. We used 2 Gy since the LD_{50} of human oocyte was reported to be <4 Gy (Wallace et al., 2003). The ionizing irradiation *in vitro* was performed using a Varian Trilogy Linac capable of delivering X-ray beams with 6 MV energy. The X-ray field size aligned at the beam central axis with a source-to-cell layer distance of 100 cm perpendicular to the Petri dish was set to 10 × 10 cm. A 6–8 mm air layer was present above the medium within the Petri dish at set up. The water equivalent depth was adjusted to the depth of maximum dose and the build-up to maximum dose was provided by slabs of Plastic Water (RW3 water equivalent phantom, PTW, Freiburg) of appropriate thickness.

Histomorphometric assessment of the ovarian samples

Follicle counts in the samples were determined using the method we described previously after staining with hematoxylin-eosin (H&E) (Oktem and Oktay, 2007). Follicle density was expressed as follicle count/mm^2. Ovarian samples were also stained with Masson's trichome (MT), VEGF and Hoechst 33 342 for the examination of ovarian stroma and vasculature (Bedaiwy et al., 2006). In brief, the ovarian samples were fixed in 4% PFA at 4°C for 24 h followed by 20% (wt/vol) and 30% (wt/vol) sucrose treatment until the tissues sunk. Cryosections of 5 μm were obtained for MT and VEGF staining. For MT staining, Masson's Goldner staining kit (Merck millipore, USA) was used according to manufacturer's instructions. The sections were treated with tap water followed respectively by Weigert's iron hematoxylin staining, azophloxine, tungstophosphoric acid orange G and light green SF solutions, and 1% acetic acid was used after each step. The sections were then washed with distilled water and mounted with an aqueous mounting medium (Abcam, USA). For VEGF staining, the sections were washed in PBS (Sigma, USA) followed by a 1:50 diluted anti-VEGF antibody (Abcam, USA). An Alexa 594 conjugated goat anti mouse antibody (Abcam, USA) was used as the secondary antibody at a 1:100 dilution. Both primary and secondary antibodies were incubated at 37°C for 90 min in a humidified chamber at dark. Hoechst 33 342 (1 μg/ml, Sigma, USA) was used in mounting medium (1:1, PBS/glycerol). Light microscopic images and fluorescent images were taken under a Zeiss Axioscope. Microvessel density was determined by averaging four microscopic fields in a defined area of each specimen.

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

HGrC1 is a human non-luteinized granulosa cell line expressing enzymes related to steroidogenesis, such as steroidogenic acute regulatory protein, aromatase and gonadotrophin receptors. These cells are not capable of undergoing luteinization, resembling the characteristics of granulosa cells belonging to follicles in the early stage. HGrC1 might also be capable of displaying the growth transition from a gonadotrophin-independent status to gonadotrophin-dependent one (Bayasula et al., 2012).

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

Non-mitotic luteinized human granulosa cells (HLGCs)

HLGCs were recovered from follicular fluid during oocyte retrieval procedure in 20 IVF patients. These cells are highly specialized primary luteinized granulosa cells, as they do not proliferate either spontaneously or after stimulation with a mitogenic agent. They produce large amounts of progesterone and estradiol hormones *in vitro*. The aspirates of follicular fluids were spun down at 500 ×g for 10 min. Then recovered cells were plated in 24-well format culture plate at a density of 5000 cells per well.

Cell culture

All cells were cultured 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 at 37°C and 5% CO_2.

cAMP-Glo™ assay

cAMP-Glo™ Assay (Promega Corporation, Madison, WI, USA) is a bioluminescent assay to measure cAMP levels in cells in response to the effects of an agonist or test compound on G protein-coupled receptors (GPCRs). The assay is based on the principle that cyclic AMP (cAMP) stimulates protein kinase A (PKA) activity, decreasing available ATP and leading to decreased light production in a coupled luciferase reaction.

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

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). The cells were treated with the chemotherapy drugs ± GnRHa at log phase and monitored every 30 min for up to 140 h. The results were expressed by normalized cell index (CI) derived from the ratio of CIs before and after the addition of the compounds. The normalization of CI arbitrarily sets CI to 1 at the indicated time points. Recording of CI and normalization CI was performed using the xCelligence 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 (1 μM) (absorbance 491 nm, emission 509 nm), whereas live cells are impermeant to it. Hoechst 33342 (1 μg/ml) was added to the mounting medium for DNA staining. 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 and the percentage of the cells expressing Yo-PRO-1 was calculated.

Detection of DNA damage and apoptosis by immunofluorescence and immunoblotting

Cleaved caspase-3 antibody was used (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 for detection of apoptosis by immunofluorescence. After rinsing with PBS, the cells were incubated with fluorochrome-conjugated secondary antibody (Alexa 486, Molecular

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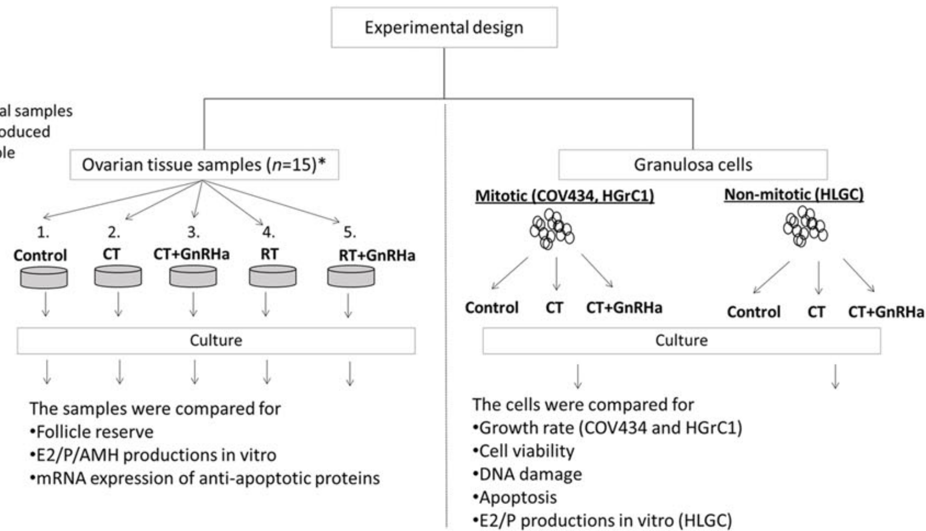
A

E2:Estradiol
P:Progesterone
AMH:Anti-mullerian hormone
CT:Chemotherapy
RT: Radiation
*: The total number of ovarian cortical samples
Each treatment arm has 15 pieces produced
from equal division of the main sample



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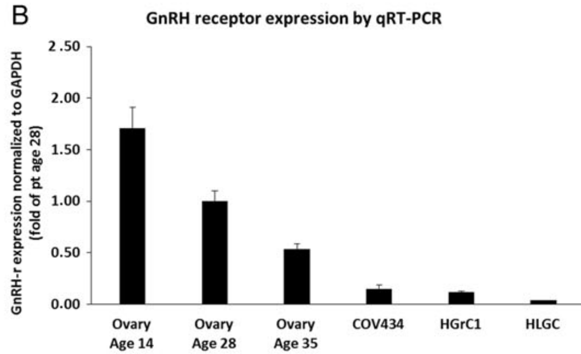

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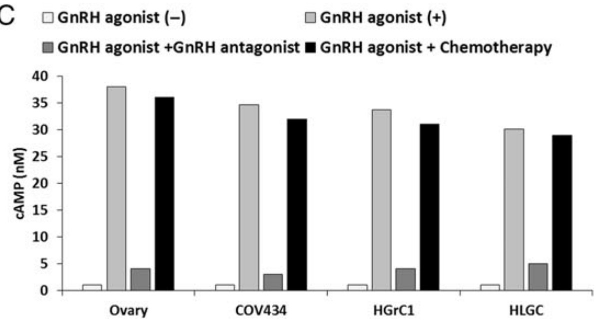

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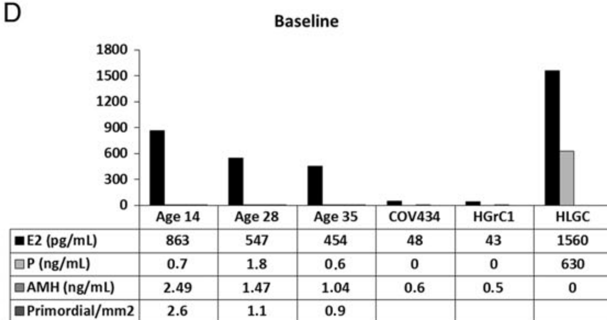
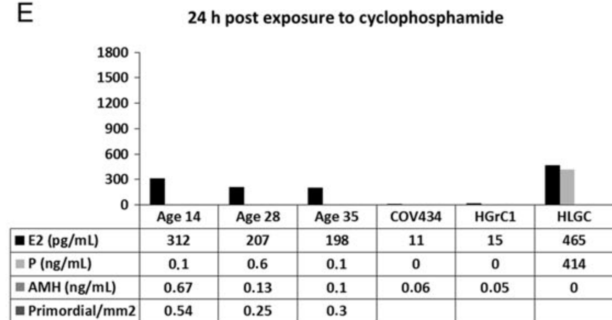


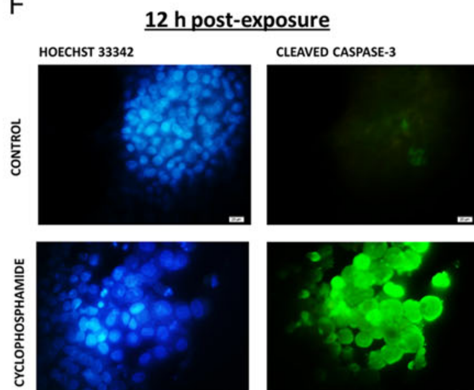
	Age 14	Age 28	Age 35	COV434	HGrC1	HLGC
E2 (pg/mL)	863	547	454	48	43	1560
P (ng/mL)	0.7	1.8	0.6	0	0	630
AMH (ng/mL)	2.49	1.47	1.04	0.6	0.5	0
Primordial/mm2	2.6	1.1	0.9			

E



	Age 14	Age 28	Age 35	COV434	HGrC1	HLGC
E2 (pg/mL)	312	207	198	11	15	465
P (ng/mL)	0.1	0.6	0.1	0	0	414
AMH (ng/mL)	0.67	0.13	0.1	0.06	0.05	0
Primordial/mm2	0.54	0.25	0.3			

F



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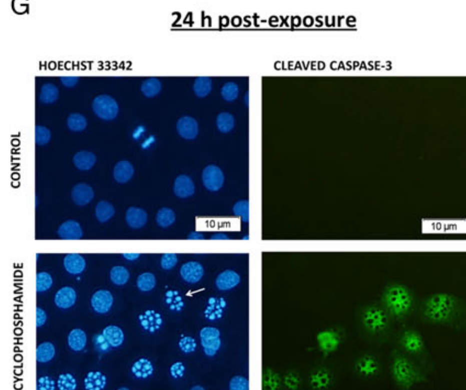

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Probes, USA) diluted in antibody dilution buffer for 1 h. This step was followed by rinsing the coverslips slides and adding Hoechst 33342 (1 μg/ml) to the mounting medium for DNA staining. The images were taken under appropriate channels using an IF microscope (Olympus IX71, Japan). The percentage of the caspase-3 positive cells was calculated after counting 500 cells at four different high magnification areas.

For analysis of DNA damage and apoptosis in western blot, the cells were incubated overnight at 4°C with anti-phospho-Histone H2A.X antibody (Ser139, clone JBW301, Millipore) and Cleaved Caspase-3 antibody (Asp175) (5A1E) (Cell Signaling Technology) at 1:1000 and 1:500 dilutions, respectively. Anti-Vinculin (Sigma-Aldrich) at a dilution of 1:10000 was used as a loading control.

Hormone assays

AMH levels in the supernatants 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 repeatability and the coefficient of variations were given as 4.6% (0.144 ng/ml), 2.4% (0.843 ng/ml), and 3.3% (4.408 ng/ml), respectively.

The levels of both hormones 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.

qRT–PCR array for profiling anti-apoptotic genes activated with chemotherapy ± GnRHa

Ovarian tissue samples and granulosa cells treated with 100 μM Cyclophosphamide (CAS-No. 39800-16-3, Niomech), with and without 50 ng/ml leuprolide acetate for 12 h. RNA isolation was performed by NucleoSpin® RNA Kit (Macharey-Nagel) following the manufacturer's instructions. RNA quantification was performed by spectrophotometric read at 260 nm by Nanodrop (Thermo Scientific). 250 ng cDNA preparation was obtained by reverse transcription of RNA using M-MLV Reverse Transcriptase (Invitrogen). Relative mRNA expression levels were detected by using Light Cycler® 480 SYBR Green I Master (Roche, Germany). The primers of the genes used for the assays are shown in Supplementary Table SI.

Statistical analysis

Follicle counts were expressed as the median ± SD. Hormone levels and cell index readouts of xcelligence system were expressed as the mean ± SD. Statistical analyses were done using SPSS for windows 20.0 statistical package program. Friedman test and paired Wilcoxon test were used to compare the follicle counts and hormone levels between ovarian samples treated with a certain chemotherapy drug and its counterpart treated with the same drug + GnRHa. The percentages of viable and apoptotic cells were compared between the groups using Fisher' exact test. For correlation analysis two-tailed Spearman's correlation test was conducted. A P-value <0.05 was considered significant for all statistical tests.

Results

We conducted two different validation experiments to test if our experimental methodology is a suitable model to study the impact of chemotherapy with and without GnRHa on the ovarian tissue samples and granulosa cells. In the first experiment, the expression of GnRH receptor and its functionality in the ovarian tissue samples and granulosa cells were analyzed (GnRH receptor assays section). In the second validation experiment we showed that chemotherapy-induced cytotoxicity on the ovarian tissue samples and granulosa cells can be demonstrated at the level of follicle reserve, steroidogenic activity, DNA damage and apoptosis.

Validation experiment 1: GnRH receptor assays

The presence of GnRH receptor in the samples was validated with quantitative RT–PCR analysis. The expression of the receptor was higher in the ovarian samples from younger donors compared with those of older ones (Fig. 1B). Stimulation of the receptor with GnRHa leuprolide acetate (50 ng/ml) increased intracellular cAMP level after 10 min in the ovarian cortical samples and granulosa cells. The blockade of GnRH receptor with its specific receptor antagonist cetrorelix acetate (5 ng/ml) given 1 h before the administration of leuprolide acetate markedly blunted the response, leading to only a slight increase in the cAMP level (Fig. 1C). The co-administration of GnRHa with cyclophosphamide did not interfere with the activation of the receptor (Fig. 1C).

Figure 1 Experimental design and preliminary experiments to test the suitability of the methodology to document *in vitro* activity of GnRHa and cytotoxicity of cyclophosphamide on the ovarian tissue samples and granulosa cells. (**A**) Experimental design of the study showing segregation of the ovarian samples and granulosa cells into treatment arms and the methods to assess and compare the cytotoxicity. (**B**) The expression of GnRH receptor in the ovarian samples and granulosa cells. The presence of GnRH receptor in the samples was validated with quantitative RT–PCR analysis. The expression of the receptor was higher in the ovarian samples from young donors compared with those of older donors and granulosa cells. (**C**) GnRH receptor activation by GnRHa. Stimulation of the receptor with GnRHa leuprolide acetate (50 ng/ml) caused a marked increase in the intracellular cAMP level after 10 min in both ovarian cortical samples and granulosa cells. The blockade of GnRH receptor with its specific receptor antagonist cetrorelix acetate (5 ng/ml) given 1 h before the administration of leuprolide acetate markedly blunted the response, leading to a slight increase in the cAMP level. The co-administration of GnRHa with cyclophosphamide did not interfere with activation of the receptor. (**D**) Primordial follicle reserve and E2, P and AMH productions of the samples at baseline. Ovarian tissue samples and granulosa cells (5000 cells/well) produced detectable amounts of E2, P and AMH after 24 h of culture. Ovarian samples from younger donors harbored more primordial follicles and produced higher amounts of E2 and AMH than those from older donors. Human luteinized granulosa cells (HLGCs) produced the highest levels of estradiol and progesterone *in vitro*. (**E**) Primordial follicle reserve and E2, P and AMH productions of the samples at 24 h post-exposure to cyclophosphamide. Treatment of the ovarian samples with active metabolite of cyclophosphamide (4-hydroperoxy cyclophosphamide) at 100 μM concentration for 24 h resulted in a dramatic decline in the number of primordial follicles with concurrent reduction in the production of AMH, E2 and P. (**F**/**G**) Cyclophosphamide induced apoptosis in the granulosa cells. There was a strong expression of cleaved caspase-3 in the cells at 12 h post-exposure. Then the intensity of the signal began to fade and nuclear fragmentation became evident at 24 h. Arrows indicates nuclear fragmentation.

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Validation experiment 2: demonstration of chemotherapy induced cytotoxicity on the ovarian tissue samples and granulosa cells

Ovarian cortical samples and granulosa cells (5000 cells/well) produced detectable amounts of E2, P and AMH after 24 h of culture (Fig. 1D). Ovarian tissue samples from younger donors harbored more primordial follicles and produced higher amounts of E2 and AMH compared with those of older donors, indicative of higher ovarian reserve in the samples from younger donors (Fig. 1D). HLGCs produced the highest amounts of E2 and P *in vitro*. These luteinized granulosa cells typically do not produce AMH since this hormone is mainly produced by the granulosa cells of pre-antral and small antral follicles (Oktem and Urman, 2010a,b). Incubation of the ovarian samples with active metabolite of cyclophosphamide (4-hydroperoxy cyclophosphamide, 100 μM) for 24 h resulted in a dramatic decline in follicle counts and hormone levels (Fig. 1E). Similar cytotoxic effects were observed in the granulosa cells exposed to the same dose of the drug. They underwent apoptosis and their steroidogenic activity was substantially reduced. There was a strong expression of cleaved caspase-3 in the cells at 12 h post-exposure. Then the intensity of the signal began to fade and nuclear fragmentation became evident at 24 h (Fig. 1F and G).

Having obtained promising results from these validation experiments, we treated ovarian tissue samples and granulosa cells with different chemotherapy agents and radiation with and without GnRHa and conducted the experiments described in the methods section and depicted in the experimental design (Fig. 1A).

Comparison of follicle reserve and steroidogenic activity in the control ovarian cortical samples and those treated with chemotherapy agents ± GnRHa

Treatment of the ovarian samples with cyclophosphamide for 24 h resulted in a significant decline in follicle reserve and steroidogenic activity of the samples. The median numbers of primordial follicles (0.33 ± 0.2 versus 2.32 ± 0.5, $P < 0.01$; respectively) and pre-antral/antral follicles (0.11 ± 0.01 versus 0.57 ± 0.02, $P < 0.01$; respectively) were significantly less than control samples (Fig. 2A–D). Also, these samples produced significantly lower amounts of E2 (788 ± 98 versus 185 ± 16 pg/ml, $P < 0.01$; respectively), P (1.76 ± 0.3 versus 0.33 ± 0.02 ng/ml, $P < 0.01$; respectively) and AMH (1.2 ± 0.09 versus 0.1 ± 0.03 ng/ml, $P < 0.01$; respectively) than control ovarian samples (Fig. 2A–D). The co-administration of GnRHa with cyclophosphamide did not prevent follicle loss in the samples. The mean number of primordial (0.33 ± 0.2 versus 0.35 ± 0.2, $P > 0.05$; respectively) and pre-antral/antral follicles (0.11 ± 0.01 versus 0.13 ± 0.1, $P > 0.05$; respectively) were comparable between cyclophosphamide and cyclophosphamide + GnRHa groups. The mean levels of E2 (185 ± 16 versus 148 ± 13 pg/ml, $P > 0.05$; respectively), P (0.33 ± 0.2 versus 0.32 ± 0.2 ng/ml, $P > 0.01$; respectively) and AMH (0.1 ± 0.03 versus 0.11 ± 0.02 ng/ml, $P > 0.05$; respectively) were almost identical between the cyclophosphamide and cyclophosphamide + GnRHa groups (Fig. 2A–D). Similar cytotoxic effects were observed after cisplatin treatment. Both primordials and pre-antral/antral follicle counts were substantially reduced with a concurrent decline in E2, P and AMH productions in the samples exposed to cisplatin. Somehow, paclitaxel appeared to be less gonadotoxic on the pre-antral/antral follicles than cyclophosphamide and cisplatin, since the extent of follicle loss and reductions in hormone levels were less prominent in the samples exposed to this drug. Notably, primordial follicles seemed unaffected by paclitaxel because the mean number of primordials in the paclitaxel-treated samples was comparable to control. On the contrary, pre-antral/antral follicles were significantly reduced after paclitaxel, albeit to a lesser extent than with cyclophosphamide and cisplatin. The addition of GnRHa to cisplatin and paclitaxel did not prevent follicle loss (Fig. 2A–D).

Correlation analyses between follicle counts and *in vitro* levels of E2 and AMH in the control ovarian samples and those treated with chemotherapy agents ± GnRHa

AMH is mainly produced by the proliferating granulosa cells of the pre-antral and small antral follicles (Oktem and Urman, 2010a,b). These cells are also the source of estrogen production. Serum AMH level is used as a hormone marker of ovarian reserve and its levels correlate well with the number of antral follicles in the ovary on ultrasonography. We curiously investigated if such a correlation also exists *in vitro*. We found that there were significant positive correlations among estradiol, AMH and pre-antral/antral follicles in the control ovaries. The correlation coefficients and the level of significance were as follows: follicle count-AMH ($r = 0.903$, $P = 0.001$); follicle count-estradiol ($r = 0.842$, $P = 0.004$); and AMH-estradiol ($r = 0.905$, $P = 0.001$) (Fig. 3A). However, in the samples exposed to chemotherapy ± GnRHa, the correlation was either insignificant [AMH versus estradiol ($r = 0.652$, $P = 0.088$)] or less significant [follicle count versus AMH ($r = 0.768$, $P = 0.035$); and follicle count versus estradiol ($r = 0.831$, $P = 0.015$)] (Fig. 3A).

The expression of anti-apoptosis genes in the control ovarian samples and those treated with chemotherapy agents ± GnRHa

In another set of experiments, we quantitatively compared the expressions of the anti-apoptotic genes (Bcl-2, Bcl-xL, Bcl-2L2, Mcl-1, BIRC2 and XIAP) among control, cyclophosphamide and cyclophosphamide + GnRHa groups to investigate if GnRHa activates anti-apoptotic genes in the ovary under the genotoxic stress of cyclophosphamide. Compared with their baseline levels, the expression of Bcl-2, Bcl-2L2 and BIRC2 were significantly decreased after cyclophosphamide treatment whereas the levels of Bcl-xL, Mcl-1, and XIAP did not change. The co-administration of GnRHa with cyclophosphamide did not increase the expression of any of the genes studied. Furthermore, the expression of Mcl-1 and BIRC2 were further reduced after cyclophosphamide + GnRHa treatment compared with those ovarian samples treated cyclophosphamide only (Fig. 3B).

Assessment of ovarian stroma and microvascular density in the control ovarian samples and those treated with chemotherapy agents ± GnRHa

The follicles and stroma preserved their structure well after 24 h culture period. Healthy follicles at primordial, transitional and pre-antral stages

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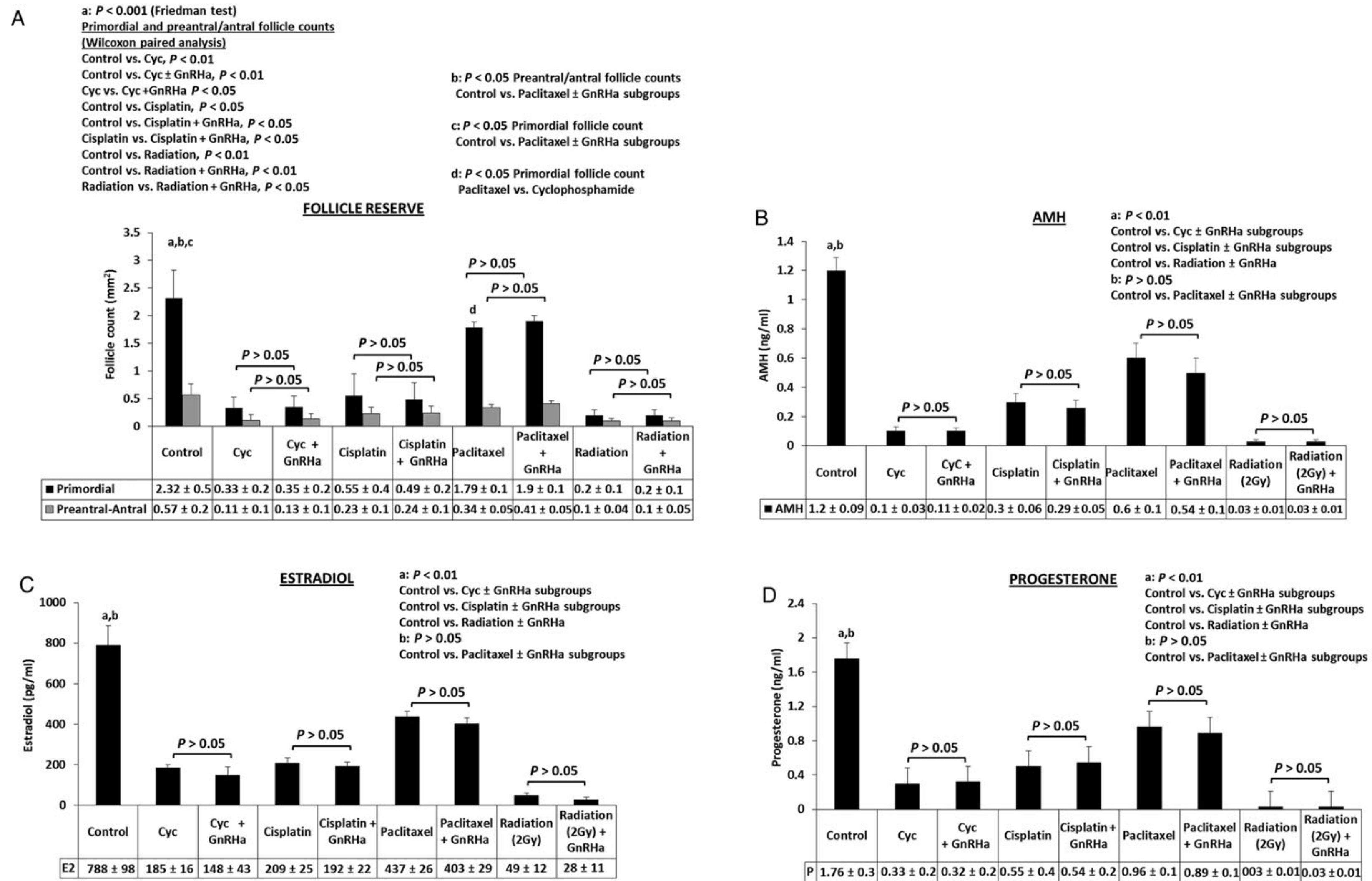

Figure 2 Quantitative and comparative analysis of the follicle reserve and steroidogenic activity in the ovarian samples before and after treatment with the indicated chemotherapy agents or radiation, with and without GnRHa. (**A**) The impact of chemotherapy drugs or radiation administered $\pm$ GnRHa on the follicle reserve. Cyclophosphamide (Cyc), cisplatin and radiation exerted a similar degree of cytotoxicity on the dormant primordials and growing follicle fraction (pre-antral/antral follicles) whereas paclitaxel impacted only pre-antral/antral follicle cohorts in the human ovary. Irradiation of the samples with 2 Gy radiation caused a massive follicle loss. More than 90% of primordials and growing follicles were lost 24 h post-irradiation. The co-administration of GnRHa with these drugs or radiation did not prevent or attenuate the follicle loss. (**B**–**D**) Comparison of the steroidogenic activity of the samples. Ovarian tissue samples produced significantly lower amounts of anti-mullerian hormone (AMH) (B), estradiol (E2) (C) and progesterone (P) (D) after exposure to cyclophosphamide, cisplatin or radiation, and to a lesser extent, after paclitaxel. The addition of GnRHa did not cause any notable change in the steroidogenic activity of the samples.

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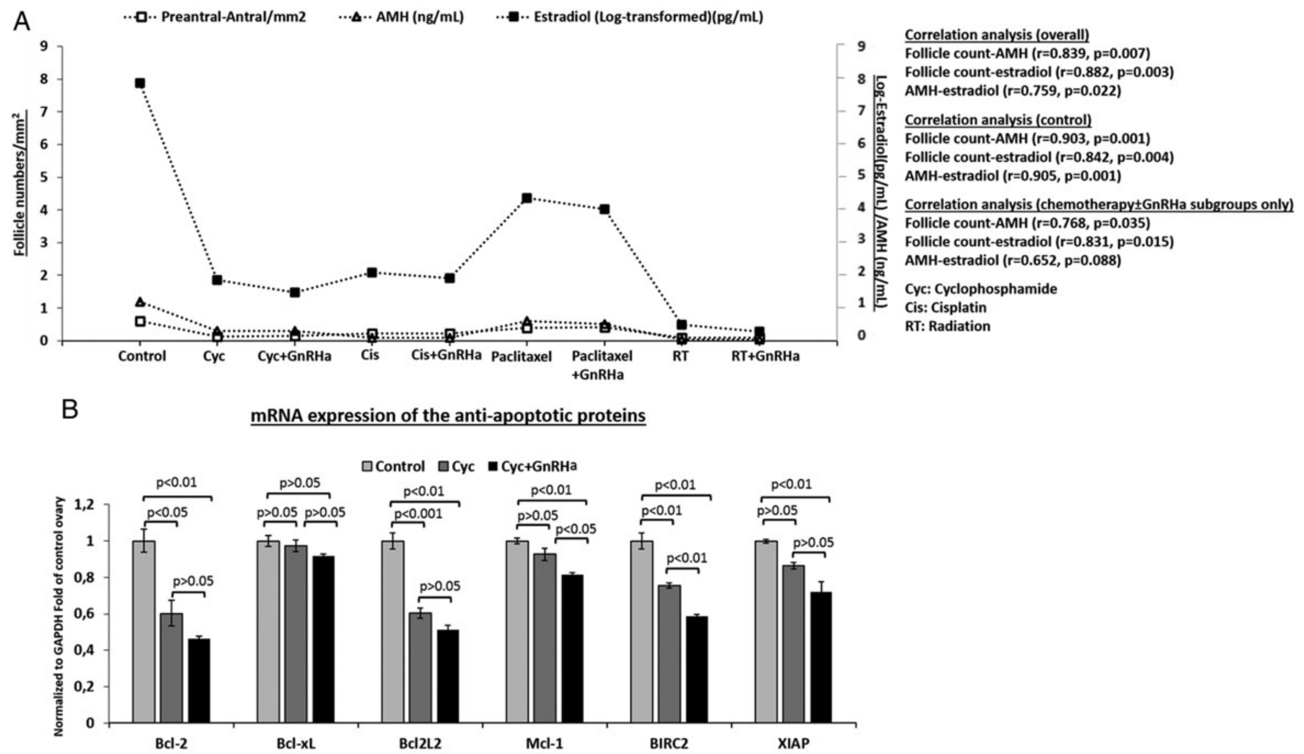

Figure 3 (**A**) Correlation analysis between the number of pre-antral/antral follicles and the levels of E2 and AMH in the control and chemotherapy treated ovarian tissue samples and mRNA expression of the anti-apoptotic genes before and after exposure to cyclophosphamide with and without GnRHa. (A) The number of growing follicles in the control ovarian samples was significantly correlated with the levels of AMH and E2 they produced *in vitro*. There was also a significant correlation between AMH and E2. However, the level of significance was either weak (follicle count versus E2; and follicle count versus AMH) or absent (AMH versus E2) in the samples exposed to chemotherapy drugs compared with control samples. E2 levels are log transformed (second *Y* axis). (**B**) The co-administration of GnRHa with cyclophosphamide did not up-regulate the transcriptional activity of the anti-apoptotic genes compared with control and cyclophosphamide treated samples. There was a further reduction in the expression of the anti-apoptotic genes Mcl-1 and BIRC2 after treatment with cyclophosphamide + GnRHa.

were easily identified. Ovarian stroma was stained uniformly HE and MT with many interstitial cells and easily identified microvascular structures. By contrast, the samples treated with cyclophosphamide and cisplatin were characterized by a less cellular stroma with a marked disarray of the cells and extracellular matrix. Interstitial cells were sparser. Atretic follicles were visible within the surrounding stroma (Fig. 4). Stromal damage was less prominent in paclitaxel-treated samples. Similar structural changes were observed in the samples treated with chemotherapy + GnRHa groups, suggesting that GnRHa did not preserve ovarian stroma. We also noticed a paucity of the vascular structures in the chemotherapy treated samples, particularly after cyclophosphamide and cisplatin. After staining with VEGF to assess microvascular structures, we observed that there was an abundance of microvascular structures in the stroma of the control samples (88%) whereas the microvascular density was decreased to 18, 22 and 56% after treatment with cyclophosphamide, cisplatin and paclitaxel, respectively. Vascular structures were preserved somewhat in paclitaxel-treated samples compared with cyclophosphamide and cisplatin. We did not observe any difference in the ovarian stroma and vascularity between chemotherapy and chemotherapy + GnRHa groups (Fig. 4).

In order to rule out the possibility that the doses of GnRH and cyclophosphamide, timing of GnRHa administration, exposure time and culture condition might potentially hinder the protective actions of GnRHa, we repeated the experiments with a total of six different dose combinations of cyclophosphamide-GnRHa, extended culture period up to 96 h, administered GnRHa 1–2 h prior to cyclophosphamide, and used a serum-free defined culture media. None of these modifications revealed any protective effect of GnRHa against the cytotoxicity of cyclophosphamide (Supplementary Fig. S1).

Irradiation of the ovarian cortical samples

Irradiation with 2 Gy of the ovarian tissue samples produced a similar degree of cytotoxicity to cyclophosphamide. In addition to massive follicle loss, there was a marked decrease in the cellularity of the stroma. As 92 and 83% of the primordial follicles and pre-antral/antral follicles, respectively, were lost after 2 Gy irradiation, the previous notion that the LD50 level of human oocyte is 2 Gy is challenged (Wallace *et al.*, 2003). Co-treatment with GnRHa did not rescue follicles from radiation induced apoptosis. The mean levels of E2, P and AMH produced by irradiated ovarian samples were significantly lower than in controls. Follicle reserve and hormone productions of the samples irradiated with GnRHa were not different from those exposed to radiation alone (Fig. 2A–D).

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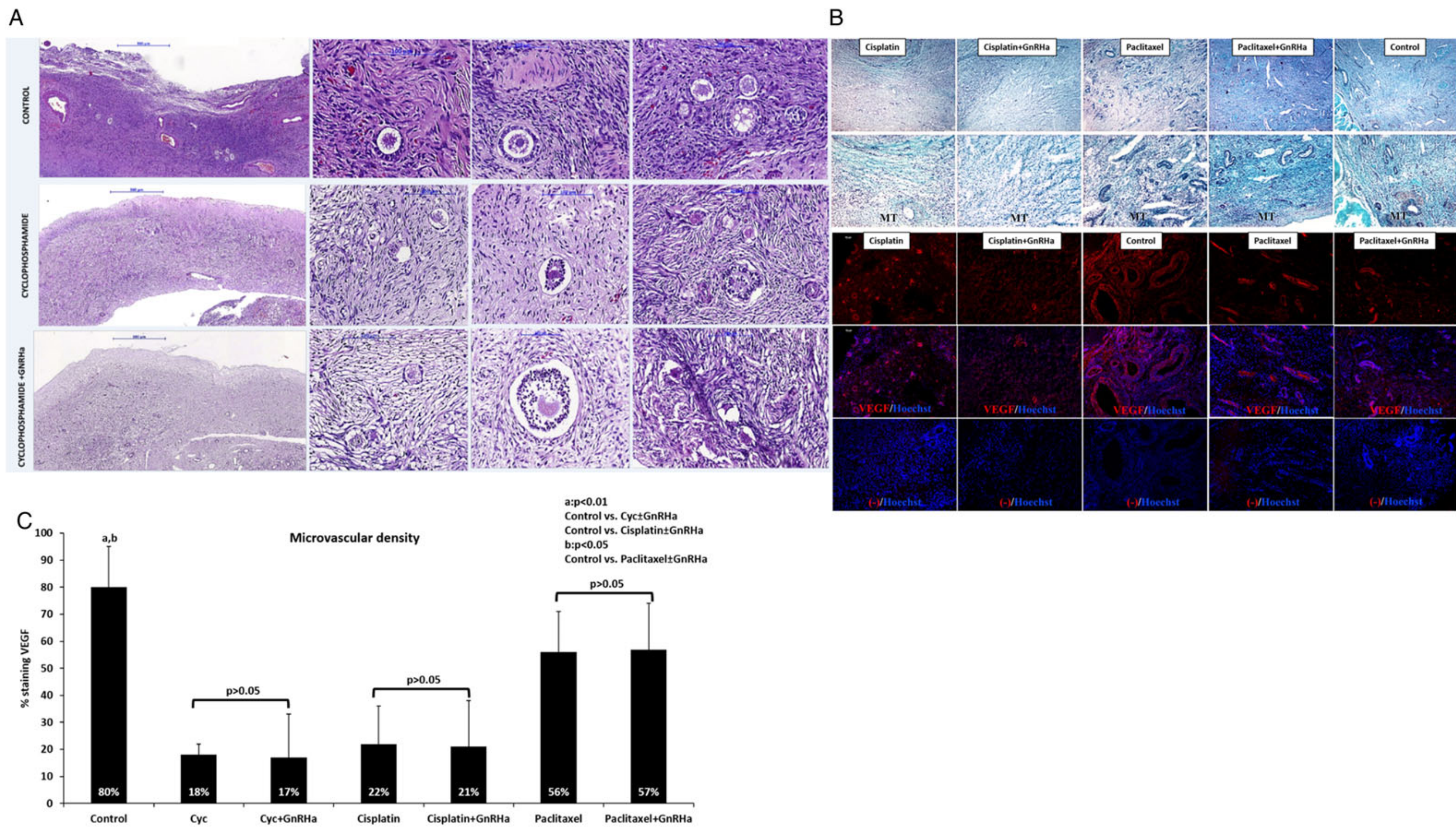

Figure 4 Histological examination of the ovarian samples after staining with hematoxylin-eosin (H&E), Masson's trichome (MT) and vascular endothelial growth factor (VEGF). (**A**) The sections of the ovarian samples before and after treatment with cyclophosphamide ± GnRHa, H&E staining. The follicles and stroma preserved their structure well after a 24 h culture period. Ovarian stroma was stained uniformly with H&E with many interstitial cells and easily identified microvascular structures. By contrast, the samples treated with cyclophosphamide were characterized by a marked disarray of the cells and extracellular matrix. Interstitial cells were more sparse. Atretic follicles with their pyknotic granulosa cells were visible within the surrounding stroma in cyclophosphamide treated samples. Similar structural alterations were observed in the samples treated with cyclophosphamide + GnRHa. (**B** and **C**) Stromal alterations and decreases in microvascular density were evident in the samples after staining with MT and VEFG, respectively. There is a marked paucity of vascular structures in the samples after treatment with cyclophosphamide or cisplatin compared with control and paclitaxel treated samples. The co-administration of GnRHa with chemotherapy did not preserve ovarian morphology and vascular structures from chemotherapy-induced cytotoxicity.

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Experiments on non-mitotic luteinized granulosa cells (HLGC)

Treatment with cyclophosphamide and cisplatin caused a significant degree of cytotoxicity on cells. There was an increase in apoptosis along with a drastic decline in the steroidogenic activity of the cells exposed to these drugs. Only 3% of the control cells were stained positive for the apoptosis marker YO-PRO-1, whereas 89 and 71% of the cells underwent apoptosis after treatment with cyclophosphamide and cisplatin, respectively ($P < 0.001$). Increased apoptosis was associated with a concurrent decrease in the steroidogenic activity of these cells. The co-administration of GnRHa with these drugs did not reduce apoptosis or improve the steroidogenic activity of the samples. Quantitative immunoblot analysis confirmed the occurrence of DNA damage and apoptosis after treatment with these drugs. The average density of the signal for cleaved caspase-3 in the quantitative immunoblot significantly increased from 1 (control) to 2.76 and 2.56 for the cells exposed to cyclophosphamide and cisplatin, respectively ($P < 0.01$) and to 2.74 and 2.54 for those with cyclophosphamide + GnRHa and cisplatin + GnRHa, respectively ($P < 0.01$). The co-administration of GnRHa with cyclophosphamide and cisplatin did not attenuate the intensity of the signal, confirming that GnRHa did not rescue the cells from DNA damage/apoptosis (Fig. 5A and B).

We also tested other chemotherapy drugs on HLGCs; paclitaxel alone, paclitaxel + cisplatin, 5-FU and TAC combination regimen. Of these drugs, paclitaxel and 5-FU were apparently devoid of any cytotoxic effects since the apoptotic fractions and E2 and P production of the cells treated with these drugs were comparable to untreated control cells (Supplementary Fig. S2). However, when paclixatel was combined with cisplatin, the cells underwent apoptosis and E2 and P production was decreased, resembling the cytotoxicity of cisplatin. The apoptosis rate and hormone production of the cells treated with cisplatin + GnRHA and cisplatin + paclitaxel + GnRHA were not any better than their counterparts treated without GnRHa. TAC combination exerted the highest magnitude of cytotoxicity on the HLGCs among the drugs tested. The cells exposed to this combination regimen had the highest number of apoptotic cells and produced the lowest amounts of E2 and P compared with controls and cells treated with other drugs. GnRHa co-administered with TAC did not alleviate the cytotoxic actions of this combination regimen (Supplementary Fig. S2).

Experiments on mitotic non-luteinized granulosa cells (COV434 and HGRC1)

Cyclophosphamide, cisplatin and TAC combination were cytotoxic to HLGCs whereas paclitaxel and 5-FU were not. We also tested these drugs at the same doses on the mitotic granulosa cells COV434 and HGrC1 to investigate if there is a difference in chemosensitivity between mitotic and non-mitotic granulosa cells. Cyclophosphamide, cisplatin and TAC markedly halted the proliferation and induced apoptosis of mitotic granulosa cells within hours. In several hours post-exposure the real-time growth curves of the cells exhibited a downward shift, indicative of rapidly induced apoptosis by these drugs. Similar toxic effects were observed after paclitaxel but it was milder than cyclophosphamide, cisplatin and TAC. 5-FU neither inhibited the proliferation nor induced apoptosis of these cells. The co-administration of GnRHa with cyclophosphamide, cisplatin, TAC and paclitaxel did not prevent the cytotoxicity of these drugs (Fig. 6 and Supplementary Fig. S3).

Discussion

Infertility and premature ovarian failure are reproductive sequelae of exposure to cytotoxic chemotherapy regimens in young females with cancer. A number of short and long-term health problems occur in women with premature menopause such as hot flushes, decreased libido, vaginal atrophy, sleep disturbances, osteoporosis and premature cardiovascular ageing.

Three fertility preservation strategies are currently available for women prior to cytotoxic chemotherapy/radiation for cancer. Of these, oocyte and embryo freezing are the established methods of fertility preservation. But ovarian tissue cryopreservation is considered still experimental due to the unknown success rate of this procedure and limited reports of pregnancies and live births achieved with this strategy (The Practice Committees of the American Society for Reproductive Medicine, 2013, 2014). Even though oocyte or embryo freezing prior to chemotherapy can help women achieve pregnancy and live birth after chemotherapy induced premature ovarian failure, these strategies cannot reverse menopause in the native ovaries. Similarly, grafting of frozen-thawed ovarian tissue appears to be remote from restoring ovarian function due to poor survival of the grafts and post-menopausal levels of sex hormones and AMH produced by these graft after transplantation (Janse *et al.*, 2011; Greve *et al.*, 2012). Furthermore, ovarian tissue transplantation carries the risk of re-introducing cancer cells, especially in hematological malignancies (Bastings *et al.*, 2013). Therefore, any drug that preserves ovarian reserve during chemotherapy can potentially sustain the normal reproductive life span and obviate the need for gamete freezing prior to chemotherapy.

Encouraged by the initial reports of animal studies and non-randomized human trials showing a beneficial effect of GnRH agonists in the preservation of ovarian function during chemotherapy, gonadotrophin-releasing hormone agonists have been proposed as a fourth potential fertility preservation strategy. But randomized controlled trials launched so far to assess the effectiveness of this method have shown contradictory results in cancer patients. Some of these trials demonstrated a protective effect of GnRH agonists in preserving ovarian function after chemotherapy (Badawy *et al.*, 2009; Sverrisdottir *et al.*, 2009; Del Mastro *et al.*, 2011; Moore *et al.*, 2015), whereas the others could not (Gerber *et al.*, 2011; Munster *et al.*, 2012; Elgindy *et al.*, 2013). Lack of molecular data in this under-studied issue led us to investigate in this study if GnRH agonist leuprolide acetate decreases DNA damage and follicular apoptosis through either activation of GnRH receptors or up-regulation of intragonadal anti-apoptotic genes during adjuvant chemotherapy. For this purpose, we conducted several specific end-point assays in this study to provide a molecular evidence for or against the role of GnRHa in the preservation ovarian function and reserve after chemotherapy.

Our experiments on the ovarian cortical samples showed that cyclophosphamide and cisplatin impacted both primordial follicles and the growing follicle fraction whereas paclitaxel was detrimental to the growing follicles only. The co-administration of GnRHa with these drugs did not preserve the follicle stockpile or improve their steroidogenic activity. Apart from follicular structures, ovarian stroma and microvessels were also destroyed by chemotherapy agents, particularly after cyclophosphamide and cisplatin. GnRHa did not preserve stroma and vessels from chemotherapy-induced damage. Furthermore, GnRHa when co-administered with cyclophosphamide did not up-regulate the

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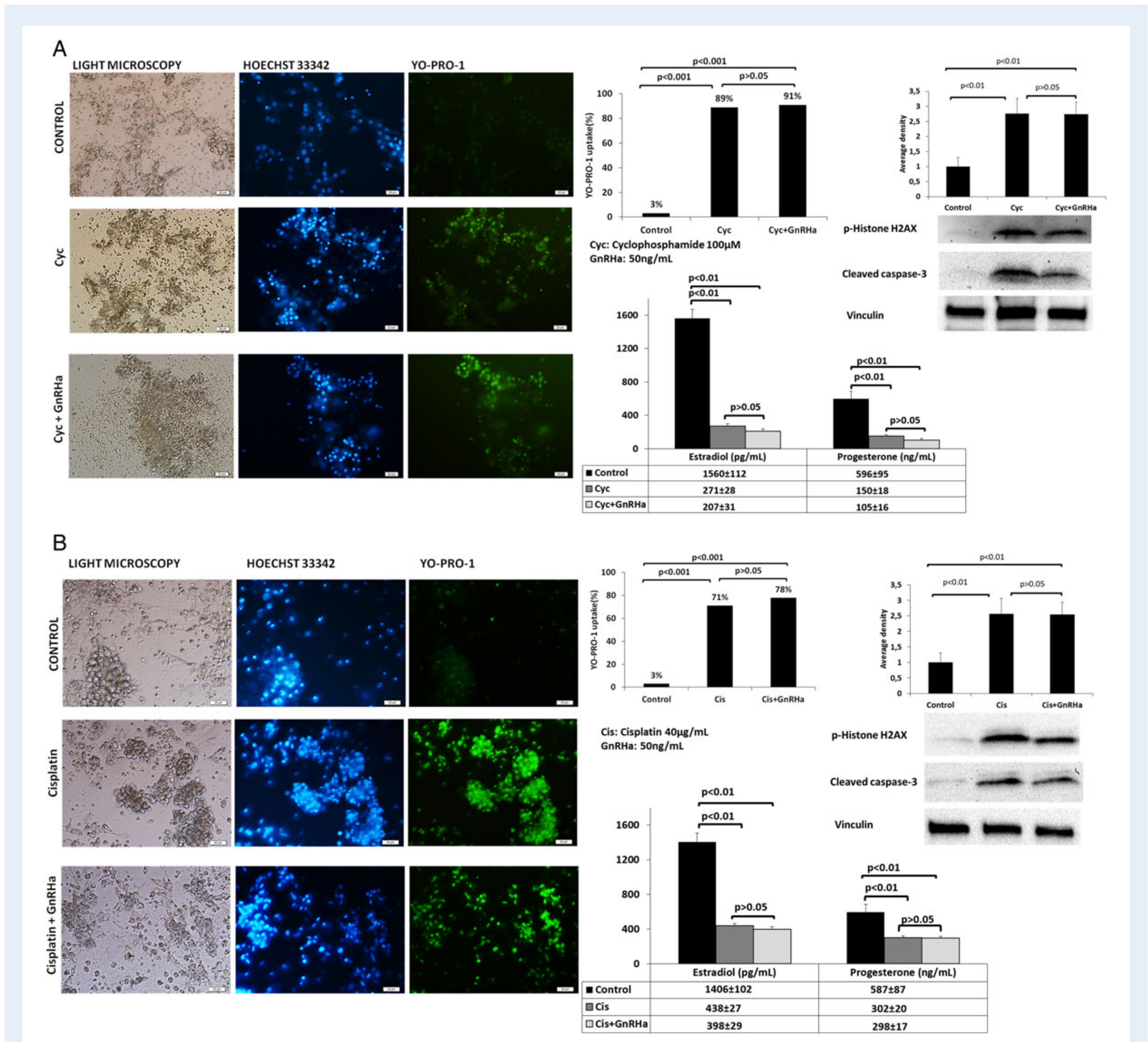

Figure 5 Human luteinized granulosa cells (HLGCs) treated with chemotherapy + GnRHa. Cyclophosphamide (**A**) and cisplatin (**B**) caused a significant degree of cytotoxicity on these cells. There was a more than 70% increase in DNA damage and apoptosis (as shown by the apoptosis marker YO-PRO-1), and decline in the steroidogenic activity of the cells incubated with these drugs for 24 h. The addition of GnRHa did not prevent or attenuate DNA damage and apoptosis induced by these drugs or improve their ability to produce E2 and P. Quantitative immunoblot analysis shown as a bar graph did not show any difference in the intensity of the signal for cleaved caspase-3 in chemotherapy versus chemotherapy + GnRHa groups.

mRNA expression of anti-apoptotic genes in the ovarian samples. Taken together, these results at least indicate that GnRHa does not confer any ovarian protection against the cytotoxic effects of chemotherapy drugs *in vitro*. Removal of ovarian tissue from its blood supply may change its sensitivity/resistance to cytotoxic stimuli and may impede the anti-apoptotic mechanism in the ovary. Therefore the *in vitro* environment may not actually represent the real *in vivo* environment in which the ovaries are exposed to cytotoxic drugs and GnRHa. This issue is particularly important when evaluating the cytotoxic effects of chemotherapy drugs and the protective actions of GnRHa on the long-term cultures of ovarian samples. In our study, the samples were treated with chemotherapy agents ± GnRHa with no delay after removal and the culture period was restricted to 24 h to minimize the changes in ovarian physiology related to *in vitro* conditions. Limited availability of ovarian cortical pieces precluded us from conducting this experiment with a larger sample size and adequate power. It should also be remembered that there is no an ideal model to harvest ovarian tissue samples from human donors containing a reasonable number of oocytes (Smitz *et al.*, 2010). Further, ovarian tissue fragments and granulosa cells obtained from ovarian cyst walls might have different sensitivity or

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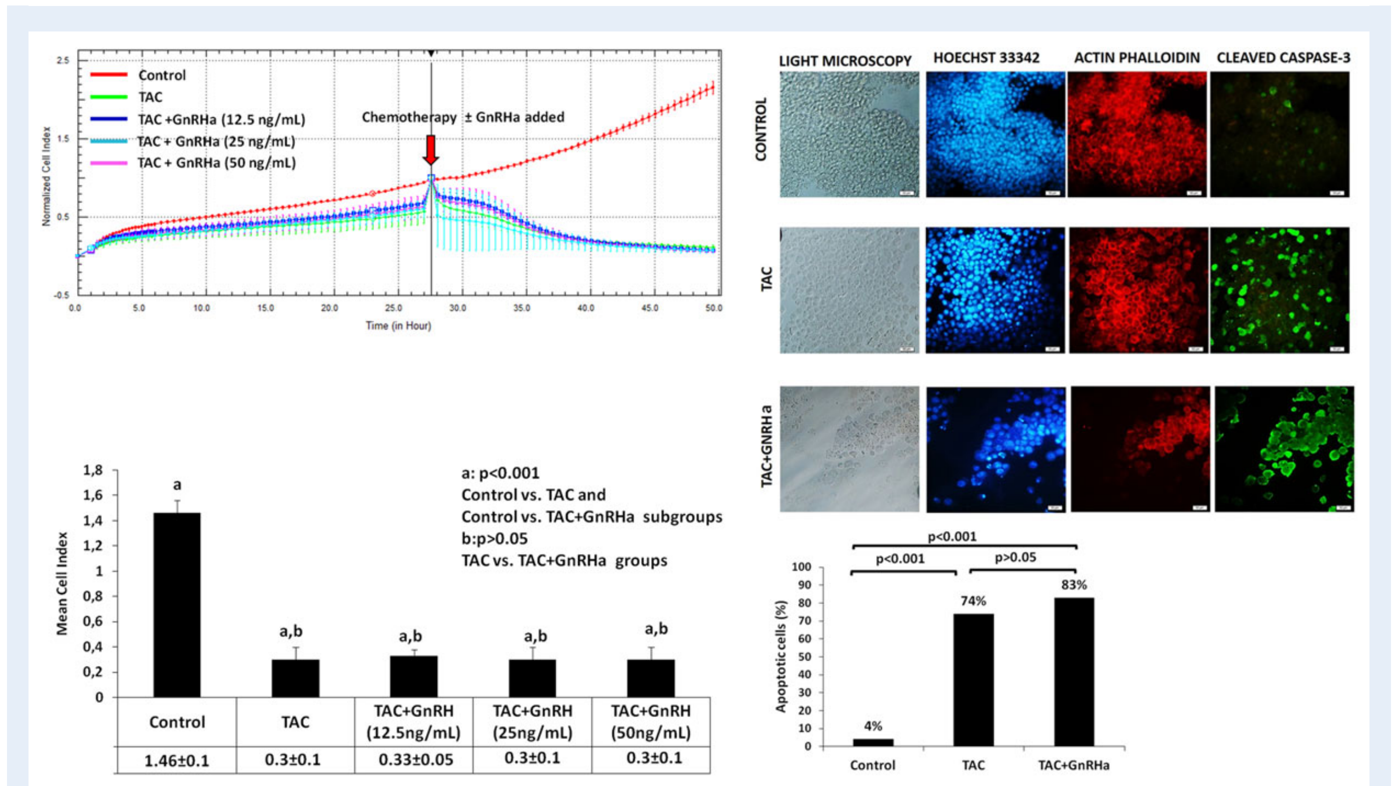

Figure 6 Real-time growth curves of the proliferating non-luteinized granulosa cells treated with different dose combinations of TAC and GnRHa. The ordinate shows normalized cell index (CI) as a measure of viable cell mass, which are derived from the ratio of CIs before and after the addition of the compounds. The absciss denotes culture period time in hours. A fixed dose of TAC regimen (10 ng/ml) was co-administered with GnRHa at three different concentrations (12.5, 25 and 50 ng/ml). Note the prominent downward shift in the growth curve of the cells exposed to TAC. GnRHa did not rescue the cells from apoptosis (as shown by the cleaved caspase-3) induced by TAC. The rate of apoptosis in the cells treated with TAC + GnRHa was not different from those treated with TAC alone.

expression of apoptotic pathways that can potentially change their sensitivity to toxic agents (Sanchez *et al.*, 2014).

We also demonstrated in this study that the number of growing follicles in the control ovarian samples was significantly correlated with the levels of AMH and E2 produced by these samples *in vitro*. However, in chemotherapy treated samples the correlations among these variables were either less significant or absent. Curiously, this finding at least raises a question as to whether the insult to granulosa cells may interfere with steroidogenetic activity of the surviving pre-antral/antral follicles.

Since GnRH receptors are mainly expressed by proliferating and luteinized granulosa cells of the growing follicles and corpus luteum respectively (Choi *et al.*, 2006), representative types of granulosa cells were intentionally included in the study for a detailed analysis of the impact of chemotherapy + GnRHa. We demonstrated, as another finding of this study, that the chemosensitivity of proliferative granulosa cells seems to be different from that of non-proliferative luteinized cells. While cyclophosphamide, cisplatin and TAC were cytotoxic to both types of granulosa cells, paclitaxel selectively impacted proliferative granulosa cells with no apparent toxicity on the luteinized non-proliferative granulosa cells. 5-FU had the least or no toxic effects on either type of the cells regardless of their ability to proliferate. These results suggest that the inhibition of depolymerization of microtubules by paclitaxel has a more profound anti-proliferative effect than the anti-metabolite drug 5-FU. When these data were collectively analyzed with the impact of these drugs on ovarian follicles, paclitaxel ranked behind cyclophosphamide and cisplatin in terms of gonadotoxic potential. These data are particularly important for paclitaxel for which human data are limited and inconsistent so far (Reh *et al.*, 2008; Abusief *et al.*, 2010).

Conclusion

GnRH agonist leuprolide acetate did not protect human ovarian samples and granulosa cells from cytotoxic effects of chemotherapy agents and radiation *in vitro*. If GnRHa had any protective effects *in vivo* through its intraovarian actions such as the inhibition of apoptosis and up-regulating anti-apoptotic genes, we could have reproduced at least some of these effects *in vitro*, either at the level of apoptosis and anti-apoptotic genes, or follicle reserve and steroidogenic activity in the ovarian samples and granulosa cells. On the other hand, our findings do not conclusively rule out the possibility that GnRHa may offer protection if any, through some other mechanisms *in vivo*.

Supplementary data

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

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Authors' roles

G.B., N.A., F.S. and G.N.S. (Reproductive Biology masters students): conducted the experiments. O.O.: conceived the work, designed the methodologies, interpreted the results and wrote the manuscript. B.B.: provided human luteal granulosa cells and prepared the cells for culture. B.U.: interpreted the results and wrote the manuscript. A.I.: provided the HGrC1 cell line. U.I. and S.K.: prepared the ovarian tissues for the histomorphologic examinations. U.S.: administrated radiation to the ovaries. C.T., E.A., K.Y., C.A., Y.G., B.U., O.O.: provided the ovarian tissue fragments. M.C., T.E., N.M.M.: provided the chemotherapy agents, designed the methodologies and interpreted the results.

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Conflict of interest

None declared.

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