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Short-term exposure of human ovarian follicles to cyclophosphamide metabolites seems to promote follicular activation *in vitro*

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KEY MESSAGE

Cyclophosphamide metabolites seem to enhance recruitment and activation of dormant primordial follicles in human ovarian tissue in vitro thus 'burning out' the ovarian reserve. This phenomenon may explain the specific toxicity of cyclophosphamide to human ovarian tissue. Developing a method to prevent follicular recruitment may possibly eliminate the toxic effect.

A B S T R A C T

How chemotherapy affects dormant ovarian primordial follicles is unclear. The 'burnout' theory, studied only in mice, suggests cyclophosphamide enhances primordial follicle activation. Using 4-hydroperoxycyclophosphamide (4hc) and phosphoramide mustard (PM), this study assessed how the active cyclophosphamide metabolites 4-hydroxycyclophosphamide (4-OHC) and PM, affect human primordial follicles. Frozen-thawed human ovarian samples were sliced and cultured with basic culture medium (cultured controls) or with 4hc/PM (3 μmol/l/10 μmol/l) (treated samples) for 24–48 h. Follicular counts and classification, Ki67 and anti-Müllerian hormone (AMH) immunohistochemistry and an apoptosis assay were used for evaluation, and 17β-oestradiol and AMH were measured in spent media samples. Generally, there was primordial follicle decrease and elevated developing follicle rates in treated samples compared with cultured ($P = 0.04$ to $P < 0.0005$) and uncultured controls ($P < 0.05$ to $P < 0.0001$). No traces of apoptosis were found. There were almost twice

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the levels of AMH and 17β-oestradiol in treated compared with untreated samples (AMH with 4hc 3 µmol/l; $P = 0.04$). All follicles stained positively for AMH included treated samples. Ki67 positive staining was noted in all samples. Cyclophosphamide metabolites seem to enhance human primordial follicle activation to developing follicles, *in vitro*. Study findings support the 'burnout' theory as the mechanism of chemotherapy-induced ovarian toxicity.

Introduction

Females are born with a fixed number of oocytes, which must remain viable over the years to maintain fertility (Faddy, 2000; Gougeon, 1996). Oocytes are surrounded by supporting granulosa cells, creating structures called follicles. Most ovarian follicles are in a quiescent state, called primordial follicles, with a single layer of flat granulosa cells surrounding the small oocytes (Gougeon, 1996). Throughout life, small numbers of primordial follicles are continuously activated, until the oocyte pool is exhausted, at which point the individual enters menopause (Faddy, 2000; Gougeon, 1996).

Oncological advances have improved survival rates, partially shifting the focus from surviving cancer to preserving optimal quality of life after completing treatments (Abir et al., 2008; Feigin et al., 2008; Meirow, 2000; Meirow and Nugent, 2001). Many patients experience severe side effects from chemotherapy, including follicular depletion leading to ovarian failure and infertility (Abir et al., 2008; Feigin et al., 2008; Meirow and Nugent, 2001). Young patients have a higher absolute number of primordial follicles, and therefore, ovarian failure is less common after toxic chemotherapy, but they do suffer a severe reduction in ovarian reserve causing early menopause and infertility (Feigin et al., 2008). The severity of ovarian damage is dependent on the chemotherapeutic agent used, the total dosage and the treatment duration. Alkylating agents such as cyclophosphamide are associated with the highest rate of ovarian toxicity (Abir et al., 2008; Feigin et al., 2008; Meirow and Nugent, 2001; Meirow et al., 2007).

It is well known that chemotherapeutic agents target metabolic pathways needed for completion of the cell cycle, such as DNA replication and microtubules of the spindle apparatus, and thus gain much of their specificity in the human body by preferentially killing rapidly proliferating cells (Mitchison, 2012). Therefore, chemotherapy destroys proliferating ovarian follicles during treatment, probably by damage caused to dividing granulosa cells (Abir et al., 2008; Ben-Aharon and Shalgi, 2012). The mechanism by which the quiescent primordial follicles are affected by chemotherapy is unclear (Abir et al., 2008; Ben-Aharon and Shalgi, 2012; Feigin et al., 2008; Raz et al., 2002).

It was long assumed that chemotherapeutic agents initiate apoptosis of primordial follicles, and thus cause ovarian failure (Tilly, 1996). Post-chemotherapy apoptotic changes have been demonstrated in mature murine oocytes exposed to doxorubicin (Bar-Joseph et al., 2010; Perez et al., 1997), in secondary and antral follicles of mice injected with doxorubicin (Ben-Aharon et al., 2010), in murine oocytes from pre-antral follicles exposed *in vivo* to cisplatin (Gonfloni et al., 2009), and in granulosa cells of human primordial follicles cultured with cisplatin (Meirow, 2000; Meirow and Nugent, 2001). Apoptosis of human oocytes from primordial follicles and of growing follicles was reported 12 and 24 h following cyclophosphamide exposure (Oktem and Oktay, 2007). However, others did not detect any sign of apoptosis in mice treated with cyclophosphamide *in vivo* (Kalich-Philosoph et al., 2013) or in ovaries of women exposed to chemotherapy, including alkylating agents, even when examined 4 days post-chemotherapy (Abir et al., 2008).

Follicular structure damage, not mediated by apoptosis, is another possible mechanism causing ovarian failure post-chemotherapy (Abir et al., 2008; Ben-Aharon and Shalgi, 2012). Transmission electron microscopy (TEM) demonstrated vacuolization of oocytes and granulosa cells and abnormally thick basal lamina in primordial follicles after combination chemotherapy for Hodgkin's disease (Familiari et al., 1993). Moreover, our group showed that human ovaries exposed to chemotherapy *in vivo* had elevated oocyte vacuolization and a reduction in normal granulosa cell nuclei (Abir et al., 2008).

Alternatively, chemotherapy may induce vascular damage of certain areas of the ovarian cortex resulting in depletion of primordial follicles (Ben-Aharon and Shalgi, 2012; Meirow et al., 2007). In this context, significant vascular damage has been identified in histological sections of ovaries from women exposed to chemotherapy, including thickening and hyalinization of small vessels, cortical proliferation of small vessels and neovascularization (Meirow et al., 2007). Studies using ultrasound Doppler demonstrated a significant reduction in ovarian blood flow, shortly following chemotherapy treatment, both in mice treated with doxorubicin (Bar-Joseph et al., 2011) and in women treated with anthracycline or taxane (Ben-Aharon et al., 2012).

Another potential mechanism of primordial oocyte loss is the 'follicular burnout' theory (Meirow et al., 2010), which suggests that exposure of ovaries to chemotherapy (in particular cyclophosphamide) causes destruction of developing follicles due to high mitotic activity. In turn, levels of anti-Müllerian hormone (AMH) (also termed Müllerian inhibiting substance, MIS) and other primordial follicular activation inhibitors, secreted from developing follicles, are reduced. The lack of inhibition induces continuous recruitment of ovarian primordial follicles to developing follicles, resulting in their destruction, thus 'burning out' the ovarian reserve. Indeed, AMH-knockout mice have increased activation of primordial follicles, with a greater number of atretic larger follicles and diminished ovarian reserve (Meirow et al., 2010). Moreover, ovaries of mice exposed to cyclophosphamide had elevated levels of primary and secondary follicles with a concomitant decrease in primordial follicles (Kalich-Philosoph et al., 2013), and increased Ki67 immunostaining levels (a granulosa cell proliferation marker), particularly in early growing follicles. So far, there is proof of the 'burnout' theory only in mice (Kalich-Philosoph et al., 2013; Meirow et al., 2010) but not in humans.

Cyclophosphamide is a prodrug activated by the hepatic cytochrome P450 enzymes to produce 4-hydroxycyclophosphamide (4-OHC). In turn, 4-OHC interconverts with aldophosphamide, which spontaneously fragments to generate phosphoramide mustard (PM) and acrolein (Ludeman, 1999). PM is considered the alkylating metabolite of therapeutic importance. PM destroys rapidly dividing cells by binding covalently to DNA, causing DNA–DNA and DNA–protein crosslinks and double-stranded DNA breaks (Helleday et al., 2008; Hurley, 2002). The plasma half-life value of both 4-OHC and PM is 14 min (Sladek et al., 1984).

Although PM is probably the active metabolite causing ovarian toxicity (Desmeules and Devine, 2006; Petrillo et al., 2011; Plowchalk and Mattison, 1991), 4-OHC is considered more ovotoxic as it is transported into cells much more readily than is the ionic PM (Ludeman,

1999). Toxicity is probably primarily associated with PM generated intracellularly from 4-OHC.

The objective of our present study was to assess for the first time the effect cyclophosphamide metabolites have on human ovarian follicles in culture and to uncover the mechanism by which cyclophosphamide metabolites affect human primordial follicles *in vitro*.

Materials and methods

Sample sources and retrieval

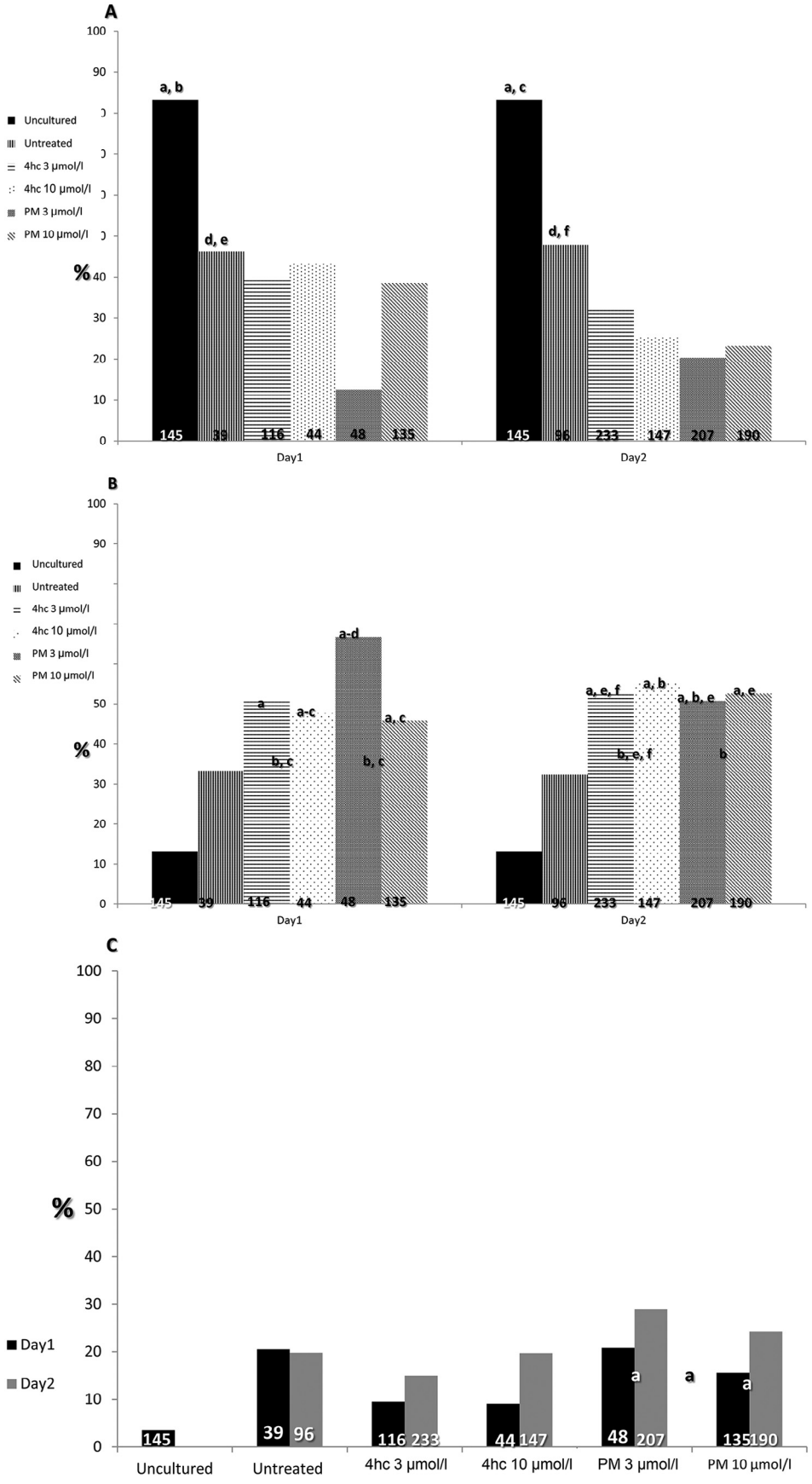
This study was performed on samples of ovarian tissue obtained from 10 girls and women (12 ± 5 years [average ± standard deviation, SD]) who underwent gynaecological laparoscopy for ovarian cryopreservation before chemotherapy (Lerer-Serfaty et al., 2013). In order to avoid problems related to follicular density that may influence results, we used ovarian tissue from young patients with high follicular content. The Ethics Committee of Rabin Medical Center approved the study protocol (approval number: 5875, 0108-07-RMC), and informed consent was obtained from every adult patient or parents of minors. One slice of each sample, measuring 1–2 mm, was immediately fixed (fresh-uncultured). All samples were handled in our laboratory within 1 h of surgery. One slice measuring ~2 mm × 1 mm with a depth of ~1 mm from each patient was fixed in 3% naturally buffered formalin (NBF) (prepared from 37% formalin solution, Sigma, St Louis, MO, USA) diluted in phosphate-buffered saline (Biological Industries, Beit Ha'emek, Israel) immediately after ovarian dissection (fresh-uncultured sample), so that follicular density could be studied histologically before culture. This practice eliminated the risk of utilizing for culture poorly populated ovarian tissue. The remaining ovarian tissue was frozen.

Cryopreservation and thawing of ovarian tissue

Each ovarian cortex sample was sliced before cryopreservation. The dimensions of each slice were 1.5 cm × 0.5 cm with a depth of ~1 mm. The slices were then transferred to cryogenic vials (Nalge Nunc International, Delta, Roskilde, Denmark) containing 1.5 mol/l dimethylsulphoxide (DMSO) (Sigma, St Louis, USA) (Lerer-Serfaty et al., 2013). Before freezing, the cryotubes were kept on ice for 30 min with DMSO (1.5 mol/l) for equilibration. All samples were frozen slowly in a programmable freezer (Kryo 10; series 10/20, Planer Biomed, Sunbury on Thames, UK), and immediately placed in liquid nitrogen.

The slices were thawed by washouts with decreasing concentration gradients of DMSO (1.0 mol/l, 0.5 mol/l, 0 mol/l) followed by incubation at 37°C. One slice of every thawed ovarian sample, similar in size to the fresh-uncultured slices, was fixed in Bouin's solution (made from compounds purchased from Sigma) immediately after thawing (thawed-uncultured samples, uncultured group in **Figures 1A, 1B and 1C**).

Figure 1 – Follicular counts and classifications in treatment groups. A. Primordial follicles. The figure presents the percentage of primordial follicles in study groups. Black column = uncultured samples. Perpendicular black lines column = untreated samples. Parallel black lines column = ovarian samples treated with 4hc 3 μmol/l. Black dots = ovarian samples treated with 4hc 10 μmol/l. Grey column = ovarian samples treated with PM 3 μmol/l. Diagonal black lines = ovarian samples treated with PM 10 μmol/l. The numbers inside the columns represent the total number of follicles counted (all follicular classes) per group. [a]Significantly more primordial follicles compared with untreated ($P = 0.0002$), 4hc 3 μmol/l ($P < 0.0001$), 4hc 10 μmol/l ($P < 0.0001$), both 4hc concentrations combined ($P < 0.0001$), PM 10 μmol/l ($P < 0.0001$), PM 3 μmol/l ($P < 0.0001$), both PM concentrations combined ($P < 0.0001$), day 1 and day 2 combined. [b]Significantly more primordial follicles compared with 4hc 10 μmol/l ($P = 0.005$), both 4hc concentrations combined ($P = 0.0002$), PM 3 μmol/l ($P < 0.0001$), PM 10 μmol/l ($P = 0.0005$) and both PM concentrations combined ($P < 0.0001$) at day 1. [c]Significantly more primordial follicles compared with 4hc 3 μmol/l ($P = 0.001$), 4hc 10 μmol/l ($P = 0.001$), PM 10 μmol/l ($P = 0.001$), both 4hc concentrations combined ($P = 0.001$), PM 3 μm ($P = 0.001$), both PM concentrations combined ($P < 0.0001$) at day 2. [d]Significantly more primordial follicles compared with 4hc 10 μmol/l ($P = 0.007$), PM 3 μmol/l ($P = 0.0005$), PM 10 μmol/l ($P = 0.03$), day 1 and day 2 combined. [e]Significantly more primordial follicles compared with PM 3 μmol/l ($P = 0.01$) at day 1. [f]Significantly more primordial follicles compared with 4hc 3 μmol/l ($P = 0.04$), 4hc 10 μmol/l ($P = 0.008$), both 4hc concentrations combined ($P = 0.02$), PM 3 μmol/l ($P = 0.03$), both PM concentrations combined ($P = 0.003$) at day 2. B. Developing follicles. The figure presents percentage of developing follicles in study groups. Black column = uncultured samples. Perpendicular black lines column = untreated samples. Parallel black lines column = ovarian samples treated with 4hc 3 μmol/l. Black dots = ovarian samples treated with 4hc 10 μmol/l. Grey column = ovarian samples treated with PM 3 μmol/l. Diagonal black lines = ovarian samples treated with PM 10 μmol/l. The numbers inside the columns represent the total number of follicles (all follicular classes) counted per group. [a]Significantly more developing follicles in 4hc 3 μmol/l ($P = 0.004$), 4hc 10 μmol/l ($P = 0.02$), both 4hc concentrations combined ($P < 0.0001$), PM 3 μmol/l ($P < 0.0001$), PM 10 μmol/l ($P = 0.002$) and both PM concentrations combined ($P = 0.001$) compared with uncultured samples, day 1 and day 2 combined. [b]Significantly more developing follicles in 4hc 10 μmol/l ($P = 0.02$), both 4hc concentrations combined ($P = 0.02$), PM 3 μmol/l ($P = 0.009$), both PM concentrations combined ($P = 0.01$) compared with untreated, day 1 and day 2 combined. [c]Significantly more developing follicles in 4hc 10 μmol/l ($P = 0.002$), both 4hc concentrations combined ($P = 0.04$), PM 3 μmol/l ($P < 0.0001$), PM 10 μmol/l ($P = 0.0005$), both PM concentrations combined ($P = 0.0003$) compared with uncultured controls at day 1. [d]Significantly more developing follicles in PM 3 μmol/l ($P = 0.002$) compared with 4hc 3 μmol/l at day 1. [e]Significantly more developing follicles in 4hc 3 μmol/l ($P < 0.0001$), both 4hc concentrations combined ($P < 0.0001$), PM 3 μmol/l ($P = 0.0005$), PM 10 μmol/l ($P = 0.008$) compared with uncultured at day 2. [f]Significantly more developing follicles in 4hc 3 μmol/l ($P < 0.05$) and both 4hc concentrations ($P = 0.02$) combined compared with untreated at day 2. C. Atretic follicles. The figure presents percentage of atretic follicles in study groups. Black column = day 1. Grey column = day 2. The numbers inside the columns represent the total number of follicles counted (all follicular classes) per group. [a]Significantly more atretic follicles in PM 3 μmol/l ($P = 0.01$), PM 10 μmol/l ($P < 0.05$) and both PM concentrations combined ($P = 0.04$) compared with uncultured, day 1 and day 2 combined. 4hc = 4-hydroperoxycyclophamide; 4-OHC = 4-hydroxycyclophosphamide; PM = phosphoramide mustard.


A
Uncultured
Untreated
4hc 3 µmol/l
4hc 10 µmol/l
PM 3 µmol/l
PM 10 µmol/l
%
a, b
d, e
a, c
d, f
145 39 116 44 48 135
145 96 233 147 207 190
Day1
Day2
B
a
a-c
a-d
a, c
b, c
b, c
a, e, f
a, b
a, b, e
a, e
b, e, f
b
C
Day1
Day2
145
39 96
116 233
44 147
48 207
135 190
Uncultured
Untreated
4hc 3 µmol/l
4hc 10 µmol/l
PM 3 µmol/l
PM 10 µmol/l

Culturing methods

Only frozen-thawed samples were used for incubation (Lerer-Serfaty et al., 2013) (see 'Sample sources and retrieval').

Each of the thawed ovarian tissues were further cut into small samples measuring ~2 mm × 1 mm in diameter (with a depth of ~1 mm), similar in size to the uncultured samples. Two slices were placed in each well of a 24-well culture plate (CELLSTAR, Greiner Bio-One, Germany).

In general, 4-hydroperoxycyclophosphamide (4hc) is often used in in vitro studies as a 4-OHC equivalent. Crystalline 4hc has a relatively long shelf-life but when dissolved in aqueous solution, it readily and spontaneously converts to 4-OHC (Desmeules and Devine, 2006; Ludeman, 1999). The concentrations of 4hc and PM and the incubation period (up to 48 h) were chosen according to an *in vitro* study on ovarian slices from mice (Desmeules and Devine, 2006) exposed to 4hc and PM at a diverse range of concentrations (0.01 to 300 µmol/l). In their study, concentrations of 1–10 µmol/l caused follicle depletion, whereas concentrations ≥30 µmol/l caused non-specific cell death. They also showed a significant reduction in primordial and primary follicles as early as 1 day following exposure to PM or 4hc at concentrations of 3–10 µmol/l, with the most significant reduction at 48 h. Samples exposed to PM or 4hc were unaffected after 12–18 h, and additional gradual follicle depletion continued until the eighth day. As the availability of human ovarian tissue is limited, we could not allow extended culture periods, for the same individual patients, beyond 48 h.

The wells were filled with one of the five following culture medium combinations (volume: 1 ml):

1. Basic culture medium (untreated samples): alpha minimal essential medium (αMEM, Biological Industries, Beit Ha'emek, Israel) supplemented with human recombinant FSH (1 IU/ml, Gonal-F, Serono, Aubonne, Switzerland), 10% human serum albumin (Irvine Scientific, Santa Ana, CA, USA), insulin, transferrin and selenium (Sigma) (Lerer-Serfaty et al., 2013).
2. Basic culture medium supplemented with 4hc (3 µmol/l) (Desmeules and Devine, 2006); 4hc was a generous gift from Dr S Ludeman.
3. Basic culture medium supplemented with 4hc (10 µmol/l) (Desmeules and Devine, 2006) (Dr S Ludeman).
4. Basic culture medium supplemented with PM (3 µmol/l) (Desmeules and Devine, 2006) (Niomech/II GmbH, University of Bielefeld, Bielefeld, Germany).
5. Basic culture medium supplemented with PM (10 µmol/l) (Desmeules and Devine, 2006) (Niomech/II GmbH, University of Bielefeld, Bielefeld, Germany).

Ovarian tissue samples from each of the 10 patients were included in all the experimental groups (uncultured controls and all cultured samples; **Figure 1**).

Media supplemented with 4hc or PM were made fresh, as these compounds are very unstable and cannot be stored in solution (Ludeman, 1999; Sladek et al., 1984). Solutions of 4hc and PM were made by dissolving the synthetic 4hc and PM in water; appropriate aliquots were added to the culture media.

The thawed ovarian tissue samples were incubated for up to 48 h in a standard incubator (95% air, 5% CO_2). One slice was removed from culture every 24 h (at 24 and at 48 h) to observe what occurs in culture after short incubation periods. Samples removed from culture were fixed immediately in Bouin's solution (made from compounds purchased from Sigma) (Lerer-Serfaty et al., 2013). Spent medium samples were collected at the end of the culture period (48 h) for subsequent 17β-oestradiol (Lerer-Serfaty et al., 2013) and AMH measurement (Xu et al., 2009).

Histological preparation

All fixed specimens were prepared for paraffin embedding, sectioning and staining with haematoxylin and eosin (Sigma) (Lerer-Serfaty et al., 2013). The number of follicles (with visible oocytes) in the uncultured and cultured samples were counted in two different section-levels per sample (50 µm between sections, to avoid counting the same follicle twice). The follicles were classified according to Gougeon (1996): primordial (with a single flat layer of granulosa cells surrounding the oocyte), primary (with a single cuboidal granulosa cell layer surrounding the oocyte) or secondary (with at least two granulosa cell layers surrounding the oocyte and a theca layer). Due to the scarcity of human ovarian tissue for research, and identification of low numbers of secondary follicles in the tissue, we primary and secondary follicles were combined to a group of developing follicles for analysis, a routine practice in our laboratory for cultured human follicles (Lerer-Serfaty et al., 2013). Atretic follicles were characterized by pyknotic cells, eosinophilia of the ooplasm, and clumping of the chromatin material (Gougeon, 1996). Unstained sections were placed on OptiPlus positively charged microscope slides for immunohistochemistry studies and terminal deoxynucleotidyl transferase (TdT, TUNEL) assay.

Immunohistochemistry for the proliferating marker Ki67 and AMH expression

Uncultured as well as cultured (day 1 and day 2) samples were processed for immunohistochemical studies for Ki67 and AMH. Ki67 is a cell cycle-associated nucleoprotein antigen that serves as a proliferation marker during the active cell-cycle phases (G_1, S, G_2 and mitosis) (Lerer-Serfaty et al., 2013). An increase in granulosa cell Ki67 staining has been reported to correlate directly with activation to cuboidal granulosa cells as well as with follicular growth. A modified Ki67 immunohistochemical staining protocol was used, as reported previously from our laboratory (Lerer-Serfaty et al., 2013): the sections were incubated overnight with a rabbit polyclonal anti-Ki67 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-15402). Sections for Ki67 immunostaining were incubated with a negative control solution that replaced the primary antibody: a normal rabbit IgG antibody (Santa Cruz Biotechnology, sc-2027) diluted to the same concentration as the primary antibody. The following morning, the samples were incubated with horseradish peroxidase polymer conjugate against mouse and rabbit primary antibodies (SuperPicture HRP, Zymed Laboratories Inc., San Francisco, CA, USA; catalogue number: 879363). Red-brown 3-amino-9-ethylcarbazole (Zymed Laboratories) staining indicated Ki67 expression. We defined a follicle as being positively stained if at least one of its granulosa cells expressed Ki67.

The sections for AMH immunohistochemistry were incubated with a goat polyclonal anti-MIS antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-6886). The negative control solution for AMH was prepared by the absorption of the primary antibody against MIS with its corresponding blocking peptide (Santa Cruz Biotechnology, catalogue number: sc-6886P). Thereafter, the samples were incubated with horseradish peroxidase polymer conjugate against goat primary antibodies (ImmPRESS REAGENT, Anti-goat Ig, Vector Laboratories, Burlingame, CA, USA; catalogue number: MP-7405). Brown diaminobenzidine (Sigma) staining indicated AMH expression.

TUNEL assay

Apoptosis was evaluated in all cultured (day 1 and day 2) and uncultured thawed control tissues (ApopTag, In Situ Detection Kit; Intergen Company, Purchase, NY). The use of this kit was successful in previous studies in our laboratory (Abir et al., 2008; Friedman et al., 2012). There were two positive controls: the first was provided with the kit and included a section from a rat mammary gland 4 days post-weaning and the second was prepared by exposure of the human ovarian sections to DNase (1 µg/ml, Sigma) for 15 min. For the negative control the equilibrium buffer provided with the kit replaced the diluted TdT enzyme.

Staining level was graded on a 4-point scale according to intensity, as previously described (Friedman et al., 2012): 0 = no TUNEL staining, 1 = low TUNEL staining intensity, 2 = medium TUNEL staining intensity, 3 = high TUNEL staining intensity. The slides were viewed independently by two of the authors (YL and RA).

17β-oestradiol accumulation in culture medium

17β-oestradiol is produced from the secondary follicle stage onwards (Gougeon, 1996) and therefore, measurement of 17β-oestradiol in spent media samples indicates presence of follicles from secondary stages onwards within the cultured ovarian tissue (Gougeon, 1996; Lerer-Serfaty et al., 2013). Spent media samples after 48 h were used for 17β-oestradiol measurements. This evaluation method is used routinely in our laboratory for evaluation of follicular growth in culture (Lerer-Serfaty et al., 2013).

17β-oestradiol concentrations were measured by a double antibody radioimmunoassay kit (Diagnostic Products Corp., Los Angeles, CA, USA), with a detection level of 1 pg/ml. Use of this kit was successful in previous studies in our laboratory (Lerer-Serfaty et al., 2013).

AMH accumulation in culture medium

AMH is produced by follicles from the primary stage onwards, and its measurement in the spent media samples indicates the presence of follicles from the primary stage onwards within the cultured ovarian tissue (La Marca et al., 2010; Xu et al., 2009). Spent media samples after 48 h were used for AMH measurements. AMH concentrations were measured by an enzyme-linked immunosorbent assay kit (GEN II ELISA kit, Beckman Coulter, Inc., Brea, CA, USA), with a detection level of 0.08 ng/ml (Xu et al., 2009). The protocol was modified at our laboratory for spent culture medium specimens (the manufacturer's instructions are for blood plasma/serum samples).

It is an enzymatically amplified two-site immunoassay. The AMH calibrators are supplied with the kit. The kit also includes a 96-well plate with mouse monoclonal anti-AMH IgG immobilized to the inside wall of each well. Both the calibrators and spent media samples were pipetted into these wells and incubated, followed by further incubation with an anti-AMH detection antibody labelled with biotin. Thereafter, the plate was incubated in the dark with strepavidin enzyme conjugate, followed by incubation in the dark with substrate tetramethylbenzidine chromogen (until a blue colour was obtained). Finally, an acidic stopping solution was added. The degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 nm and 600 nm. The absorbance measured is directly proportional to the concentration of AMH in the samples. A set of AMH calibrators was used to plot a calibration curve of absorbance versus AMH concentration. The AMH concentrations in the samples were then calculated from this calibration curve.

Statistical analysis

Data was analysed by analysis of variance, chi-squared test and Fisher's exact test, as required. *P*-values less than 0.05 were considered statistically significant.

Follicular counts and classifications were compared between controls and treated samples cultured at 24 h (day 1) and 48 h (day 2). Due to the scarcity of human ovarian tissue for research purposes day 1 and day 2 were combined, and the two concentrations (3 µmol/l and 10 µmol/l) for 4hc and PM were combined as well as analysed separately.

Results

The follicular counts and classifications of the uncultured thawed controls and the cultured untreated and treated groups are presented in **Figure 1**. **Figure 1A, 1B and 1C** present the percentage of primordial, developing and atretic follicles, respectively. Follicles at various stages of the experiment are shown in **Figure 2**.

Nearly all uncultured samples had a significantly higher rate of primordial follicles compared with all cultured samples (with the exception of PM 10 µm at day 2); day 1 and day 2 combined: untreated ($P = 0.0002$), 4hc and PM at both concentrations and combined concentrations of 4hc or PM ($P < 0.0001$); at day 1: 4hc 3 µm ($P < 0.05$), 4hc 10 µM ($P = 0.005$), PM 3 µM ($P < 0.0001$), PM 10 µmol/l ($P = 0.0005$) and combined concentrations of PM ($P < 0.0001$); at day 2: 4hc at both concentrations ($P = 0.001$), PM 3 µM ($P = 0.001$) and combined concentrations of PM ($P < 0.0001$).

In general there was a higher rate of primordial follicles in untreated samples compared with all treated samples (**Figure 1A**); day 1 and day 2 combined: 4hc 10 µmol/l ($P = 0.007$), PM 3 µmol/l ($P = 0.0005$) and PM 10 µmol/l ($P = 0.03$); after one day: PM 3 µmol/l ($P = 0.01$) at day 2: 4hc 3 µmol/l ($P = 0.04$), 4hc 10 µmol/l ($P = 0.008$), combined concentrations of 4hc ($P = 0.02$), PM 3 µmol/l ($p = 0.03$), and combined concentrations of PM ($P = 0.003$).

In parallel with the decrease in primordial follicles in general there was a higher rate of developing follicles in treated samples compared with uncultured, and many were found statistically significant (**Figure 1B**); day 1 and day 2 combined: 4hc 3 µmol/l ($P = 0.004$), 4hc 10 µmol/l ($P = 0.02$), combined concentrations of 4hc ($P < 0.0001$), PM 3 µmol/l ($P < 0.0001$), PM 10 µmol/l ($P = 0.002$) and combined concentrations of PM ($P = 0.001$); at day 1: 4hc 10 µmol/l ($P = 0.002$), combined concentrations of 4hc ($P = 0.04$), PM 3 µmol/l ($P < 0.0001$), PM 10 µmol/l ($p = 0.0005$), combined concentrations of PM ($P = 0.0003$); at day 2: 4hc 3 µmol/l ($P < 0.0001$), combined concentrations of 4hc ($P < 0.0001$), PM 3 µmol/l ($p = 0.0005$), PM 10 µmol/l ($P = 0.008$).

In general, there was a higher rate of developing follicles in treated compared with untreated samples, and many were found statistically significant (**Figure 1B**); day 1 and day 2 combined: 4hc 10 µmol/l ($P = 0.02$), combined concentrations of 4hc ($P = 0.02$), PM 3 µmol/l ($P = 0.009$), combined concentrations of PM ($P = 0.01$); at day 2: 4hc 3 µmol/l ($P < 0.05$) and combined concentrations of 4hc ($P = 0.02$). There was also a significantly higher rate of developing follicles at day 1 in the PM 3 µmol/l sample group compared with 4hc 3 µmol/l ($P = 0.002$), yet at day 2 this difference disappeared.

The majority of atretic follicles in cultured samples (**Figure 2D**) were in stages of development (not primordial). A trend was observed towards a higher rate of atretic follicles at day 2 compared with day 1 in all treated groups (NS, **Figure 1C**). In the cultured

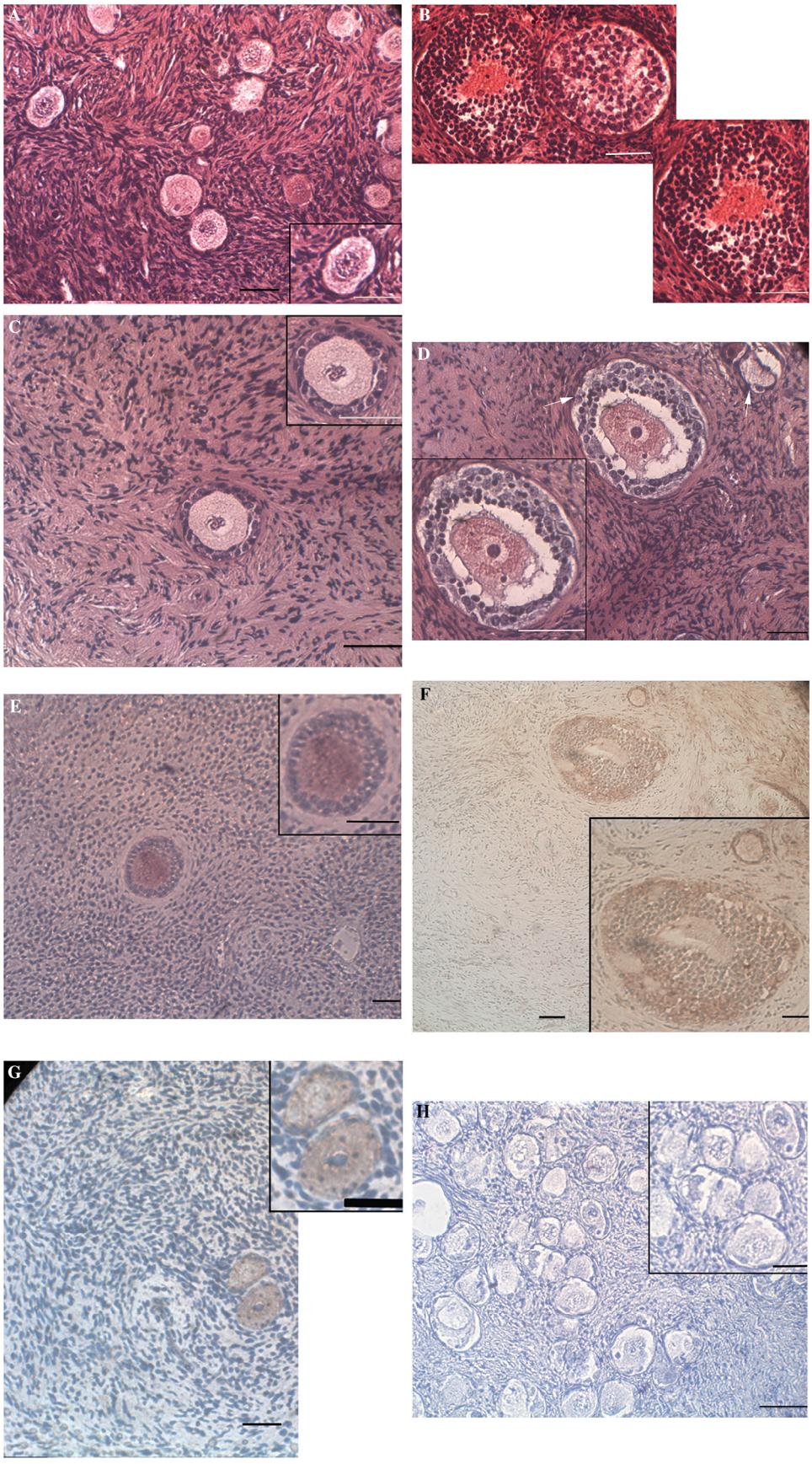

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Figure 2 – Histological and immunohistochemical staining of uncultured and cultured ovarian samples. (A) Histological section of an uncultured frozen-thawed ovarian sample. Note the numerous primordial follicles. Hematoxylin and eosin, scale bar = 30 μm. Insert on the bottom right-hand side of an enlarged image of a primordial follicle. Insert scale bar = 30 μm. (B) Histological section of ovary on day 2 cultured with 4hc 3 μmol/l. Note the two morphologically normal secondary follicles. Haematoxylin and eosin, scale bar = 30 μm. Insert on the bottom right-hand side an enlarged image of one of the secondary follicles. Insert scale bar = 35 μm. (C) Histological section of ovary on day 1 cultured with PM 10 μmol/l. Note the morphologically normal secondary follicle. Haematoxylin and eosin, scale bar = 30 μm. Insert on the upper right-hand side an enlarged image of the secondary follicle. Insert scale bar = 30 μm. (D) Histological section of ovary from the same patient as in (A) on day 2 cultured with 4hc 10 μmol/l. Note the atretic secondary and primordial follicles (arrows). Hematoxylin and eosin, scale bar = 30 μm. Insert on the bottom left-hand side of the enlarged secondary atretic follicle. Insert scale bar = 35 μm. (E) Histological section of ovary on day 2 cultured with 4hc 3 μmol/l, stained for Ki67. Note the secondary follicle with the weak positive red-brown 3-amino-9-ethylcarbazole staining (AEC) Ki67 staining in the follicle. Scale bar = 30 μm. Insert on the upper right-hand side of the enlarged follicle. Insert scale bar = 30 μm. (F) Histological section from the same sample as in (C) stained for Ki67. Note the secondary atretic follicle and the primordial-primary follicle stained positively for Ki67 in their granulosa cells (red-brown AEC). Scale bar = 30 μm. Insert on the lower right-hand side of the enlarged follicles. Insert scale bar = 30 μm. (G) Histological section of ovary on day 2 cultured with PM 10 μmol/l. Note the two primary-primordial follicles stained positively with brown diaminobenzidine staining for AMH mostly in the oocytes but also in their granulosa cells. Scale bar = 30 μm. Insert on the upper right-hand side of the enlarged follicles. Insert scale bar = 30 μm. (H) Histological section from the same sample as in (C) stained by TUNEL assay for apoptosis identification. Note the numerous primordial and primary follicles stained negatively for apoptosis (only blue haematoxylin background staining). Scale bar = 30 μm. Insert on the upper right-hand side of an enlarged image of primordial and primary follicles. Insert scale bar = 30 μm.

untreated samples there were no differences in the atretic follicle rate between day 1 and day 2. There was a significantly higher rate of atretic follicles with PM 3 μmol/l ($p = 0.01$), PM 10 μmol/l ($P < 0.05$) and combined concentrations of PM ($P = 0.04$) compared with uncultured samples, day 1 and day 2 combined. There was a non-significant trend towards a difference ($P = 0.057$) between combined concentrations of 4hc compared with uncultured.

In general, there were no significant differences between the two concentrations of PM or 4hc (3 μmol/l/10 μmol/l) and between the two culture days.

A total of 529 follicles were stained for ki67. In all cultured samples, including treated, >93% of follicles stained positively for ki67 in their granulosa cells (**Figures 2E, 2F**), apart from those cultured with 4hc 3 μmol/l at day 2 (80%) and PM 3 μmol/l at day 2 (60%, only five follicles were found in this group of ki67 staining) and the uncultured samples (65%). These differences were not significant.

Positive staining for AMH was found in all samples, including treated, cultured untreated and uncultured controls (**Figure 2G**). In general, staining in primordial follicles was mostly in the oocytes and staining in the developing follicles appeared both in oocytes and granulosa cells. Staining was also observed in atretic follicles.

In sections evaluated by TUNEL assay for apoptosis, no traces of apoptosis were found in treated samples or in cultured and uncultured controls (**Figure 2H**), similar to the negative controls. Brown TUNEL staining was identified in the positive controls.

Figure 3 presents AMH (**Figure 3A**) and 17β-oestradiol (**Figure 3B**) levels secreted in spent media samples. There were significantly higher AMH levels in samples treated with 4hc 3 μmol/l ($P = 0.04$) compared with untreated samples. The general trend of all other treatments was more than doubled levels of AMH and 17β-oestradiol compared with untreated samples, yet these differences were not significant.

Discussion

Although there seemed to be a trend of activation of primordial follicles to developing follicles and subsequent atresia in all cultured groups including the untreated samples noted by the elevated rate of developing follicles (non-significant elevation in developing follicles in the untreated group) and atretic follicles in parallel with a decreased rate of primordial follicles compared with uncultured samples, these findings were more prominent and statistically significant in samples treated with 4hc or PM. In most of the treated samples, between days 1 and 2 there seemed to be a trend of more apparent follicular changes (differences in primordial, developing and atretic follicles), a process that seemed to cease after the first day of culture in the untreated samples. There were no traces of apoptosis in both cultured and uncultured follicles, including treated groups. Almost all cultured and uncultured follicles stained positively for AMH and there were no differences in Ki67 staining in granulosa cells of all the experimental groups. There was a general trend of secretion of almost double AMH and 17β-oestradiol levels compared with untreated samples; with a significantly higher AMH level in samples treated with 4hc 3 μmol/l compared with untreated samples.

Results from this study showed a higher rate of primordial follicles in the untreated group compared with some of the treated groups in parallel with an increase in developing follicles in some of the treated groups compared with the untreated group. These findings suggest that cyclophosphamide metabolites 4-OHC and PM both enhance in-vitro recruitment of dormant primordial follicles to developing follicles that subsequently undergo atresia. Activation of primordial follicles in untreated samples may be explained by slicing of the tissue (Kawamura et al., 2013), or by spontaneous follicular growth in culture (Lerer-Serfaty et al., 2013). Yet, follicular activation was clearly enhanced by 4hc and PM.

The differences in follicular number between the various groups are due to uneven distribution throughout the human ovary (Abir et al., 2008; Feigin et al., 2008; Schmidt et al., 2003). Cultured samples were very small, thus we may have inadvertently cultured limited numbers of follicles in some of the treated groups relative to the uncultured control samples. Moreover, it is difficult to obtain human ovarian tissue for research, especially from young patients (as in this study) and the amount of donated tissue is small. Therefore, the study included only 10 ovarian samples and each sample was sliced into small specimens in order to include slices from each patient in all study groups.

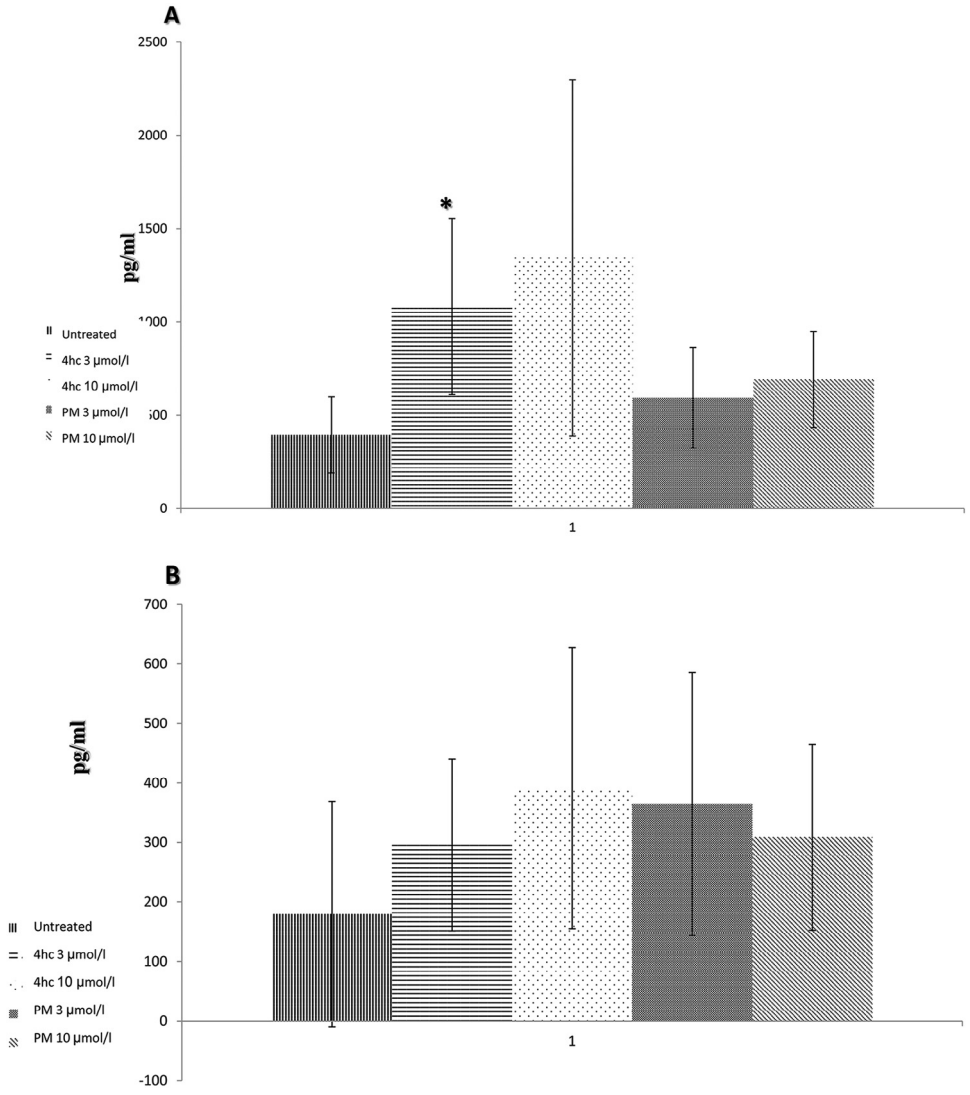

Figure 3 – AMH (A) and 17β-oestradiol (B) secretion from spent medium samples. Perpendicular black lines column = untreated samples. Parallel black lines column = ovarian samples treated with 4hc 3 µmol/l. Black dots = ovarian samples treated with 4hc 10 µmol/l. Grey column = ovarian samples treated with PM 3 µmol/l. Diagonal black lines = ovarian samples treated with PM 10 µmol/l. *P = 0.04 significantly higher than untreated.

The consistently positive Ki67 staining found both in treated and untreated follicles indicates profound granulosa cell proliferation even after culture with cyclophosphamide metabolites. This finding suggests that the cyclophosphamide metabolites 4-OHC and PM do not inhibit granulosa cell proliferation *in vitro*.

The lack of apoptosis identified in any of the cultured samples, including those treated with 4hc and PM, demonstrates that this is most probably not the mechanism causing depletion of oocytes in response to cyclophosphamide treatment. Our findings are in line with Morgan et al. (2013) who showed no evidence for primordial follicle apoptosis after *in vitro* exposure to doxorubicin and cisplatin.

AMH-knockout (AMHKO) adult mice were found to have fewer primordial follicles in parallel with more pre-antral and small antral follicles compared with wild type mice (Durlinger et al., 1999). These changes are most probably due to lack of AMH-regulated inhibition causing increased follicular recruitment in the AMHKO mice. Similarly, mouse ovaries cultured *in vitro* for 2 or 4 days in the absence or presence of AMH revealed that AMH causes a 40–50% decrease in the number of growing follicles after 2 and 4 days of culture (Durlinger et al., 2002a), most probably by inhibiting initiation of follicle growth (Durlinger et al., 2002b).

Findings of our current study suggest that at early culture stages cyclophosphamide metabolites cause recruitment of primordial follicles to developing follicles, causing secretion of AMH and 17β-oestradiol in spent media, as well as AMH and Ki67 expression in the follicles. AMH and 17β-oestradiol secretion in spent media of treated samples hints that development and proliferation of follicles occurs in response to cyclophosphamide metabolites; specifically, the significantly higher AMH secretion after exposure to 4hc 3 µmol/l compared with untreated samples. Most of the differences in the AMH and 17β-oestradiol levels of the treated samples compared with the untreated samples were not statistically significant because of the large standard deviations between the individual spent media sample measurements. It is possible that with a larger group of ovarian specimens we would have obtained more statistically significant differences.

It is well known that spontaneous activation of primordial follicles occurs in culture, but the exact timing at which it occurs is unknown (Lerer-Serfaty et al., 2013), and might be during the first 2 days of culture, immediately after release from the *in vivo* ovarian milieu. Our present study suggests that this process is accelerated with addition of cyclophosphamide metabolites. Moreover, the effect cyclophosphamide metabolites have on follicular recruitment is not responsive to the inhibitory effect expected by elevated levels of AMH (Durlinger et al., 1999). It is, however, necessary to be cautious with this conclusion, as the inhibitory effect of AMH on follicular activation may have occurred, had the culture period been extended longer than the 48 h of the present study. It is noteworthy also that AMH has actually been proven as an inhibitor of primordial follicle activation only in mice, and its effect in humans is in dispute. One group showed that in humans elevated AMH had a stimulatory effect on follicular recruitment *in vitro* (Schmidt et al., 2005), yet others found an inhibitory *in vitro* effect of AMH in humans similar to mice (Carlsson et al., 2006). Moreover, the minimal level of AMH that was shown to have an inhibitory effect maintaining the human primordial follicular pool in a quiescent stage *in vitro* was 100 ng/ml and lower doses did not show this effect. In our present study the AMH levels produced by the cultured human follicles were much lower and reached a maximal level of almost 2.5 ng/ml (2500 pg/ml) with 4hc 10 µm. We suggest that the AMH and 17β-oestradiol produced from the follicles indicate follicular viability rather than AMH inhibitory function, similar to AMH and Ki67 follicular expression.

The results also suggest that the induction of PM (3 µmol/l) on ovarian primordial follicles is more rapid than that of 4hc with maximal effect detected already after 24 h. However, we did expect that 4hc will be much more cytotoxic than PM, presumably because of its efficiency in getting into cells (Ludeman, 1999). Be that as it may, PM is the final active metabolite, whereas 4hc requires breakdown to active metabolites, and may therefore take longer to reach maximal effect in human follicles.

As shown previously in our laboratory (Lerer-Serfaty et al., 2013), our present study demonstrates follicular activation in cultured samples from young girls (including pre-pubertal), inconsistent with findings reported by others (Anderson et al., 2014).

The 'burnout' theory suggests that cyclophosphamide causes uncontrolled recruitment of primordial follicles to developing follicles that subsequently become atretic, and thus burnout of the ovarian reserve (Kalich-Philosoph et al., 2013; Meirow et al., 2010). Our findings seem to support that cyclophosphamide metabolites cause activation of primordial follicles *in vitro* to developing follicles with subsequent atresia causing further growth promotion of additional primordial follicles with time. This present report is the first study that supports the 'burnout' theory as the cause of cyclophosphamide follicular toxicity in humans. Yet, the mechanism by which cyclophosphamide metabolites cause seemingly uncontrolled recruitment of human primordial follicles *in vitro* has not been investigated to date, and further experiments should be conducted.

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REFERENCES

Abir, R., Ben-Haroush, A., Felz, C., Okon, E., Raanani, H., Orvieto, R., Nitke, S., Fisch, B., 2008. Selection of patients before and after anticancer treatment for ovarian cryopreservation. Hum. Reprod. 23, 869–877.

Anderson, R.A., McLaughlin, M., Wallace, W.H., Albertini, D.F., Telfer, E.E., 2014. The immature human ovary shows loss of abnormal follicles and increasing follicle developmental competence through childhood and adolescence. Hum. Reprod. 29, 97–106.

Bar-Joseph, H., Ben-Aharon, I., Rizel, S., Stemmer, S.M., Tzabari, M., Shalgi, R., 2010. Doxorubicin-induced apoptosis in germinal vesicle (GV) oocytes. Reprod. Toxicol. 30, 566–572.

Bar-Joseph, H., Ben-Aharon, I., Tzabari, M., Tsarfaty, G., Stemmer, S.M., Shalgi, R., 2011. In vivo bioimaging as a novel strategy to detect doxorubicin-induced damage to gonadal blood Vessels. PLoS ONE 6, e23492.

Ben-Aharon, I., Shalgi, R., 2012. What lies behind chemotherapy-induced ovarian toxicity? Reproduction 144, 153–163.

Ben-Aharon, I., Bar-Joseph, H., Tzarfaty, G., Kuchinsky, L., Rizel, S., Stemmer, S.M., Shalgi, R., 2010. Doxorubicin-induced ovarian toxicity. Reprod. Biol. Endocrinol. 8, 20.

Ben-Aharon, I., Meizner, I., Granot, T., Uri, S., Hasky, N., Rizel, S., Yerushalmi, R., Sulkes, A., Stemmer, S.M., 2012. Chemotherapy-induced ovarian failure as a prototype for acute vascular toxicity. Oncologist 17, 1386–1393.

Carlsson, I.B., Scott, J.E., Visser, J.A., Ritvos, O., Themmen, A.P., Hovatta, O., 2006. Anti-Müllerian hormone inhibits initiation of growth of human primordial ovarian follicles in vitro. Hum. Reprod. 21, 2223–2227.

Desmeules, P., Devine, P.J., 2006. Characterizing the ovotoxicity of cyclophosphamide metabolites on cultured mouse ovaries. Toxicol. Sci. 90, 500–509.

Durlinger, A.L., Kramer, P., Karels, B., de Jong, F.H., Uilenbroek, J.T., Grootegoed, J.A., Themmen, A.P., 1999. Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. Endocrinology 140, 5789–5796.

Durlinger, A.L., Gruijters, M.J., Kramer, P., Karels, B., Ingraham, H.A., Nachtigal, M.W., Uilenbroek, J.T., Grootegoed, J.A., Themmen, A.P., 2002a. Anti-Müllerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 143, 1076–1084.

Durlinger, A.L., Visser, J.A., Themmen, A.P., 2002b. Regulation of ovarian function: the role of anti-Müllerian hormone. Reproduction 124, 601–609.

Faddy, M.J., 2000. Follicle dynamics during ovarian ageing. Mol. Cell. Endocrinol. 163, 43–48.
Familiari, G., Caggiati, A., Nottola, S.A., Ermimi, M., di Benedetto, M.R., Motta, P.M., 1993. Ultrastructure of human ovarian primordial follicles after combination chemotherapy for Hodgkin's disease. Hum. Reprod. 8, 2080–2087.
Feigin, E., Freud, E., Fisch, B., Orvieto, R., Kravarusic, D., Avrahami, G., 2008. Fertility preservation in female adolescents with malignancies. In: *Cancer in Female Adolescents*. USA Science Publishers Inc, Hauppauge, NY, pp. 38–101.
Friedman, O., Orvieto, R., Fisch, B., Felz, C., Freud, E., Ben-Haroush, A., Abir, R., 2012. Possible improvements in human ovarian grafting by various host and graft treatments. Hum. Reprod. 27, 474–482.
Gonfloni, S., Di Tella, L., Caldarola, S., Cannata, S.M., Klinger, F.G., Di Bartolomeo, C., Mattei, M., Candi, E., De Felici, M., Melino, G., Cesareni, G., 2009. Inhibition of the c-Abl-TAp63 pathway protects mouse oocytes from chemotherapy-induced death. Nat. Med. 15, 1179–1185.
Gougeon, A., 1996. Regulations of follicular ovarian development in primates: facts and hypotheses. Endocr. Rev. 17, 121–154.
Helleday, T., Petermann, E., Lundin, C., Hodgson, B., Sharma, R.A., 2008. DNA repair pathways as targets for cancer therapy. Nat. Rev. Cancer 3, 193–204.
Hurley, L.H., 2002. DNA and its associated processes as targets for cancer therapy. Nat. Rev. Cancer 2, 188–200.
Kalich-Philosoph, L., Roness, H., Carmely, A., Fishel-Bartal, M., Ligumsky, H., Paglin, S., Wolf, I., Kanety, H., Sredni, B., Meirow, D., 2013. Cyclophosphamide triggers follicle activation and' 'burnout'; AS101 prevents follicle loss and preserves fertility. Sci. Transl. Med. 15, 185.
Kawamura, K., Cheng, Y., Suzuki, N., Deguchi, M., Sato, Y., Takae, S., Ho, C.H., Kawamura, N., Tamura, M., Hashimoto, S., Sugishita, Y., Morimoto, Y., Hosoi, Y., Yoshioka, N., Ishizuka, B., Hsueh, A.J., 2013. Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment. Proc. Natl. Acad. Sci. U.S.A. 110, 17474–17479.
La Marca, A., Sighinolfi, G., Radi, D., Argento, C., Baraldi, E., Artenisio, A.C., Stabile, G., Volpe, A., 2010. Anti-Mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART). Hum. Reprod. Update 16, 113–130.
Lerer-Serfaty, G., Samara, N., Fisch, B., Shachar, M., Kossover, O., Seliktar, D., Ben-Haroush, A., Abir, A., 2013. Attempted application of bioengineered/biosynthetic supporting matrices with phosphatidylinositol-trisphosphate-enhancing substances to organ culture of human primordial follicles. J. Assist. Reprod. Genet. 30, 1279–1288.
Ludeman, S.M., 1999. The chemistry of the metabolites of cyclophosphamide. Curr. Pharm. Des. 5, 627–643.
Meirow, D., 2000. Reproduction post-chemotherapy in young cancer patients. Mol. Cell. Endocrinol. 169, 123–131.
Meirow, D., Nugent, D., 2001. The effects of radiotherapy and chemotherapy on female reproduction. Hum. Reprod. Update 7, 535–543.
Meirow, D., Dor, J., Kaufman, B., Shrim, A., Rabinovici, J., Schiff, E., Raanani, H., Levron, J., Fridman, E., 2007. Cortical fibrosis and blood vessels damage in human ovaries exposed to chemotherapy. Potential mechanisms of ovarian injury. Hum. Reprod. 22, 1626–1633.
Meirow, D., Biederman, H., Anderson, R.A., Wallace, W.H.B., 2010. Toxicity of chemotherapy and radiation on female reproduction. Clin. Obstet. Gynecol. 53, 727–739.
Mitchison, T.J., 2012. The proliferation rate paradox in antimitotic chemotherapy. Mol. Biol. Cell 23, 1–6.
Morgan, S., Lopes, F., Gourley, C., Anderson, R.A., Spears, N., 2013. Cisplatin and doxorubicin induce distinct mechanisms of ovarian follicle loss; imatinib provides selective protection only against cisplatin. PLoS ONE 8.
Oktem, O., Oktay, K., 2007. A novel ovarian xenografting model to characterize the impact of chemotherapy agents on human primordial follicle reserve. Cancer Res. 67, 10159–10162.
Perez, G.I., Knufdon, C.M., Leykin, L., Korsmeyer, S.J., Tilly, J.L., 1997. Apoptosis associated signaling pathways are required for chemotherapy mediated female germ cell destruction. Nat. Med. 3, 1228–1232.
Petrillo, S.K., Desmeules, P., Truong, T.Q., Devine, P.J., 2011. Detection of DNA damage in oocytes of small ovarian follicles following phosphoramide mustard exposures of cultured rodent ovaries in vitro. Toxicol. Appl. Pharmacol. 253, 94–102.
Plowchalk, D.R., Mattison, D.R., 1991. Phosphoramide mustard is responsible for the ovarian toxicity of cyclophosphamide. Toxicol. Appl. Pharmacol. 107, 472–481.
Raz, A., Fisch, B., Okon, E., Feldberg, D., Nitke, S., Raanani, H., Abir, R., 2002. Possible direct toxicity effects of cyclophosphamide on cultured human follicles: an electron microscopy study. J. Assist. Reprod. Genet. 19, 498–504.
Schmidt, K.L., Byskov, A.G., Nyboe-Andersen, A., Muller, J., Yding-Andersen, C., 2003. Density and distribution of primordial follicles in single pieces of cortex from 21 patients and in individual pieces of cortex from three entire human ovaries. Hum. Reprod. 18, 1158–1164.
Schmidt, K.L., Kryger-Baggesen, N., Byskov, A.G., Andersen, C.Y., 2005. Anti-Müllerian hormone initiates growth of human primordial follicles in vitro. Mol. Cell. Endocrinol. 234, 87–93.
Sladek, N.E., Powers, J.F., Grage, G.M., 1984. Half-life of oxazaphosphorines in biological fluids. Drug Metab. Dispos. 12, 553–559.
Tilly, J., 1996. Apoptosis and ovarian function. Rev. Reprod. 1, 162–172.
Xu, M., Barrett, S.L., West-Farrell, E., Kondapalli, L.A., Kiesewetter, S.E., Shea, L.D., Woodruff, T.K., 2009. In vitro grown human ovarian follicles from cancer patients support oocyte growth. Hum. Reprod. 24, 2531–2540.