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ARTICLE

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

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Yechezkel Lande MD graduated with honours from the Sackler Faculty of Medicine, Tel-Aviv University, Israel. Dr Lande completed his residency in Obstetrics and Gynaecology, with honours, at the Rabin Medical Center. He participated in infertility studies, and is active in the training of medical students in the field of Obstetrics and Gynaecology, delivering many lectures to students. Dr Lande is currently performing basic science research on the effect different compounds have on ovarian follicles in the fertility preservation programme and research laboratory at the infertility and IVF unit, Beilinson Women's Hospital, Rabin Medical Center, Israel.

Abstract 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 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.

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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 preantral 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

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