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Morphological and morphometrical study of cyclophosphamide-induced changes in the ovary and uterus in the Syrian mice

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Summary

The objective of this research was to evaluate the cause of fertility reduction after chemotherapy. Cyclophosphamide is a common chemotherapy and immunosuppressive agent used for the treatment of a wide range of neoplastic and some auto-immune diseases. In the present study, morphometrical changes in the ovary and uterus of 12 5–6-week-old female Syrian mice after intraperitoneal injection of 75 mg cyclophosphamide/kg BW were assessed. Control animals (n = 8) were injected with sterile distilled water using similar method. The results of this study revealed that cyclophosphamide eliminated almost more than 50% of the primordial follicles (PMF) reserve. The mean ± SE number of PMF in the control group was 1210 ± 135 compared to 464 ± 55 in the test group. The mean number of secondary, tertiary follicles and corpora lutea also showed significant ($P<0.05$) reduction in the treatment group. The histomorphometric studies also showed that the mean ± SE diameter of the ovary in the control group was 1703 ± 78 µm as compared to 900.9 ± 86 µm in the test group ($P<0.01$). The thickness of the uterine wall was also significantly different with a mean ± SE of 745.7 ± 13 µm in the control and 393.1 ± 23 µm in the test group. The mean ± SE thickness of endometrium in the control group was 392.1 ± 16 µm, whereas in the test group it was 194 ± 10 µm ($P<0.001$). The results of this study revealed that chemotherapy with cyclophosphamide causes destruction of PMF as well as other growing follicles; accordingly, the reproductive potential was negatively affected. The method used in this study can be most likely used as a sensitive and inexpensive tool to predict the damage to fertility caused by new chemotherapy protocols. In conclusion, chemotherapy brings about a) reduction in ovarian follicular populations, especially PMF, b) it causes reduction in diameter and size of ovary and c) decreases thickness of uterine wall, especially endometrium.

Key words: Cyclophosphamide, Ovarian follicles, Uterus, Endometrium, Mice

Introduction

Studies on chemotherapy treatment have shown that in spite of the increased successes in cancer treatment, it induces gonadal failure in the cancer survivors (Sutton *et al*., 1990; Richman and Green, 2004). Cyclophosphamide is a common chemotherapy and immunosuppressive agent used for the treatment of a wide range of neoplastic and some auto-immune diseases (Pryor *et al*., 2000).

Cyclophosphamide is an effective antimitogenic agent that inhibits mitotic cell division and prevents cancer cell growth. It can be inverted in the liver to alkylating metabolites (Trissel, 1992). Alkylating agents act by transferring alkyl groups to guanine compound of the DNA, which results in miscoding and DNA breakage (Becker and Sconeich, 1982). There has been growing concern about long-term side-effects of this alkylating agent and other antineoplastic drugs (Byrne *et al*., 1987).

These effects may be expressed in the gonads as sterilization or germ cell DNA damage (Mahecha *et al*., 2002). Sterilization may be acute, or identified by the occurrence of premature menopause (Apperley and Reddy, 1995). DNA damage may be identified by an increased risk for chromosomal syndromes, single gene defects or major

congenital malformations in the offsprings (Armitage and Antman, 1992; Green, 1997).

Damage to the gonads by chemotherapy depends on the patient's gender, age at the time of treatment, and total dose and type of chemotherapy delivered (Harrouk *et al*., 2002). Moreover, animals' studies have shown that these alkylating agents could damage the primordial follicles (PMF) population (Meirow *et al*., 2001).

In human, exposure to alkylating agents is associated with premature ovarian failure (Apperley and Reddy, 1995). The degree of ovarian dysfunction seems to be inversely correlated to the age at drug exposure (Byrne *et al*., 1987). Older women had a much higher incidence of complete ovarian failure and permanent infertility than did younger women. Ovarian damage results in both sterilization and loss of hormone production, because ovarian hormonal production is closely correlated to the presence of ova and maturation of the primary follicles. These functions are not as intimately in the testis. As a result, men have normal androgen production in the presence of azoospermia (Mahecha *et al*., 2002). Loss of PMF may not be proportionally enough to cause immediate ovarian failure. The reduction in reserve in addition to the natural atretic follicular loss is the explanation for the increased risk of premature menopause in patients (Kumar *et al*., 1972).

Important landmarks of ovarian development in mice are similar to those in the human; however, the timing is greatly compressed. At birth, the mice ovary consists of cords and oogonia. Primordial follicles are formed by day three of age; well-developed secondary follicles are found by day seven. The regular oestrous cycles continue until around 5–6 weeks of age when the cycles become prolonged and irregular (McGee *et al*., 2000). As the pool of PMF is non-renewable, ovarian life span is tightly limited by the size of the follicle stockpile; the exhaustion of the PMF pool is referred to as reproductive senescence in mammals, termed menopause in women (Kumar *et al*., 1972). The aim of this research was to investigate the effect of cyclophosphamide on ovarian and uterine morphometry.

Materials and Methods

Female mice, 5–6 weeks of age (n = 12), were received single intraperitoneal injection of cyclophosphamide 75 mg/kg body weight (Baxter Oncology GmbH, Frankfurt, Germany). This dose was chosen based on research of Meirow *et al*. (2001). Control animals (n = 8) were injected with sterile water in a similar way. Then, the mice were housed separately. Seven days later, mice were sacrificed with CO_2 gas. Then, both ovaries and uteri from each mouse were removed and fixed in 4% paraformaldehyde in PBS. Ovaries and uteri were embedded in paraffin and serially sectioned at 5-µm. Care was taken to ensure that both ovaries were removed from each mouse entirely for histological processing. These tissues were stained with haematoxylin and eosin. Ovarian follicles were counted in every section by ×400 with graduated lenses devices in each section. The follicles were classified into five types based on the classification of Erickson (2003): a) primordial—containing an oocyte surrounded by a single layer of flattened cells, b) primary—characterized by a single layer of cuboidal pregranulosa cells, c) secondary—containing 2–5 complete layers of granulosa cells, d) tertiary—containing multiple layers of granulosa cells with some small antrum and e) graafian—with the cavity occupying most of the total follicular volume. In the study of follicles, if the oocyte appeared to have a definitive nuclear membrane within the germinal vesicle (GV), they were taken into account and by this technique, each follicle was counted for once; finally, the total number of each follicle types was found out. Then, using histomorphometrical techniques, the diameters of ovary were measured. In the second step of the study, the thickness of uteri and endometrium was also measured. Data were subjected to analysis by SPSS. The Student's t-test conducted to determine probable differences between the test and control groups.

Results

The statistical analysis of total distribu-

tion of follicles in the ovaries revealed that there was significant (P<0.001) differences in number of PMF between the control and test groups. The nuclei of oocytes in these follicles were identified. They were surrounded by a single layer of flattened squamous follicular cells and located almost in the ovarian cortex. They were small (15-µm in diameter) without a theca layer (Fig. 1). The mean ± SE number of PMF in the ovaries of the test group (464 ± 55) was lesser than that in the control group (1210 ± 135) (Table 1).

Fig. 1: Cross section of an ovary of a mouse in the test group. PMF = primordial follicles in extreme cortex. These follicles made up of an oocyte which surrounded by a single layer of flattened squamous pregranulosa cells. Some growing follicles are obvious (PM = primary follicles, SE = secondary follicles) (H&E, ×400)

Thus, when the mice were exposed to 75 mg/kg BW of cyclophosphamide, the PMF reserve was reduced by almost one-half. The mean distribution of secondary and tertiary follicles was also significantly (P<0.01) reduced in the test group (Table 1). Moreover, the mean distribution of corpora lutea was significantly (P<0.05) reduced in the test in comparison to the control group (Table 1). Although there were decreases in the mean number of primary and graafian follicles in the test as compared to the control group, the differences were not of statistical significance.

The histomorphometrical study revealed significant (P<0.001) reduction in the mean ovarian diameter of the test group in comparison to the control group (Table 2). The second part of this investigation revealed that the mean ± SE thickness of uterus was also reduced significantly (P<0.001) from an average of 745.7 ± 13 µm in the control to 393.1 ± 23 µm in the test group. The endometrial thickness was decreased from the mean ± SE of 392.1 ± 16 µm in the control group to 194 ± 10 µm in the test group (Table 2). There was significant (P<0.001) reduction in the uterine thickness as well as endometrial thickness in the test group.

Discussion

We found that reduction of ovarian follicular population was occurred when mice were treated with cyclophosphamide. The results of this investigation clearly indicated that the most important ovarian

Table 1: The mean distributions of the ovarian follicles and corpora lutea in the test and control groups

Group	Follicle					
	Primordial Mean ± SE	Primary Mean ± SE	Secondary Mean ± SE	Tertiary Mean ± SE	Graafian Mean ± SE	Corpora lutea Mean ± SE
Control	1210 ± 135	113 ± 22	50 ± 4	21 ± 2	2 ± 0	8 ± 1
Test	464 ± 55***	90 ± 23	36 ± 2*	15 ± 0*	1 ± 0	4 ± 0*

*P<0.05 and ***P<0.001

Table 2: Mean ± SE thicknesses of uterine wall, endometrium and diameters of the ovaries in the test and control groups

Group	Thickness		
	Uterus (µm)	Endometrium (µm)	Diameters of the ovary (µm)
Control	745.7 ± 13	392.1 ± 16	1703 ± 78
Test	393.1 ± 23***	194 ± 10***	900.9 ± 86**

**P<0.01 and ***P<0.0001

contents such as primordial follicular population decreased significantly after cyclophosphamide injection. Reduction of PMF reserve in treated mice, suggests that ovarian damage could occur by exposure to chemotherapy. In rodent studies, treatment with busulfan, a chemotherapeutic agent, decreases the resting follicle pool by more than 90% in rats and substantially accelerates follicle depletion (Sanders *et al*., 1996). Our studies showed similar effect of cyclophosphamide on the mice ovary. Ovarian function depends on the follicular reserve and PMF populations sustain ovarian function. Therefore, chemotherapy with cyclophos-phamide at dose of 75 mg/kg, destroys more than 50% of PMF population. The depletion of PMF reserve, therefore, explains the ovarian failure in treated mice with cyclophosphamide (Apperley and Reddy, 1995; Meirow *et al*., 1999). Ovarian failure more likely leads to sterilization and loss of hormone production in ovaries. Since cyclophosphamide is an antimitogenic agent, it brings about reduction in mitotic division in fast dividing cells such as endometrial cells and that is why the reduction in endometrium as well as uterine thickness are seen in the test group. A study by Meirow *et al*. (1999) on effects of cyclophosphamide administration on the mouse ovary revealed a dose-related reduction in PMF reserve which is in accordance with results of our study. The mechanism of PMF damage induced by therapy is presented as well as the role of apoptosis signaling pathways underlying follicular destruction (Meirow *et al*., 1999). Other studies on radiotherapy revealed that the oocytes contained in PMF are particularly susceptible to environmental chemicals and chemotherapeutic drugs, whereas growing oocytes are relatively resistant (Mandl, 1959).

Assessment of other follicular distribution (total count) at different stages of growth revealed that there was significant reduction of such follicular population in the test group compared with the control group. Results of statistical analysis of this study clearly indicated that the distribution of all follicular types, i.e., primordial, primary, secondary, tertiary, graafian and also corpora lutea in the control and test groups had significant differences. This indicates that injection of cyclophosphamide brings decrement in follicular distribution in the test group. The distribution of the PMF was highly ($P<0.001$) decreased; the secondary and tertiary follicles differences was also significant ($P<0.05$). The differences of primary and graafian follicles in the control and test groups was however not significant ($P>0.05$). The diameter of ovaries in the treated mice was significantly decreased compared to the controls. Histologic studies on human ovarian tissue, which examined the effects of therapy on human ovaries following treatment, have shown that the end result of therapy was ovarian atrophy with marked loss of PMF (Kuhjada *et al*., 1982). Our results showed reduction in ovarian diameter of mice which were treated with cyclophosphamide. Many studies have demonstrated that cyclophosphamide and many other chemotherapy agents cause gene mutation, chromosomal breaks and rearrangements, and aneuploidy in somatic cells in human cancer survivors (Sandoval *et al*., 1993; Ben-Yehuda *et al*., 1996).

Moreover, animal studies have shown clear evidence that cyclophosphamide causes injury to germ cells as well as induction of transmissible genetic damage (Generoso *et al*., 1971; Becker and Sconeich, 1982), so this has raised serious concerns regarding the risk of abortions, birth defects, genetic or neoplastic diseases in offsprings of cancer survivors who regain fertility after treatment. Thus, for patients who will receive therapy, in order to preserve fertility, centers offer patients the option of oocyte retrieval and embryo cryopreservation before commencement of therapy (Grundy *et al*., 2001). The direct mechanisms of PMF destruction by chemo-therapy are still unclear. A possible explanation is that the drug induces apoptosis in the supporting granulosa cells of the follicle, or probably due to oocyte atresia, which becomes smaller and atretic. *In vitro* studies on human ovarian tissue showed that granulosa cells underwent apoptosis as a result of exposure to chemotherapy (Meirow *et al*., 1999).

A number of studies suggest that in parallel with chemotherapy, treatment with GnRH agonist protocol causes a reduction in ovarian damage induced by chemotherapy

by reducing the rate of PMF attrition (Damewood and Grochow, 1986; Meirow *et al*., 2004; Oktay *et al*., 2004), which will be the topic of our following studies. However, this protocol does not show a protective effect of GnRH agonist treatment on radiation-induced follicular injury (Gosden *et al*., 1983).

In conclusion, chemotherapy brings about a) reduction in ovarian follicular populations, especially PMF, b) it causes reduction in ovarian diameter and size and c) it decreases thickness of uterine wall, especially endometrium. Further investigations should be done to make clear the relationship between the activation of the oocyte death program after chemotherapy and the follicular stage. Secondly, the mechanism of PMF destruction must be clearly revealed. Finally, the treatment with GnRH analogues protocol in parallel to chemotherapy must be investigated.

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