Effect of Cyclophosphamide on the Immature Rat Ovary

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ABSTRACT

To investigate the early ovarian changes after cyclophosphamide treatment, immature rats primed for 48 h with pregnant mare serum gonadotropin were given injections i.p. of cyclophosphamide (100 mg/kg) at 1, 2, 4, 16, and 24 h before decapitation. Serum estradiol dropped significantly after 24 h of exposure to cyclophosphamide (P < 0.001). Following 16 and 24 h of cyclophosphamide exposure, (a) the number of granulosa cells expressed from each ovary decreased (P < 0.05 and P < 0.01, respectively); (b) the number of nucleated bone marrow cells decreased (P < 0.01 and P < 0.01), and their median nuclear size was significantly reduced (P < 0.05 and P < 0.05) as measured by Coulter Counter and C-256 channelizer (Hialeah, FL); and (c) the mean follicular diameter and the number of follicles with diameters greater than 300 μm were significantly lower than in control. After 4, 16, and 24 h of exposure, median granulosa cell nuclear size significantly increased (P < 0.05, P < 0.01, and P < 0.001, respectively). DNA cross-links in granulosa cells, measured by alkaline elution, reached a maximum at 2 h of exposure and decreased thereafter. The above findings demonstrate that cyclophosphamide has significant effects on the rat ovary structure and function and that the granulosa cell is an important target of cyclophosphamide-induced ovarian toxicity.

INTRODUCTION

Cyclophosphamide is the most commonly used chemotherapeutic agent in the treatment of cancer (1). It is used alone or in combination with other antineoplastic drugs in Hodgkin’s disease, Burkitt’s lymphoma, acute lymphoblastic leukemia of childhood, and breast cancer (2–5). It is also used in the treatment of some connective tissue and autoimmune diseases, minimal lesion glomerulonephritis, and for the control of organ rejection after transplantation (6–13).

A number of reports described the adverse effects of cyclophosphamide on fertility in humans and animals of both sexes (14, 15). Men treated with cyclophosphamide, 1 to 2 mg/kg for more than 4 mo, develop oligospermia or azospermia (16–18). The induced testicular damage appears to be dose dependent (19). In rodents, cyclophosphamide results in testicular damage (20, 21) and ovarian toxicity (22–26). In women this drug causes ovarian failure with associated amenorrhea and infertility (27–29). Koyama et al. (5) estimated that doses of 10.4, 9.3, or 5.2 g of cyclophosphamide resulted in amenorrhea in patients in their 20s, 30s, and 40s, respectively. With longer life expectancy in many patients, gonadal toxicity from chemotherapy is an undesirable complication. Understanding the mechanisms of this toxicity is essential for a rational approach to its prevention.

The advent of combination chemotherapy has made it more difficult to evaluate the toxic effects of individual drugs on the gonads and other tissues. Thus, animal models to study each drug separately become a necessity in understanding the mechanisms of gonadal toxicity. In this report, we describe the effect of cyclophosphamide on ovarian granulosa cells obtained from immature rats primed with PMSG. PMSG has been shown to stimulate follicular development and increase granulosa cell proliferation within a short time (48 h). In adult rodents, the time required for a follicle to reach the preovulatory stage is approximately 15 to 17 days (30), a rather long time. We have studied the effects of long-term administration of cyclophosphamide on adult rat ovaries (22). While those studies demonstrated a reduction in the number of follicles, they do not define the mechanisms involved at the cellular and subcellular levels. The advantages of using our present model of immature rats include minimal prior exposure of the ovaries to endogenous gonadotropins. Variability in follicular development and ovarian steroid secretion in different days of the estrus cycle characteristic of cycling adult rats is avoided. The time relationship of serum estradiol to endogenous gonadotropin surges that result in ovulation is difficult to control for. In addition, adult rat cycle length can vary unpredictably, thus adding to potential variability in reproductive and endocrine parameters (31). For comparative purposes the response of the bone marrow, a known target of cytotoxic agents, was also examined.

MATERIALS AND METHODS

Chemicals. Cyclophosphamide (Cytoxan for injection), donated by Bristol-Myers (Syracuse, NY), was dissolved in 0.9% NaCl solution. PMSG was purchased from Sigma (St. Louis, MO) and dissolved in 0.9% NaCl solution. Medium M199 and HBSS were purchased from Gibco (Grand Island, NY). BSA was purchased from Sigma (St. Louis, MO). Two liters of buffer for alkaline elution were prepared by adding distilled water to 16 g of NaCl, 0.8 g of KCl, 2 g of dextrose, 0.7 g of NaHCO3, and 3.722 g of disodium EDTA, supplemented with 1% BSA and the pH adjusted to 7.4. Zapoglobin and Isotone (Coulter Diagnostics, Hialeah, FL) were used for cell counts using a Model ZM Coulter Counter (Coulter Electronics, Hialeah, FL). Serum estradiol was measured using radioimmunoassay kits from Radioassay Systems Laboratories (Carson, CA) after extraction with ether (10:1).

Treatment Regimens. Immature Sprague-Dawley rats (Charles River, Portage, MI) were housed 5 per cage under controlled temperature (20–22°C) and light (14 h of light, 10 h of dark). At 24 to 25 days of age, they were given injections i.p. of 20 IU of PMSG and sacrificed 48 h later by decapitation. At 1, 2, 4, 16, and 24 h before sacrifice, 100 mg/kg of cyclophosphamide were injected i.p. Trunk blood was collected and allowed to clot. Serum was separated and frozen at −20°C until assayed for estradiol. The ovaries were excised, cleaned, weighed, and placed in Medium 199, containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid with 1% bovine serum albumin. Bone Marrow. Bone marrow from one femur of each rat was prepared as follows. The femur was cleaned of muscle and connective tissue, and the two ends were removed without actually exposing the marrow cavity. Using a 23-gauge needle, a hole was made at either side, and HBSS with 1% BSA and 0.15% EDTA was used to irrigate the bone marrow cavity. The solution (1.5 ml) and air (1 ml) were injected from either femur side and recovered through the other end into a vial. In each experiment, bone marrow of all rats within each treatment group was pooled. The marrow was then finely suspended and pushed through

Received 5/24/88; revised 10/5/88, 12/29/88; accepted 1/4/89.

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1 This research was funded by the American Cancer Society (Grant CH-365), Wayne State University Institute of Chemical Toxicology, and Children’s Leukemia Foundation of Michigan.

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3 The abbreviations used are: PMSG, pregnant mare serum gonadotropin; HBSS, Hank’s balanced salt solution; BSA, bovine serum albumin; CLF, cross-link factor; FSH, follicle-stimulating hormone; EGTA, ethyleneglycol bis(β-aminoethoxy) ethyl)-N,N,N′,N′-tetraacetic acid.

Bone Marrow. Bone marrow from one femur of each rat was prepared as follows. The femur was cleaned of muscle and connective tissue, and the two ends were removed without actually exposing the marrow cavity. Using a 23-gauge needle, a hole was made at either side, and HBSS with 1% BSA and 0.15% EDTA was used to irrigate the bone marrow cavity. The solution (1.5 ml) and air (1 ml) were injected from either femur side and recovered through the other end into a vial. In each experiment, bone marrow of all rats within each treatment group was pooled. The marrow was then finely suspended and pushed through
a 25-gauge needle. Nucleated cell count, nuclear size distribution, and its median for the different treatment groups were obtained using a Model ZM Cytometer Counter with C-256 channelizer.

Granulosa Cells. The ovaries of each treatment group were pooled together and incubated with 6.8 mm EGTA for 15 min in hypertonic sucrose (0.5 M) for 5 min. Granulosa cells were expressed into the medium containing equimolar amounts of EGTA and calcium by puncturing the follicles with a 25-gauge needle as described by Campbell (32) and modified by Clark et al. (33). The ovaries were then gently compressed in a multiwell glass plate using a curved metallic spatula. The cell suspension was then filtered through a 100-μm nylon mesh. The cells were well dispersed as judged by microscopic examination. After washing the cells with Medium 199 (without EGTA), cell viability was evaluated by the trypan blue dye exclusion test. Granulosa cell counts were obtained by a Model ZM Cytometer Counter, and their nuclear size distribution and median size were determined using a C-256 channelizer after cell lysis with Zapoglobin (6 drops/20 ml of Isotone). Granulosa cell number per ovary was obtained by dividing the total cell count by the number of ovaries which varied between 4 and 8 ovaries per treatment group in each experiment. Meanwhile, the granulosa cell suspensions were kept on ice until 10 cells were taken for alkaline elution to evaluate the degree of granulosa cell interstrand DNA and DNA-protein cross-links as described by Kohn (34). The cells were irradiated at 0°C with 500 R from a Gammacell 170Cs irradiator (Mark I, Model 68; G. L. Shepherd & Associates, Glendale, CA). The fraction of their DNA retained on the filter is directly related to the degree of cross-links. A CLF was calculated using the formula

\[
CLF = \log \left( \frac{f_{500}}{f_0} \right) / \log \left( \frac{f_x}{f_0} \right)
\]

where \(f_{500}\) is the fraction of DNA eluted after control cells (not exposed to cyclophosphamide) were irradiated, \(f_0\) is the fraction eluted from control nonirradiated cells, and \(f_x\) is the fraction eluted from granulosa cells of different treatment groups. DNA was measured fluorometrically.

Ovarian Histology. Ovaries were obtained 48 h after PMSG injection from control rats and from those exposed to cyclophosphamide (100 mg/kg) for 16 or 24 h. One ovary from each rat was fixed, embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin. Every eighth section was examined using a light microscope attached to a BioQuant image analysis computer system (22). Antral follicles were counted when a nuclear membrane and/or nucleolus of the oocyte was observed. The mean follicle diameter was calculated after measuring the longest and shortest follicle diameters (22).

Statistics. Contrast analysis and the Dunette test were used to evaluate the significance of differences between treatment groups and their controls. \(P < 0.05\) was considered to indicate statistical significance.

RESULTS

Effect of Cyclophosphamide on Bone Marrow Nucleated Cell Counts. Cyclophosphamide given 16 and 24 h before sacrifice resulted in a significant reduction in bone marrow nucleated cell counts per femur of 63 ± 5% and 79 ± 3%, respectively (\(P < 0.01\) for both), in comparison to control. No effect was noted at 1, 2, and 4 h of exposure to cyclophosphamide (Fig. 1A). The controls had 51 ± 4 million cells/femur.

Effect of Cyclophosphamide on Median Bone Marrow Cell Nuclear Size. Significant reductions in median bone marrow cell nuclear size of 6.2 ± 2.6% and 8.0 ± 1.6% (\(P < 0.05\)) were obtained when cyclophosphamide was given 16 and 24 h before sacrifice in comparison to control (Fig. 1B). Control rats had a median bone marrow cell nuclear size of 64 ± 2 femtoliters. No significant effect was noted when cyclophosphamide was given 1, 2, and 4 h before sacrifice.

Effect of PMSG and Cyclophosphamide on Ovarian Weight. As expected, PMSG significantly increased ovarian weight (\(P < 0.001\)) from 14.1 ± 0.4 (for rats not treated with PMSG, mean ± SEM, \(n = 12\)) to 68.7 ± 4.1 mg (for PMSG-treated rats, \(n = 13\)). Cyclophosphamide did not have a significant effect on ovarian weight in any of the groups compared to control.

Effect of Cyclophosphamide on Granulosa Cell Number and Viability. As shown in Fig. 1C, cyclophosphamide given 16 and 24 h before sacrifice reduced granulosa cell number per ovary by 24 ± 5% and 51 ± 4% in comparison to control (\(P < 0.05\) and \(P < 0.01\), respectively). No significant change in cell number was noted when cyclophosphamide was given 1, 2, or 4 h before sacrifice. The control group had 4.7 ± 0.7 million cells per ovary. No significant change in the percentage of cell viability was observed in any of the treatment groups compared to control as evaluated by trypan blue exclusion.

Effect of Cyclophosphamide on Median Granulosa Cell Nuclear Size. Cyclophosphamide given at 4, 16, and 24 h before sacrifice resulted in 11 ± 0.8%, 21 ± 4%, and 17 ± 4% increase in granulosa cell nuclear size in comparison to control (\(P < 0.05\), \(P < 0.01\), and \(P < 0.01\), respectively). No significant change in granulosa cell nuclear size was noted when cyclophosphamide was given 1 and 2 h before sacrifice (Fig. 1D). The control group granulosa cell median nuclear size was 77 ± 4 femtoliters.

Effect of Cyclophosphamide on Granulosa Cell DNA Cross-Links. CLF obtained from alkaline elution data reached a maximum at 2 h and decreased thereafter as shown in Fig. 2. The cross-link factor was 1.39 ± 0.17 at 1 h, 2.77 ± 0.90 at 2 h, 1.77 ± 0.42 at 4 h, 1.73 ± 0.3 at 16 h, and 1.61 ± 0.10 at 24 h. No statistical comparison was performed, since the formula for CLF always gives a value of 1 for control.

Effect of Cyclophosphamide on Serum Estradiol. A significant reduction in serum estradiol was obtained when cyclophosphamide was injected 24 h before sacrifice (\(P < 0.005\)). Serum estradiol was 1116 ± 83 pg/ml for control and 326 ± 70 pg/ml for the 24-h group. No significant effect was noted when cyclophosphamide was given 1, 2, 4, or 16 h before sacrifice (Fig. 3).

Effect of Cyclophosphamide on Antral Follicular Development. The number of antral follicles counted per ovary was 36 ± 3 for ovaries exposed to cyclophosphamide for 16 h (n = 7) and 27 ± 4 for ovaries exposed for 24 h (n = 6). None was significantly different from control (32 ± 3, n = 10). The mean diameter (μm) of antral follicles was lower in the groups exposed for 16 (264 ± 9, \(P < 0.001\)) and 24 h (237 ± 11, \(P < 0.001\)) in comparison to control (317 ± 8). The number of large antral follicles (>300 μm in diameter) was lower in the groups exposed for 16 h (11 ± 1, \(P < 0.05\)) and 24 h (7 ± 2, \(P < 0.01\)) in comparison to control (17 ± 2). No evidence of luteinization was observed.

DISCUSSION

Granulosa cell proliferation and ovarian hormone (estradiol) secretion were significantly modified by cyclophosphamide. A statistically significant decrease in granulosa cell number per ovary as well as mean follicular diameter was observed 16 and 24 h after cyclophosphamide injection. Alkaline elution studies showed maximum DNA cross-linkage in granulosa cells at 2 h after injection of cyclophosphamide. Alterations in granulosa cell structure and function detectable by our methods were relatively delayed. Four h after cyclophosphamide injection, a significant increase in median granulosa cell nuclear size was detected consistent with a G2-phase block.
**A. EFFECT OF CYCLOPHOSPHAMIDE ON BONE MARROW NUCLEATED CELL COUNTS**

![Graph A](image1)

**B. EFFECT OF CYCLOPHOSPHAMIDE ON BONE MARROW NUCLEAR SIZE**

![Graph B](image2)

**C. EFFECT OF CYCLOPHOSPHAMIDE ON GRANULOSA CELL NUMBER/OVARY**

![Graph C](image3)

**D. EFFECT OF CYCLOPHOSPHAMIDE ON GRANULOSA CELL NUCLEAR SIZE**

![Graph D](image4)

Fig. 1. Effect of cyclophosphamide on bone marrow nucleated cell counts (A), bone marrow cell median nuclear size (B), granulosa cell number per ovary (C), and granulosa cell median nuclear size (D). Control is considered 100%. Other treatment groups are expressed as the percentage of control. *, P < 0.05; **, P < 0.01. Numbers in parentheses, number of experiments. Bars, SE.

Alkylating agents are known to have two important effects on intact living mammalian cells: cell killing and delay in cell cycle progression with accumulation of living cells in the G2 phase (35). These effects have been observed in vivo and in vitro. More of the surviving granulosa cells had bigger nuclei as they accumulated more DNA in the preceding S phase of the cell cycle. The decrease in nuclear size of bone marrow cells probably reflects killing of the actively dividing giant stem cells of the bone marrow. This may have masked the effects of the G2-phase block which is expected to increase nuclear size. Unlike the bone marrow, the ovary does not have giant stem cells.

The expected decrease in bone marrow nucleated cell count was observed. Consistent with the cytotoxic effects of cyclophosphamide on proliferating cells, similar reductions in the number of granulosa cells per ovary and mean ovarian follicular diameter were also observed. Mean follicle diameter is known to correlate strongly with the number of granulosa cells per follicle (36), and both are expected to increase following PMSG injection (37). The decrease in serum estradiol obtained 24 h after injecting cyclophosphamide may represent an interference.
with granulosa cell function. Granulosa cells are the main source of serum estradiol which increases with larger follicle diameter and granulosa cell number (38). In fact, serum estradiol and follicle diameter measurements are used routinely during monitoring of follicle maturation and ovulation in women (38). The above changes in granulosa cell nuclear size, number, and serum estradiol may be related to cross-linking of DNA. On the other hand, cyclophosphamide metabolites can also alkylate other macromolecules, such as proteins and enzymes, that may be involved in mitosis and steroid synthesis and secretion.

The early events leading to ovarian failure after cyclophosphamide treatment have not been well characterized previously. Most previous studies documented the rather late effects of cyclophosphamide treatment: accelerated depletion of ovarian follicles (3, 22–24). Histological sections of ovaries from patients treated with cyclophosphamide were found to be devoid of follicles (3). Significant reductions (P < 0.01) of primordial and maturing follicles as well as corpora lutea have been observed in cyclophosphamide-treated mice compared to control (24). Burkhl and Schiechtl (23) studied the early disturbances in follicle growth induced by busulphan and cyclophosphamide in rats using histological techniques. Secondary and antral follicles disappeared within a short period of time after treatment. Degenerating cells containing DNA inclusions were frequently seen in the follicular epithelium. The growth of primary oocytes in primary follicles continued with apparently normal cytoplasmic structures. The growth of the normal oocyte is known to take place very rapidly in the early stages of follicular growth and is nearly completed in the late secondary follicle (39, 40). The number of layers of granulosa cells in treated rats did not increase as it should under physiological conditions (23). These findings in granulosa cells are consistent with ours, indicating that granulosa cells represent a primary target ovarian compartment involved in the eventual follicle depletion. Other studies in our laboratory indicate that oocytes may also be involved, however. Oocyte fertilizability and early cleavage were significantly impaired by cyclophosphamide metabolites (41). Thus, although the oocyte morphology may appear normal (23), their function may be adversely affected (41).

The dynamics of accelerated follicular atresia during chemotherapy have not been extensively studied. Starting with a defined total number of follicles in the ovary, follicular recruitment and development from the pool of primordial follicles are a dynamic continuous process occurring all the time. We have shown that cyclophosphamide can reduce estradiol levels in immature PMSG-treated rats. A similar decrease can possibly occur in adult rats and in women. This would be consistent with our findings of decreased follicular diameters in ovaries of adult (22) and PMSG-treated immature rats (42). Our experiments show that exposure of granulosa cells to the effects of cyclophosphamide, as measured by DNA cross-links, continues up to and probably beyond 24 h. A similar situation may also occur with other macromolecules (enzymes, proteins) that are alkylated by cyclophosphamide metabolites. Thus, with repeated administration of the drug (sometimes given daily), rather prolonged exposure of granulosa cells to the alkylating effects of cyclophosphamide is ensured. The endogenous gonadotropins, being related to estrogens by a negative feedback mechanism (38), are expected to increase in response to decreasing estrogen levels. The increased endogenous FSH secretion may accelerate further follicular recruitment into the developing cyclophosphamide-sensitive pool of follicles (22, 43). The S phase and other phases of the granulosa cell division cycle have been shown to be shortened by FSH (43). This mechanism permeates a vicious cycle, whereby cyclophosphamide destroys the developing follicles by attacking rapidly dividing granulosa cells, reducing their steroid secretion, leading to increased pituitary gonadotropin production that enhances further recruitment of follicles into the pool of maturing follicles susceptible to cyclophosphamide. Thus, follicular recruitment and atresia are accelerated, resulting in eventual premature depletion of the limited number of follicles in the ovaries. A similar mechanism may also apply to humans, where estrogens and FSH are related by a negative feedback mechanism (38). In this context, we have shown that cyclophosphamide metabolites can reduce steroidogenesis in human granulosa cells (44).

In conclusion, granulosa cells appear to be important targets for toxicity in the ovaries of rats treated with cyclophosphamide. This is further supported by the recent demonstration of a direct in vitro effect of 4-hydroperoxycyclophosphamide, an activated form of cyclophosphamide, on rat granulosa cells (from PMSG-primed immature rats) and human granulosa cells (obtained from women undergoing follicle aspiration for in vitro fertilization) (44, 45). In those studies, granulosa cell survival and progesterone accumulation were significantly reduced in a dose-related manner. Whether these cells are the targets of damage in women in vivo remains unknown.

ACKNOWLEDGMENTS

We thank Maryann Milewski for secretarial assistance and Magaly Muskus and Carleen Yost for technical assistance.

REFERENCES

8. Fosdick, W. M., Parsons, J. L., and Hill, D. F. Preliminary report: long-


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