Does controlled ovarian stimulation prior to chemotherapy increase primordial follicle loss and diminish ovarian reserve? An animal study

E. Maman, K. Prokopis, J. Levron, A. Carmely, J. Dor, and D. Meirow

IVF Unit, Division of Obstetrics and Gynecology, Chaim Sheba Medical Center, Tel-Hashomer, Israel

1Correspondence address. Tel: +972-3-5302882; Fax: +972-3-5341589; E-mail: meirow@post.tau.ac.il

INTRODUCTION

Background:
Storage of embryos for fertility preservation before chemotherapy is widely practiced. For multiple oocyte collection, the ovaries are hyperstimulated with gonadotrophins that significantly alter ovarian physiology. The effects of ovarian stimulation prior to chemotherapy on future ovarian reserve were investigated in an animal model.

Methods:
Cyclophosphamide (Cy) in doses of 0, 50 or 100 mg/kg was administered to 38 adult mice (control, unstimulated). A second group of 12 mice were superovulated with equine chorionic gonadotrophin (eCG, 10 IU on Day 0) before Cy administration; hCG (10 IU) was administered (Day 2) followed by 0, 50 or 100 mg/kg Cy (Day 4). In both groups ovaries were removed, serially sectioned (7-day post-Cy), primordial follicles were counted and differences between groups evaluated.

Results:
Follicle number dropped from 469 ± 24 (mean ± SE) to 307 ± 27 and 234 ± 19 with 50 or 100 mg/kg Cy, respectively (P < 0.0001). In the eCG pretreated group, follicle count dropped from 480 ± 31 to 345 ± 16 and 211 ± 26 when 50 or 100 mg/kg Cy were administered (P < 0.0001). There were no significant differences in follicle count between the pretreated eCG group and controls for each chemotherapy dose.

Conclusions:
This animal study indicates that ovarian stimulation before administration of Cy does not adversely affect ovarian reserve post-treatment. These results provide support for the safety of fertility preservation using ovarian stimulation and IVF–embryo cryopreservation procedures prior to chemotherapy.

Key words: fertility preservation / chemotherapy / IVF / ovarian stimulation / primordial follicles

Introduction
Young patients treated with chemotherapy may suffer from treatment related ovarian damage (Chemaitilly et al., 2006; Brydøy et al., 2007). Chemotherapy compromises future fertility and can result in immediate ovarian failure post-treatment (Meirow, 2000; Lobo, 2005). For patients who retain ovarian function after completion of cancer treatment premature menopause is common (Byrne et al., 1992; Sklar, 2005; Wallace et al., 2005). Moreover, restoration of menstrual cycles post-chemotherapy does not ensure normal fertility due to low-ovarian reserve.

The group of alkylating agents is one of the mainstays of chemotherapy regimens, cyclophosphamide (Cy) in particular, which is commonly used in the treatment of cancer and autoimmune diseases. Cy is toxic to the ovaries and reduces primordial follicle reserve, as demonstrated in clinical studies and animal experiments.
(Warne et al., 1973; Plowchalk and Mattison, 1992; Meirow and Nugent, 2001; Desmeules and Devine, 2006; Oktem and Oktay, 2007). In a previous study using a rodent model, we have shown that the effect of chemotherapy on the ovary is not an ‘all or none’ phenomenon; the total number of follicles remaining in both ovaries is inversely related to Cy dosage (Meirow et al., 1999).

Studies that investigated the mechanisms responsible for follicle loss following exposure to chemotherapy have shown apoptosis in follicles. TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling) staining of rodent ovaries exposed to chemotherapy demonstrated apoptotic granulosa cells and germ cells (Perez et al., 1997; Tsai-Turton et al., 2007). Human ovaries exposed to chemotherapy in vitro also showed apoptosis of follicles (Meirow, 2000). In addition, chemotherapy has an effect on the whole ovary and not only directly on follicles. Human ovaries exposed to non-sterilizing doses of chemotherapy showed damage and obstruction of cortical blood vessels, areas of cortical focal fibrosis and disappearance of primordial follicles (PMF) in those areas (Meirow et al., 2007).

Subjects at risk of developing complete ovarian failure or partial ovarian damage due to significant follicle loss may benefit from fertility preservation techniques prior to chemotherapy administration. Various strategies of fertility preservation are used depending on the risks and probabilities of gonadal failure, the patient’s general health at diagnosis and the partner’s status (Meirow, 1999; Falcone and Bedaiwy, 2005). These strategies include IVF and embryo cryopreservation, ovarian tissue cryopreservation, oocyte freezing, in vitro maturation and hormonal manipulation. The most readily available method today is IVF and embryo cryopreservation, which is a standard established procedure with predictable and well-documented outcomes in terms of pregnancies and childbirth rates (Lee et al., 2006). The necessity to start chemotherapy early is a time limiting factor—hence patients start chemotherapy treatments only a few days after oocyte collection—in many cases even within 24 h.

To preserve future fertility, storage of a significant number of embryos is indicated, thus the ovaries are stimulated with gonadotrophins to enable recruitment and growth of multiple follicles. The volume of hyperstimulated ovaries is significantly larger than quiescent ovaries and blood flow to the whole ovary is significantly increased (Engmann et al., 1999; Pan et al., 2003). Administration of chemotherapy at the hyperstimulated stage might theoretically lead to a greater delivery of toxic agents to the ovaries. However, the question that deals with the extent of chemotherapy-induced ovarian damage post-ovarian stimulation has not been investigated. There are no data to indicate whether chemotherapy-induced ovarian damage is altered when enlarged hyperstimulated ovaries with elevated blood supply are exposed to cytotoxic agents. Neither are there data to show whether ovarian stimulation (for IVF) prior to chemotherapy treatment has an effect on ovarian reserve (PMF stockpile) post-treatment. Nevertheless, IVF and embryo cryopreservation just prior to chemotherapy are commonly practiced in cancer patients. The aim of this study was to investigate the effects of ovarian stimulation before administration of Cy on future ovarian reserve, using a rodent model.

Materials and Methods

The study was performed using young, highly inbred BALB/c adult female mice (Harland Laboratories, UK), 6–7 weeks of age (50 animals in total) weighing ~25 g that were kept under specific pathogen-free conditions. Ethical approval for animal experimentation was received from the institutional Ethical Committee.

The first group (control, unstimulated n = 38) was given a single i.p. injection of Cy (CAS no. 6055–19–2, Sigma, St Louis, MO, USA) dissolved in phosphate-buffered saline (PBS), at doses of 100 mg/kg body weight (n = 8 mice) or 50 mg/kg body weight (n = 12 mice) or PBS vehicle alone (n = 18 mice). The doses of Cy that were used in this study were chosen according to previous animal studies indicating significant measurable loss of the PMF with increasing doses of Cy. However, these Cy doses did not cause complete ovarian atrophy and were tolerated by the treated animals (Meirow 1999; Meirow et al., 2004). The second group of mice (n = 12) was superovulated with an i.p. injection of 10 IU equine chorionic gonadotrophin (eCG; Sigma) followed by 10 IU of hCG after 48 ± 2 h (Sigma). On the 4th day post-eCG administration, a single i.p. injection of 50 mg/kg Cy (n = 4), 100 mg/kg Cy (n = 4) or PBS alone (n = 4) was administered. The treatment protocol and the time frame of the experiment are presented in Fig. 1.

Seven days after Cy (or vehicle) injection, all mice were sacrificed and both ovaries were removed for evaluation. The ovaries were fixed in 4% paraformaldehyde, embedded in paraffin and serially sectioned into 5 μm slices. During dissection and laboratory preparation care was taken to ensure that all the tissue from the ovaries was completely removed and prepared for histological evaluation to allow counting of the entire PMF population.

Sections were stained with hematoxylin–eosin and the number of PMF was counted in every 5th section. A primordial follicle was defined as a sphere with a single layer of flattened squamous epithelial cells, a single layer of follicular cells and an intact nucleus surrounded by a single layer of flattened squamous epithelial cells, with a single layer of follicular cells, a single layer of flattened squamous epithelial cells, with a single layer of flattened squamous epithelial cells. The diameters of the follicular cells were measured using a microscope equipped with an eyepiece graticule. The mean diameter of the follicular cells was calculated as the average of the diameters of 25 follicles.

The second group of mice (n = 12) was superovulated with an i.p. injection of 10 IU equine chorionic gonadotrophin (eCG; Sigma) followed by 10 IU of hCG after 48 ± 2 h (Sigma). On the 4th day post-eCG administration, a single i.p. injection of 50 mg/kg Cy (n = 4), 100 mg/kg Cy (n = 4) or PBS alone (n = 4) was administered. The treatment protocol and the time frame of the experiment are presented in Fig. 1.

Seven days after Cy (or vehicle) injection, all mice were sacrificed and both ovaries were removed for evaluation. The ovaries were fixed in 4% paraformaldehyde, embedded in paraffin and serially sectioned into 5 μm slices. During dissection and laboratory preparation care was taken to ensure that all the tissue from the ovaries was completely removed and prepared for histological evaluation to allow counting of the entire PMF population.

Statistical analysis

The results of PMF counting in each group were presented as mean ± SE.

One- and two-way analyses of variance with Bonferroni correction were performed for statistical analysis of the different groups of Cy treatments. A parametric Mann–Whitney test was used to compare pairs of equivalent doses of Cy with and without eCG.

Results

All 50 female mice that started the experiment survived were equally active and gained weight. The total number of PMF counted in the different groups of Cy treatment indicated a significant decrease in the primordial follicle pool, which correlated with increasing Cy dose. The number of PMF without Cy was 469 ± 24 and decreased to 307 ± 27 and 234 ± 19 with treatment with 50 mg/kg Cy and 100 mg/kg, respectively. The decrease in the number of PMF with increasing Cy dose was highly significant (P < 0.0001). The group of mice pretreated with eCG also showed a significant decrease in PMF count after Cy administration in a dose–response manner: without Cy 480 ± 31, 345 ± 16 when treated with 50 mg/kg Cy and 211 ± 26 post 100 mg/kg Cy (P < 0.0001).

Ovarian stimulation did not alter primordial follicle count by the observer as there were no significant differences between hyperstimulated and unstimulated ovaries in the pair groups not exposed to
chemotherapy. Statistical analysis indicated that there were no significant differences between the hyperstimulated and unstimulated ovaries exposed to 50 and 100 mg/kg Cy (Fig. 2).

Discussion

The cytotoxic agent Cy causes ovarian damage manifested by depletion of the primordial follicle stockpile. The response is dose related—the higher the dose administered the lower the remaining ovarian follicle reserve (Meirow et al., 1999). The mechanisms of ovarian follicle destruction, as shown in animal and human studies, suggest a direct toxic effect of chemotherapy on follicles (Perez et al., 1997), and injury to the entire ovary resulting in blood vessel occlusion, focal cortical fibrosis and disappearance of PMF from fibrotic zones (Meirow et al., 2007). Following the eCG stimulation protocol used here, the volume of ovaries at the time of Cy administration was 10-fold more than the volume of quiescent ovaries and histology showed congestion of blood vessels: these physiological alterations were shown in ovaries removed on the same day as Cy administration following the stimulation protocol (an additional four animals, data not shown). Ovarian stimulation to induce ovulation induction as practiced in IVF protocols significantly alters ovarian physiology, as ovarian volume and blood flow are dramatically increased (Pan et al., 2003). Hence, it was

![Figure 1](https://example.com/figure1.png)

**Figure 1** Study design: a group of mice (n = 12) was superovulated with i.p. injection of 10 IU equine chorionic gonadotrophin (eCG, Day 0), followed by 10 IU of hCG (Day 2). On the 4th day post-eCG administration, a single i.p. injection of cyclophosphamide (Cy) 50 or 100 mg/kg or only PBS was administered. The control unstimulated group was given a single i.p. injection of Cy 50 or 100 mg/kg or only PBS on the same day as the superovulated group. From both groups, ovaries were removed and sectioned for primordial follicle count at 7-day post-Cy administration.

![Figure 2](https://example.com/figure2.png)

**Figure 2** Comparison of ovarian reserve (as represented by primordial follicle count) between the two groups of mice: those superovulated (hyperstimulated) before chemotherapy administration and the non-stimulated group. There were no significant differences between the hyperstimulated and non-stimulated ovaries exposed to 0 (control), 50 or 100 mg/kg Cy.
hypothesized that with hyperstimulation prior to chemotherapy the extent of ovarian damage and the number of follicles lost will be affected. One hypothesis was that ovarian reserve might be lower due to more chemotherapy reaching the ovary causing increased follicle destruction. On the other hand, an alternative hypothesis suggested that increased blood flow might prevent obliteration of blood vessels and fibrosis, thus reducing the number of follicles lost after chemotherapy.

Our results clearly indicated a significant decrease in primordial follicle number with increasing Cy dose. The reduction of the primordial follicle pool was documented in ovaries exposed to chemotherapy under physiological conditions as well as in the ovaries that were hyperstimulated prior to Cy administration. Comparison between superovulated and non-superoovulated ovaries indicated that at each Cy dose administered, there was no significant difference in ovarian reserve post-treatment between the two groups. The number of residual follicles was similar in both groups.

The results of this study suggest that in the mouse model ovarian stimulation before Cy administration will not change the extent of damage to the follicles and will not reduce ovarian reserve post-treatment. Further studies on the mechanism of follicle destruction by chemotherapy are needed in order to shed more light on these results.

Over the years, cancer treatment in young patients has achieved increasing cure rates, hence it is important to improve the quality-of-life of cancer survivors. As cancer treatment often poses a threat to fertility, and the ability to have their own, biological children is of great importance to many patients, the field of fertility preservation has become an essential step in all treatment plans for young cancer patients.

The most readily available method today for fertility preservation is IVF and embryo cryopreservation (Oktay et al., 2005; Lee et al., 2006). One of the options is to perform a natural cycle IVF (Brown et al., 1996), however, the likelihood of fertility and live birth due to freezing of only one embryo is very low. To increase the odds for future fertility, the ovaries are stimulated with FSH to induce ovulation induction, multiple oocytes are collected and fertilized and several embryos are stored for future use. In most cases, the chemotherapy protocol is initiated shortly following the fertility preservation procedure.

There are several problems and dilemmas in IVF–embryo cryopreservation fertility preservation (Oktay and Meirow, 2007): first the need for a male partner or the necessity for donor sperm; the second problem is the time required to accomplish fertility treatment (usually a few weeks) with the resultant delay in initiating chemotherapy (Stern et al., 2006). Thirdly, for women with hormone-sensitive tumors, alternative hormonal stimulation protocols (Oktay et al., 2006) are indicated to reduce the potential risk of estrogen exposure.

The question that deals with the extent of chemotherapy-induced ovarian damage post-ovarian stimulation has been investigated in this study for the first time.

The results of this animal study show that ovarian stimulation before administration of Cy does not adversely affect ovarian reserve post-treatment. These results provide support for the safety of fertility preservation procedures (IVF–embryo cryopreservation) in cancer patients undergoing ovarian stimulation prior to chemotherapy administration.

References


Submitted on June 25, 2008; resubmitted on July 27, 2008; accepted on July 30, 2008.