Experimental Researces

Prevention of cyclophosphamide-induced ovarian damage by concomitant administration of GnRHa in mice: A dose-dependent relationship?

M.A. Yüce[1], *M.D.*; **P. Balkanlı Kaplan**[1], *M.D.*; **F. Gücer**[1], *M.D.*; **L. Doğanay**[2], *M.D.*; **S. Altaner**[2], *M.D.*; **T. Canda**[1], *M.D.*; **T. Yardım**[1], *M.D.*

Departments of Obstetrics and Gynecology[1] and Pathology[2], Faculty of Medicine, University of Trakya, Edirne (Turkey)

Summary

Objective (s): This experimental study investigates the dose-related effects of cyclophosphamide (Cy) on primordial follicular reserve in young mice, and examines whether the concomitant administration of a gonadotropin-releasing hormone agonist (GnRHa) may protect gonadal reserve, even at different doses of Cy.

Methods: Forty sexually mature virginal Balb/c mice aged five to six weeks were administered different doses (0, 50, 75,100 mg/kg) of Cy. Another 40 animals were treated with increasing doses (0, 50, 75, 100 mg/kg) of Cy in combination with GnRHa. GnRHa treatment was initiated one week prior to chemotherapy and also continued after chemotherapy for one week. The ovaries were removed seven days after Cy administration and the total number of primordial follicles in both ovaries was counted.

Results: Primordial follicular destruction occurred at all levels of Cy exposure. There was a positive correlation between increasing doses of Cy and higher proportion of follicular loss ($p < 0.0001$). GnRHa was not able to protect against the chemotherapy-induced negative effect on primordial follicular count at low doses (50 mg/kg and 75 mg/kg). Mean ± SD primordial follicle count in the 100 mg/kg Cy-treated group was significantly lower than in the 100 mg/kg Cy + GnRHa treatment group (73.9 ± 33.1 vs 89 ± 17.9, $p = 0.047$).

Conclusion: Our data suggest a possible ovarian protective effect of GnRHa cotreatment only at high doses of Cy treatment. However, in spite of co-administration of GnRHa, loss of primordial follicular reserve occurred at all doses of Cy in mice.

Key words: Chemotherapy; GnRHa; Ovarian toxicity; Premature ovarian failure; Primordial follicles; Cyclophosphamide; Primordial follicular reserve.

Introduction

With novel advances in cancer therapy, increasing numbers of cancer patients are achieving long-term survival. One of the more commonly recognized adverse effects of anti-cancer treatment is ovarian failure as a result of depletion of the number of primordial follicles (PMF) leading to premature ovarian failure (POF) [1]. Whereas the chemotherapy-induced damage is reversible in rapidly dividing cells, it seems to be irreversible in the ovary, where the number of germ cells is limited, fixed since the fetal stage, and cannot be regenerated [2].

A few alternatives have been attempted for preservation of fertility in young women receiving chemotherapy. An attempt was made to minimize the gonadotoxic effect of chemotherapy by co-treatment with a gonadotropin-releasing hormone agonist (GnRHa) to induce a temporary prepubertal milieu.

Cyclophosphamide (Cy), an alkylating agent, is commonly employed in the treatment of many malignancies and results in a high rate of gonadal damage [3-5]. The aim of the present study was to evaluate the dose-related effects of Cy on the primordial follicular reserve in young mice, and to investigate whether the concomitant administration of GnRHa may protect gonadal reserve, even at different doses of Cy.

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Materials and Methods

Animals

Adult female inbred mice aged five to six weeks, weighing 20-25 g were fed mice chow and water ad libitum and housed 10/cage under controlled temperature (20-22°C) and light (12-h light, 12-h dark). The guidelines for the care and use of the animals approved by the local institution were followed.

Chemicals

Cy for injection was donated by Endoxan (Hoechst Marion Roussel Ltd, Astra Medica, AG, Frankfurt, Germany). It was prepared in 0.9% NaCl solution. GnRHa was supplied by Abbott France S.A. (Lucrin daily, Saint remy. Sur Avre., France) and was dissolved in 0.9% NaCl solution for injection.

Treatment Regimens

Experiment I (only Cy treatment protocol): Forty inbred Balb/c mature female mice aged five to six weeks were randomly divided into four groups of ten mice each. Cy was administered intraperitonealy (IP) in normal saline using a sterile technique as a single injection at doses of 50, 75 or 100 mg/kg in three groups. Ten control mice received only sterile saline in the same volume. The length of the mouse estrous cycle is approximately five days. Therefore, to evaluate the toxic effect of Cy in the subsequent cycle, ovaries were removed seven days after Cy administration. According to the demonstrated primordial follicular toxicity in mice [6], 50, 75, 100 mg/kg doses of Cy were chosen for the study.

Experiment II (GnRHa + Cy treatment protocol): A second experiment was conducted to assess the protective effect of GnRHa on chemotherapy-induced primordial follicular destruction. It has been shown that suppression of ovarian mitotic activity occurs within five days of GnRHa treatment [7]. Treatment with GnRHa was initiated one week prior to Cy administration and also continued after chemotherapy. Forty mice were divided into four groups of ten animals each. Group I (GnRHa only) received daily subcutaneous injections of 0.1 mg/kg GnRHa solution for seven days and then a single injection of IP saline. Group II (GnRHa +Cy 50 mg/kg), Group III (GnRHa + Cy 75 mg/kg) and Group IV (GnRHa + Cy 100 mg/kg) received the same GnRHa treatment as Group I, and on the seventh day of GnRHa injection, a single IP injection of Cy at increasing doses of 50, 75 and 100 mg/kg, respectively. GnRHa administration was continued after Cy for seven days. Ovaries were removed seven days after Cy administration.

Histological examination: Ovaries were fixed in 10% formalin and embedded in paraffin. Serial sections were prepared in 5-mm slices and stained with hematoxylin and eosin. Care were taken to ensure that both ovaries were removed from each mouse in their entirety for histological processing. Although primordial follicle toxicity can be adequately established by counting five random sections [8], the primordial follicles were counted in every fifth section. Therefore, the presented numbers are not absolute total primordial follicle numbers, but are relative to the counting procedure. Moreover comparable primordial follicular numbers in control groups have been found in other reports and our previous work [6, 9]. Primordial follicles were identified when the nucleus was clearly identified surrounded by a single layer of flattened squamous pregranulosa cells without a theca layer.

The differences in the mean number of primordial follicular counts among the study groups were compared by one-way analysis of variance (ANOVA) using SPSS software (version 9.0, Statistical Package for Social Sciences, Inc., Chicago, IL). Regression models were used to analyze the relationship between the dose of Cy with or without GnRHa co-administration and primordial follicular counts; p values < 0.05 were considered statistically significant.

Results

Mean ± standard deviation (SD) number of primordial follicular counts in the study groups are shown in Table 1. The number of PMF decreased in all groups that received Cy with or without GnRHa co-administration (Table 1 and Figure 1). Mean + SD number of PMF in the control group of only the Cy treatment protocol and in group I (GnRHa + 0mg/kg Cy) of the GnRHa + Cy treatment protocol were 215.2 ± 54.7 and 194.8 ± 42.9, respectively (p > 0.05).

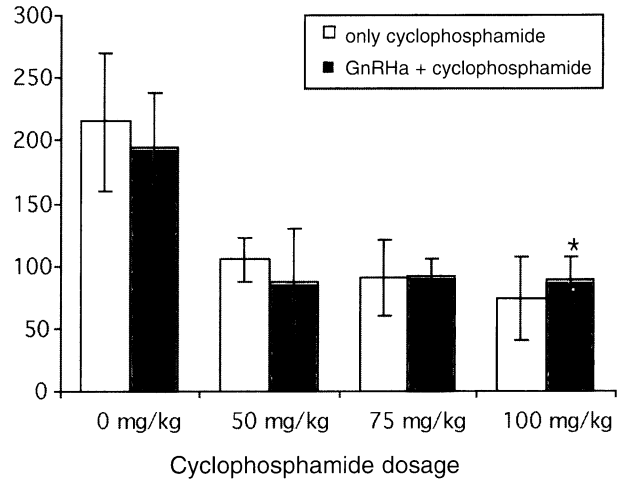

Figure 1. — Mean number (bars) and standard deviation (lines) of total counted primordial follicles in ovaries of mice treated with cyclophosphamide at doses of 0, 50, 75, 100 mg/kg with or without GnRHa co-administration. * denotes statistically significant difference compared to the only 100 mg/kg cyclophosphamide group (p = 0.047).

If the control group (0 mg/kg Cy) was excluded from statistical analysis of experiment I (only Cy treatment protocol), there was no statistically significant difference with respect to mean number of primordial follicular counts between the 50, 75, 100 mg/kg Cy treatment groups. Also, similarly, there was no statistically significant difference in the mean number of PMF among groups II, III, and IV in the GnRHa + Cy treatment protocol (experiment II), if group I (GnRHa + 0mg/kg Cy) was excluded from statistical analysis.

Comparing both treatment protocols – depending on Cy doses at 0, 50, 75 mg/kg – there was no significant difference in the mean number of PMF between Cy only (experiment I) and Cy + GnRHa (experiment II) groups. However, the mean number + SD of PMF in 100 mg/kg Cy + GnRHa group was significantly higher than the 100 mg/kg Cy group (89 ± 17.9 vs 73.9 ± 33.1, p = 0.047).

Table 1. — *Mean ± standard deviation (SD) of primordial follicles in ovaries of mice treated with cyclophosphamide at doses of 0, 50, 75, 100 mg/kg with or without GnRHa co-administration.*

	Only cyclophosphamide (Cy) Protocol				GnRHa+Cyclophosphamide (Cy) Protocol			
	0 mg/kg Cy	50 mg/kg Cy	75 mg/kg	100 mg/kg Cy	GnRHa+ 0 mg/kg Cy	GnRHa+ 50 mg/kg Cy	GnRHa+ 75 mg/kg	GnRHa+ 100 mg/kg
Mean ± SD number of primordial follicles	215.2 ± 54.7 (controls)	104.5 ± 17.3[a]	89.6 ± 30.2[a]	73.9 ± 33.1[a]	194.8 ± 42.9[b]	86.9 ± 42.8[b]	92 ± 13.9[b]	89 ± 17.9[b, c]

[a]Statistically significant difference comparing the number of primordial follicles of the 0 mg/kg group using only the cyclophosphamide protocol.
[b]Statistically significant difference comparing the number of primordial follicles of the GnRHa + 0 mg/kg cyclophosphamide group with the GnRHa + cyclophosphamide protocol.
[c]Statistically significant difference comparing the number of primordial follicles of only the 100 mg/kg cyclophosphamide group (p = 0.047).

For both treatment protocols, linear regression analysis showed a statistically significant inverse relationship between the increasing doses of Cy and primordial follicular counts, independent of co-administration of GnRHa (for both protocols, ANOVA, $p < 0.0001$).

Discussion

In our study, primordial follicular loss occurred at all administered doses of Cy, even at the lowest dose of 50 mg/kg. Also, linear regression analysis showed a statistically significant inverse relationship between the dose of Cy and the number of PMF counted in the ovaries. Our results in mice are in line with other animal studies [6, 10, 11] in that the higher the dose of Cy, the lower the remaining number of PMF in the ovaries. However, there are some controversial experimental studies on the effects of chemotherapy on ovaries and the use of GnRHa co-administration for preservation of ovarian function. Jarrell *et al.* [12] claimed that the acute administration (single dose) of Cy did not cause loss of PMF in rat ovaries. Studies in multiple dose Cy-treated rats showed that administration of Cy induced a significant reduction in the total number of follicles and this reduction was due to reduction in the number of medium to large follicles without significantly affecting the number of small follicles [2, 7]. In contrast to these findings, as in our study, other authors have reported a significant loss of primordial follicular reserve in response to Cy administration in mouse models. Mattison *et al.* [11] found a 63% reduction in the number of PMF in the ovaries of mice treated with a single dose of 100 mg/kg (IP) Cy injection. Also, Meirow *et al.* [6] reported that when Balb/c mice were exposed to a single injection of Cy at a dose of 75 mg/kg, a reduction of primordial follicular reserve by about half (54%) occurred. According to the above-mentioned investigations, PMF of rats do not appear to be as sensitive as to Cy as those of mice [2].

Ataya *et al.* [13] reported that chronic GnRHa treatment resulted in a significant suppression of ovarian mitotic activity when given in combination with chemotherapy. Although the mechanism of putative protection is not known, it has been suggested that the administration of GnRH analogues suppresses mitotic activity in the granulosa cells of ovarian follicles [13].

Ataya *et al.* documented improved reproductive performance and a protective effect of GnRHa on Cy-induced ovarian damage measured as pregnancy rate in the rat model [14]. However, the pregnancy rate does not appear to be an accurate marker for assessing ovarian toxicity of chemotherapeutic agents. Meirow *et al.* showed that despite a significant loss of primordial follicular reserve (approximately 50%), the short-term reproductive potential of mice treated with Cy was not affected [6]. This is also consistent with clinical observations that an apparent resumption of ovarian function such as regular menses does not rule out a significant reduction in ovarian reserve and the risk of subsequent premature ovarian failure. The issue is further complicated by the fact that rat oocytes may respond to GnRHa differently from human oocytes due to the various biological differences. Furthermore, Montz *et al.* [15] found a protective effect of GnRHa co-treatment in conjunction with chemotherapy on rat fertility but not on rat fecundity.

Histologic studies examining the effects of chemotherapy on human ovaries have shown that the end result is ovarian atrophy with a marked loss of PMF [16]. Also we found a statistically significant decrease in the mean number of primordial follicles in both Cy only groups and Cy + GnRHa groups. Furthermore, at 0, 50, 75 mg/kg doses of Cy, there was no significant difference in the mean number of PMF between Cy only and Cy + GnRHa groups. On the other hand, there was a statistically significant protective effect of GnRHa at a 100 mg/kg dose of Cy. Our data suggest a possible ovarian protective effect of GnRHa co-treatment only at high doses of Cy treatment (100 mg/kg). This protective effect can be attributed to the strong depletion of primordial follicular reserve at a 100 mg/kg dose in that the difference of mean number of PMF between only the 100 mg/kg Cy group and the 100 mg/kg Cy + GnRHa group reached statistical significance.

To which degree our results would occur in humans and primates is unknown because of the biological differences and controversial presence of GnRH receptors in human ovaries [17, 18]. On the other hand, in rhesus monkeys Ataya *et al.* showed a protective effect of GnRHa on PMF reserve [19].

As in experimental studies, the clinical results of GnRHa co-treatment during chemotherapy for prevention of gonadal damage are controversial. In recent studies, the use of GnRHa in young women as an adjuvant co-treatment before and during chemotherapy for hematological malignancies and systemic lupus erythamatosus has been reported to be quite promising [20, 21]. In Blumenfeld's prospective clinical study [20], 98.4% of the survivors of chemotherapy who received the GnRHa (decapeptyl) co-treatment resumed ovulatory menses, whereas 60% of the patients who were treated with chemotherapy without GnRHa had POF. A recent prospective clinical study by Pereyra Pacheco *et al.* [21] confirmed that GnRHa (leuprolide acetate) treatment before and during chemotherapy enhances ovarian function and preserves fertility. In contrast to these findings, in a randomized controlled study Waxman *et al.* [22] unsuccessfully attempted to protect the ovaries of eight patients receiving chemotherapy by administering intranasal buserelin up to one week before initiation of chemotherapy. Also, invariably occurrence of ovarian failure in patients receiving high dose ablative chemotherapy in conjunction with bone marrow transplantation, irrespective of the suppression of endogenous hormone secretion during chemotherapy, has been also reported [23]. Therefore, it is considered by some authors that there is no conclusive evidence to support the protective effects of GnRHa co-treatment during chemotherapy [24].

Conclusion

Our results show that:

1) the administration of Cy to young mice causes the linear depletion of primordial follicular reserve in a dose-dependent manner, independent of co-administration of GnRHa;

2) GnRHa co-treatment is protective only at high doses of Cy treatment, since stronger depletion of PMF reserve occurs with high doses of Cy;

3) even if GnRHa co-treatment protects PMF from high- dose chemotherapy-induced damage, there is still a risk of premature menopause, since PMF reserve depletion also occurs at high-dose chemotherapy with concomitant GnRHa treatment (more than 50% depletion in our study).

Future research is needed to confirm the exact mechanisms of PMF destruction under chemotherapy treatment. Gonadal protection during chemotherapy is an important theme for the future perspectives of patients, not only for patients with hematological malignancies, but also for patients with gynecological malignancies for whom chemotherapy is an issue, especially for those with unilateral oophorectomy and fertility desire and limited primordial follicular reserve. Ovarian cryopreservation with transplantation of cryopreserved tissue after completion of cancer treatment is another alternative way to preserve ovarian function which is under development. However, this method may involve additional cost and not be available worldwide for all patients. Initiation of GnRHa treatment prior to chemotherapy may have an impact on some patients, at least for a part of them, since the damaging effect of chemotherapy on the PMF reserve seems to be milder.

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Address reprint requests to:
M.A. YÜCE, M.D.
Department of Obstetrics
and Gynecology
Faculty of Medicine Trakya University
22030 Edirne (Turkey)