Subclinical depletion of primordial follicular reserve in mice treated with cyclophosphamide: clinical importance and proposed accurate investigative tool

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Studies have shown that ovarian failure is a common side-effect of chemotherapy treatment; however, continuation of regular menses post-treatment does not necessarily imply that the ovaries have escaped damage. This animal study measures directly the primordial follicle (PMF) loss following exposure to chemotherapy and evaluates reproductive outcome following significant destruction of the PMF population. Inbred Balb/c mice aged 5–6 weeks were administered different doses of an alkylating agent, cyclophosphamide, and the total number of PMF remaining in both ovaries was counted. Results show that cyclophosphamide causes PMF destruction in proportion to increasing dose (P = 0.0001). Reproductive performance was assessed after exposure to 75 mg/kg cyclophosphamide, a dose which destroys ~50% of PMF reserve, by evaluation of ovulation, mating and pregnancy rates. Reproductive potential of treated mice was not affected compared with controls despite the significant loss of PMF. Our results indicate that reproductive performance is not an accurate parameter for assessing ovarian injury. Rather, histological counting of PMF number more directly reflects the damage caused by chemotherapy to the ovary. This method can be used as a sensitive, inexpensive tool to gauge the damage to fertility caused by new chemotherapy agents or protocols.

Key words: chemotherapy/mouse/ovarian failure/primordial follicles/reproduction

Introduction

The development and widespread use of high dose chemotherapy and radiotherapy have opened new vistas for long-term survival of young patients with cancer (Byrne et al., 1987; Boring, 1994). Frequently, however, the price that is paid by female patients for successful cancer treatment is ovarian failure and infertility. This is of particular concern because of the young median age of these patients (Horning et al., 1981; Nicholson and Byrne, 1993; Meirow et al., 1997a). A number of studies have used indirect clinical measurements such as menstrual history, endocrine profiles and pregnancy rates to examine the effects of chemotherapy on the ovary, necessarily defining the outcome in dichotomous terms—whether or not ovarian failure occurred. Such clinical studies fail to indicate chemotherapy effects, if any, on patients who continue cycling regularly following therapy. Nor do these studies demonstrate the effects of increasing doses of chemotherapy within non-sterilizing range on the primordial follicle (PMF) population and ovarian reserve. These studies, however, have defined several factors which predispose patients to chemotherapy-induced ovarian failure. Different chemotherapeutic agents have been shown to affect fertility to varying degrees (Damewood and Grochow, 1986), with alkylating agents being among the most damaging (Chapman et al., 1979; Shalet, 1980). The extent of ovarian dysfunction is also determined by the patient’s age and the total dose administered, with older women at an increased risk of chemotherapy-induced ovarian failure even when subjected to comparatively low doses (Kumar et al., 1972; Uldall et al., 1972; Koyama et al., 1977; Fisher et al., 1979; Sanders et al., 1996).

Histological studies of human ovarian tissue, which examined the effects of chemotherapy on human ovaries following treatment, have shown that the end result of therapy is ovarian atrophy with a marked loss of PMF (Warne et al., 1973; Himelstein-Braw et al., 1978; Marcello et al., 1990; Familiari et al., 1993).

Animal studies have demonstrated that irradiation at higher intensities caused depletion of PMF reserve in a dose-related manner (Gosden et al., 1997).

The aims of this study were to analyse the effects of cyclophosphamide administration at different doses on the ovarian PMF population in a uniform highly inbred cohort of young mice, in order to isolate the dose–effect relationship from other confounding factors such as animal age. In addition, the correlation between significant reduction in PMF number post-chemotherapy and reproductive performance was investigated following maturation of the exposed follicle population, as expressed by ovulation, mating and pregnancy rates. Data of this nature reflect directly the relationship between ovarian injury as expressed by clinical observations, and the loss of PMF which was counted directly. This experiment may indicate whether regular ovulation post-treatment attests to the fact that the ovaries remained uninjured despite exposure to chemotherapy.

For this study, the alkylating agent cyclophosphamide was used. Cyclophosphamide is commonly employed in the treatment of many malignancies including lymphomas—Hodgkin’s and non-Hodgkin’s, leukaemias and many solid tumours, and results in a high rate of ovarian failure. Alkylating agents act by transferring alkyl groups to biologically important cellular constituents, in particular alkylation of guanine compound of...
the DNA which results in miscoding, crosslinking and DNA breakage. The doses used in this study were within the regular experimental range used for single dose intraperitoneal injection in animal studies.

Materials and methods

Forty-one inbred Balb/c young mature female mice, aged 5–6 weeks, were used to study the dose related effects of cyclophosphamide on the ovaries of this population. Twenty-nine mice, were given a single i.p. injection of cyclophosphamide at doses of 20 (n = 13), 50 (n = 7), 75 (n = 3) or 100 (n = 6) mg/kg body weight. Twelve control mice were injected with sterile water. Seven days later both ovaries from each animal were removed and fixed in 4% paraformaldehyde in PBS. Ovaries were embedded in paraplast and serially sectioned to 5 μM slices. Care was taken to ensure that both ovaries were removed from each mouse in their entirety for histological processing. The tissues were stained with haematoxylin–eosin, and the ovarian PMF number was counted in every fifth section by one examiner, in order to eliminate observer variation. The numbers were then multiplied by 5 to reach a value representative of the total number of PMF in both ovaries. Primordial follicles were counted when the nucleus was clearly identified surrounded by a single layer of flattened squamous follicular cells. In this species, PMF are located almost exclusively in the ovarian cortex; they are very small (~15 μm diameter) with a single layer of squamous pregranulosa cells without a theca layer (Figure 1). The data were statistically analysed using regression models to determine the variance in the distribution of follicle number.

In the second part of the study, ovarian performance following significant PMF depletion was assessed, as expressed by ovulation, mating and pregnancy rates. One hundred and one young mature female mice, aged 5–6 weeks, were injected i.p. with 75 mg/kg body weight of cyclophosphamide, a dose observed in part one to cause depletion of ~50% of the PMF reserve. Thirty-four control animals were injected with sterile water in similar volumes. The treated mice along with the control mice were mated with proven fertile males at weekly intervals post-treatment: 1 week (n = 27), 2 weeks (n = 29), 3 weeks (n = 30) and 4 weeks (n = 15). In the mouse the time period for maturation of PMF to ovulatory oocytes is 19 days; hence ovulations which occurred 3 or 4 weeks post-treatment represent follicles which were exposed to chemotherapy at the primordial stage. Follicles which ovulate 1 week following exposure to chemotherapy were exposed to chemotherapy at mature stage and follicles which ovulate 2 weeks following chemotherapy represent follicles which were exposed to chemotherapy at growing stage (Figure 2). The mice were examined for 3 days (length of the mouse oestrus cycle) for vaginal plugs, indicating successful mating. Once mating was established, females were separated, and the pregnancies allowed to progress for 12 days before being killed. Females which did not show evidence of mating were separated at the end of the 5 day period, and were killed 9 days later. Both ovaries were removed from each female and examined microscopically to establish the number of corpora lutea. Differences in ovulation, mating and pregnancy between the treated and control groups were assessed with the Kruskal–Wallis ANOVA test.

Ethical approval of animal experimentation was received from the Ethical Committee of Hadassah Medical Center-Hebrew University.

Results

A significant inverse relationship (P < 0.0001) was observed between the dose of cyclophosphamide and the total number of follicles counted in the ovaries. Results indicated that damage occurred at all administered doses of cyclophosphamide, even at the lowest dose of 20 mg/kg. The higher the dose of cyclophosphamide received by the animals, the lower the remaining number of follicles observed (Figure 3). The mean number of primordial follicles in the ovaries decreased as the dose of cyclophosphamide increased, from an average of 2034 (±199 SE) follicles in the control group, to a mean of 907 (±139) in the group which received 75 mg/kg (a reduction of 54%), and a mean of only 630 (±130) in mice subjected to a dose of 100 mg/kg. Linear and non-linear solutions to the regression analysis were investigated...
by examining linear, quadratic, cubic and exponential lines of best fit. The association between dose and follicles number was best expressed by the exponential solution which explained 42% of the variance in follicle number distribution as a function of dosage ($P < 0.0001$).

When Balb-c mice were exposed to 75 mg/kg of cyclophosphamide, a dose which was found to reduce PMF reserve by about half (54%), the reproductive performance expressed by ovulation, mating and pregnancy rates following maturation of exposed PMF was not compromised compared with controls (Figure 4). Similar percentages of mice in each group (between 31 and 40%) did not show corpora lutea, this includes animals that mated without conceiving, as well as those which did not mate at all. Of the animals that did ovulate, there were no significant differences between the average number of corpora lutea observed in treated and control mice, or in pregnancy rates (Table I). However, cyclophosphamide had a deleterious effect on the reproductive outcome of those females treated one week before mating, i.e. of mature follicles exposed to chemotherapy. This group showed significantly fewer number of pregnancies ($P = 0.001$), and appeared to have fewer corpora lutea (but the difference was not significant) with mating rates similar to controls.

**Discussion**

This study of highly inbred young mature mice of the same age shows that PMF destruction occurred at all levels of cyclophosphamide exposure that were tested. The results clearly indicate a relationship between increasing doses and higher portion of follicular destruction. The relationship was best approximated by an exponential curve, however extrapolation shows that the doses at which the loss of PMF would begin to plateau are greatly beyond the therapeutic range. Significant damage to the PMF population resulted even following administration of low doses of cyclophosphamide (20 mg/kg). A study by Gosden and associates on the effects of radiation on the mouse ovary observed a dose-related reduction in PMF reserve which correlates with this study (Gosden et al., 1997).

Clinical studies on the effects of chemotherapy on female fertility which have defined the outcome in dichotomous terms, whether ovarian function was regained or not, have highlighted age as a significant factor in determining the effects of the chemotherapy on subsequent ovarian function. Older women had a much higher incidence of complete ovarian failure and permanent infertility than did younger women (Kumar et al., 1972; Uldall et al., 1972; Koyama et al., 1977; Shalet, 1980; Fisher and Cheung 1984; Sanders et al., 1996).

The young age of the mice used in this study, and the results that indicate follicular destruction at all levels of exposure, suggest that ovarian damage occurs in all age groups and is

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**Table I.** Comparison of reproductive performance, as measured by mating rate, number of corpora lutea (CL) and pregnancies, in control and treated animals mated at 4-weekly intervals. Statistical significance as compared with control values is indicated by * ($P < 0.001$). Other data were not significantly different from control values.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>34</td>
<td>27</td>
<td>29</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>No. (%) of animals without CL</td>
<td>(35)</td>
<td>(37)</td>
<td>(31)</td>
<td>(40)</td>
<td>(33)</td>
</tr>
<tr>
<td>Mean no. of CL in mated females</td>
<td>10.4 ± 0.6</td>
<td>8.1 ± 1.0</td>
<td>11.2 ± 0.4</td>
<td>9.6 ± 0.8</td>
<td>10.4 ± 0.7</td>
</tr>
<tr>
<td>Mean no. of pregnancy sacs/pregnant animal</td>
<td>8.3 ± 0.8</td>
<td>4.8 ± 1.0*</td>
<td>7.4 ± 0.9</td>
<td>7.0 ± 0.8</td>
<td>7.9 ± 1.2</td>
</tr>
<tr>
<td>No. (%) of pregnant animals</td>
<td>21 (61)</td>
<td>12 (44)</td>
<td>18 (62)</td>
<td>17 (56)</td>
<td>9 (60)</td>
</tr>
</tbody>
</table>
not restricted to older females. The clinical observation that older females appear to be more affected by exposure to chemotherapy can be explained by the fact that older females naturally have a smaller ovarian reserve of follicles. At birth in the human female, there are \( \sim 2 \times 10^6 \) follicles in the ovary; however, due to a continual process of atresia this reserve is progressively eroded over time (Gosden and Faddy, 1994). At the age of 45~50 years, the PMF population falls below a key threshold number required for ovarian function, at which point the menstrual cycle ceases and natural menopause occurs. The chemotherapeutic destruction of an already low follicular reserve reduces the number of follicles in the ovary below a certain ‘threshold’ number required to sustain ovarian function, thus resulting in ovarian failure, that is menopause. Thus the depletion of the PMF store, as presented in this study, may explain the higher incidence of ovarian failure in older women treated with chemotherapy.

Several studies have observed that a significant proportion of younger patients who did regain ovarian function after chemotherapy were at risk of undergoing premature menopause a number of years after treatment (Rose and Davis, 1977; Chapman et al., 1979; Byrne et al., 1992; Wallace et al., 1993), indicating that their ovaries sustained damage which became evident years later. It is this particular observation which indicates that the effects of chemotherapy on the ovary can be concealed and require more research beyond the limitation of clinical studies. In young females, with a larger reserve of follicles, the chemotherapeutic-induced loss of PMF may not be proportionally enough to cause immediate ovarian failure. However, the reduction in reserve in addition to natural atretic follicular loss, is the explanation for the increased risk of premature menopause in these patients.

As the results of this study clearly demonstrate, exposure to doses of chemotherapy strong enough to destroy half of the ovarian PMF reserve, does not affect reproductive performance following short term maturation of the exposed primordial follicles (week 3 and 4). There was no influence of treatment on ovulation, mating and pregnancy rates. These results strongly indicate that clinical information such as regular menses and normal reproductive outcome after chemotherapy are not reassuring parameters from which it is possible to conclude that the ovaries survived treatment unaffected (Figure 4).

The results of this study support the recommendation that patients who regain ovarian function post-radiotherapy or chemotherapy treatments should not delay child bearing, given the very real risk of premature ovarian failure. An alternative is to try and preserve the future fertility of young cancer patients through cryopreservation and storage of ovarian tissue, containing many PMF, or embryos prior to radiochemotherapy treatments and consequent PMF destruction.

The significant reduction in the number of pregnancy sacs and the reduced number of corpora lutea that were observed in females mated 1 week following the chemotherapy injection, might suggest that mature follicles are more sensitive to chemotherapy-induced damage. Alternatively, this short interval of time between exposure to cyclophosphamide and conception may in itself inhibit conception due to general effects of the toxin on the mother, in particular on uterine receptivity. These results require further investigation, and are in accordance with observations made by other studies (Kuhajda et al., 1982; Familiari, 1993; Gosden et al., 1997).

The direct mechanisms of PMF destruction by chemotherapy are unclear. A possible explanation is that the drug induces apoptosis in the supporting granulosa cells of the follicle, without which it cannot survive. In-vitro studies on human ovarian tissue show that granulosa cells undergo apoptosis as a result of exposure to chemotherapy (Meirow et al., 1997b). Future research is needed to confirm the exact mechanisms of PMF destruction and to determine the apoptotic pathways induced by chemotherapy.

New treatment regimens have and will continue to emerge, and will continue to increase survival rates among young cancer patients. The described animal model may serve as a more accurate tool to study the effects of existing and newly developed chemotherapeutic agents, as well as different protocols, on the gonads. Currently, the standard method for assessing the toxic effects of a new agent on fertility is to check the ability of treated females to conceive and carry pregnancies post-treatment. As this study has shown, conception and pregnancy are not accurate parameters by which to assess the damage caused by a drug to the ovary. Histological counting of primordial follicle number, as described in this study, reflects directly the effects of chemotherapy on the ovaries. This animal assay is a sensitive and inexpensive method of gauging and comparing the damage to fertility caused by new agents or combination of agents, and determining the least toxic agent or protocol before it is used in a clinical setting. This method can also be used to evaluate the practical effectiveness of proposed protectants, such as gonadotrophin-releasing hormone antagonists (Gosden et al., 1997) or antioxidants in circumventing chemotherapy induced gonadal failure.

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References

Primordial follicle loss following chemotherapy


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