A Luteinizing Hormone-releasing Hormone Agonist for the Prevention of Chemotherapy-induced Ovarian Follicular Loss in Rats

Khalid M. Ataya,2 James A. McKanna, Alan M. Weintraub, Martin R. Clark, and William J. LeMaire

ABSTRACT

In an attempt to prevent chemotherapy-induced ovarian follicular loss, [α-Leu6, des-Gly10-NH2]-luteinizing hormone-releasing hormone ethylamide (LHRHa) was given subcutaneously to Sprague-Dawley cycling female rats in two daily doses of 2.5 μg starting 2 days prior to and concomitant with cyclophosphamide (CTX) (5 mg/kg/day for 21 days). Four groups of female cycling rats (10 in each) received either no treatment, CTX alone, CTX + LHRHa, or LHRHa alone. One ovary from each animal was serially sectioned, stained, and examined for the number and size of follicles.

CTX produced a significant reduction in the total number of follicles. The pool of growing follicles (medium to large, >30 μm in diameter) appeared to be vulnerable to the cytotoxic effect of CTX. LHRHa resulted in a significant reduction in the number of medium-to-large follicles and an increase in the number of small follicles. When given in combination with CTX, LHRHa significantly further reduced the number of medium-to-large follicles, significantly increased the number of small follicles, and resulted in an increase in the total number of follicles. Chronic LHRHa treatment resulted in functional deprivation of follicles from gonadotropins, thus halting the process of recruitment from the quiescent pool of primordial follicles into the CTX sensitive pool and thereby preserving the functional potential of the ovary.

INTRODUCTION

With the advent of cancer chemotherapy, 50 to 70% of patients with Hodgkin's disease may achieve long-term survival (9, 10, 21). Similar statements can be made about patients with leukemia and non-Hodgkin's lymphoma, as well as patients with other tumors (2). The fact that increasing numbers of cancer patients are achieving long-term survival following chemotherapy makes the issue of the long-term side effects of such therapy increasingly significant.

Chapman et al. (7) reported that, of 41 women treated by combination chemotherapy for Hodgkin's disease, 69% developed ovarian failure if their age was less than 29 at the onset of therapy, while 86% developed ovarian failure if their age was 30 years or older. Ovarian failure manifests itself as hypergonadotropic hypogonadism resulting in amenorrhea and, usually, irreversible infertility. Once all of the follicles with their oocytes have been irreversibly damaged, there is no chance of conception ever occurring. The hormonal failure associated with this condition results in multiple manifestations of estrogen deficiency which may include annoying acute problems such as hot flushes, vaginal dryness, and breast atrophy but which, more importantly, also may lead to osteoporosis (17). It is therefore not a trivial consideration to attempt to minimize or prevent the loss of the two gonadal functions, reproduction and hormone secretion, resulting from cancer chemotherapy.

In this study, we chose CTX3 as a representative of the cytotoxic drugs. This alkylating agent has been used extensively for cancer chemotherapy, either alone or in combination with other drugs. In addition, it is also utilized in the treatment of certain connective tissue diseases, minimal lesion glomerulonephritis and for the control of organ rejection after transplantation (5, 12–14, 33). This compound has been shown to produce gonadal failure in humans (22, 27, 31, 32) and animals (4, 23, 25, 26). Mattison et al. (23) found a 63% reduction in the numbers of small follicles in the ovaries of mice treated with a single 100 mg/kg i.p. injection of CTX.

The aim of our study was to try to prevent ovarian failure induced by CTX using prior and concomitant treatment with LHRHa. Similar agonists when given to female rats interrupt their cyclicity, and the rats enter persistent diestrus (19). The analogues are known to suppress gonadotropin receptors in the ovaries (29), which would halt follicular development (34) and follicular cell division, thus possibly protecting the dormant ovarian follicle from destruction, since cytotoxic agents mostly attack rapidly dividing cells (5).

MATERIALS AND METHODS

Animals

Adult female Sprague Dawley rats, weighing 175 to 240 g, were fed rat chow and water ad libitum and housed in plastic transparent cages with 2 rats/cage under controlled temperature (20–22°C) and light (14 h light, 10 h dark). Vaginal smears were obtained daily. Only rats demonstrating at least 2 consecutive normal 4-day vaginal estrus cycles were included in the experiments.

Chemicals

CTX for injection was generously donated by Mead Johnson (Evansville, IN). It was prepared every other day in 0.9% NaCl solution at a concentration of 1 or 2 mg/ml. LHRHa was generously supplied by Abbott Laboratories (North Chicago, IL) and was dissolved in 0.9% NaCl solution for injection.

Treatment Regimens

Experiment 1. Twenty-six rats were divided into 3 groups. Group I (n = 8) received a loading dose of CTX (50 mg/kg) followed by daily i.p.

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2To whom requests for reprints should be addressed, at REPSCELD Laboratories, P. O. Box 016960, Miami, FL 33101.
3The abbreviations used are: CTX, (Cytoxan) cyclophosphamide; LHRHa, (Leu-prolinate) luteinizing hormone-releasing hormone agonist ([α-Leu6,des-Gly10-NH2]-LHRHa, (Leu-prolinate) luteinizing hormone-releasing hormone agonist).
Injections of 5 mg/kg. Group II (n = 10) received the same loading dose followed by daily i.p. injections of CTX (10 mg/kg). Group III (n = 8) received daily i.p. injections of 0.9% NaCl solution (control). After 21 days, treatment was stopped, and one-half of the animals in each group was sacrificed immediately, while the other half was sacrificed 3 weeks later. Upon sacrifice, the ovaries, thymus, and a piece of bone from the femur were taken for histological examination.

Experiment 2. Forty rats were divided into 4 groups of 10 animals each. Group I (control) received daily i.p. injections of 0.9% NaCl solution as well as twice daily s.c. injections of 0.3 ml of 0.9% NaCl solution. Group II (CTX) received an initial i.p. loading dose of CTX (50 mg/kg), followed by daily i.p. injections of 5 mg/kg, as well as, twice daily s.c., 0.3 ml of 0.9% NaCl solution. Group III (CTX + LHRHa) received the same CTX treatment as did Group II and, in addition, 2.5 µg of LHRHa twice daily s.c. Group IV (LHRHa) received daily i.p. injections of 0.9% NaCl solution and 2.5 µg of LHRHa twice daily s.c.

The treatment with LHRHa or its vehicle was initiated on the day of metestrus, and the CTX therapy was started 2 days later. The CTX therapy was continued for 21 days. All cycling animals were sacrificed on the first diestrus following discontinuation of CTX or its vehicle treatment. Noncycling animals were sacrificed on the day of discontinuation of CTX treatment. Treatment with LHRHa or its vehicle was continued until the day of sacrifice. The ovaries and uteri were then removed, and their wet weight was recorded.

Histological Examination

In Experiment 1, 2 sections of the thymus and femur of each animal were prepared for histological examination. The ovaries of all animals were fixed in Bouin's solution for 24 h, prepared for serial sectioning (6 µm thickness), and stained with hematoxylin and eosin. One ovary from each animal was examined by the same investigator without knowledge of the treatment. In Experiment 1, every 40th section was examined microscopically using a light microscope, and all follicles with a nucleolus in the the oocyte were counted. The total number of follicles was estimated using the method of Dornfeld et al. (11). Statistical analysis was carried out using the Student's t-test.

In Experiment 2, all sections (8 µm thickness) were mounted on slides, and every 10th section was examined and measured using light microscopy combined with the Bioquant image analysis system (E. Leitz). Under the microscope, each follicle was identified and measured. Those having a single layer of granulosa cells surrounding a well-recognized oocyte were counted. Profiles of larger follicles containing oocytes with intact nuclear membranes were counted and measured using Bioquant video-overlay analysis. The image of the microscopy field is captured by a high-resolution black and white video camera and displayed on the computer monitor along with cross-hairs corresponding to the position of the cursor on the digitizing tablet. The average diameter was determined as the mean of the longest and shortest diameters of each follicle, measured as straight-line distances between opposite points on the basement membrane. The Bioquant programs provided for data storage on floppy disks, data retrieval, calculations, correlation, distribution, and statistics. For comparison of follicle size and number in the various groups in Experiment 2, statistical analysis was carried out using the Student's t-test, using pooled or separate variance estimates, as appropriate.

RESULTS

Experiment 1. Three animals in Group I (CTX [5 mg/kg/day]) died on Days 10, 12, and 30 of the experiment, and 1 animal of Group II [CTX (10 mg/kg/day)] died on Day 14. The animal in Group I that died on Day 30 was included for analysis, while the other 2 in Group I and the one in Group II were excluded because of tissue decomposition by the time the death was discovered. Four animals in Group II stopped cycling. Two of these were sacrificed at the end of CTX therapy, while the other 2 resumed cycling after CTX was stopped. All other animals exhibited continued normal cycling vaginal cytology.

Histological examination of the bone marrow of the animals sacrificed at the end of CTX therapy showed severe depression of all marrow elements in Group II animals and moderate depression in Group I animals. The thymus showed marked atrophy and depletion of lymphocytes in both CTX groups. This effect was more prominent in Group II. In the animals sacrificed 3 weeks after termination of therapy, the histology of the bone marrow and the thymus were found to be normal.

Table 1 shows the findings of the measurement of follicular number in the various groups. In each instance, the number of follicles in the ovaries of animals sacrificed 3 weeks after termination of therapy was lower than for the animals sacrificed immediately (Subgroups d and i, respectively, in Table 1), but these differences were not significant. Therefore, the data within each group for these 2 subgroups were combined. The number of follicles in both treatment Groups I and II was significantly lower than in the control group (III), as estimated by the method of Dornfeld et al. (11). Similar results were obtained by the arithmetic correction of the number of follicles counted for the number of sections examined. Since, in this first experiment, the animals were sacrificed on different days of the estrous cycle, no attempt was made at correlating follicle size in the different treatment groups because the size distribution of follicles within the ovary varies considerably during the estrous cycle (18).

Experiment 2. Two animals in Group II (CTX only) died on Days 10 and 11 of CTX therapy. One of these was discovered immediately and was included for histological examination. Five animals in Group III (CTX + LHRHa) died, 2 on Day 15 and 1 each on Days 16, 17, and 21 of the CTX therapy. All of these animals could be included for histological examination. There was no significant difference between the numbers of follicles in rats that died and those that survived until sacrifice. Thus, they were treated as one group. The ovaries of 3 animals of Group IV were not included because of inadequate quality of histological preparation. In this experiment, 8 of 10 animals of Group II (CTX only) stopped cycling, and all animals receiving LHRHa (Groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Subgroup</th>
<th>Group</th>
<th>Follicles/ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3</td>
<td>i</td>
<td></td>
<td>480 ± 330</td>
</tr>
<tr>
<td>CTX (5 mg/kg)</td>
<td>3</td>
<td>d</td>
<td></td>
<td>400 ± 160</td>
</tr>
<tr>
<td>II</td>
<td>4</td>
<td>i</td>
<td></td>
<td>420 ± 140</td>
</tr>
<tr>
<td>CTX (10 mg/kg)</td>
<td>5</td>
<td>d</td>
<td></td>
<td>510 ± 60</td>
</tr>
<tr>
<td>III</td>
<td>4</td>
<td>i</td>
<td></td>
<td>1430 ± 260</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>d</td>
<td></td>
<td>860 ± 140</td>
</tr>
</tbody>
</table>

*p = significance as compared to Group III (control). 
* As the difference between Subgroups i and d was not significant, these subgroups were combined in each group.
* i, immediate posttreatment sacrifice.
* d, sacrifice 3 weeks after therapy is terminated.

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cells surrounding the oocyte. For the purpose of this study, all follicles measuring more than 30 μm were grouped together and called "medium to large." CTX alone (II) reduced the total number of follicles compared to control (I) \( P < 0.05 \). This was apparently due to a reduction of the number of medium to large follicles \( P < 0.01 \) without significantly affecting the number of small follicles. This finding is supported by the observation that the mean follicular diameter in the CTX group \( [47 ± 2 \text{ (SE)}] \) was significantly smaller \( P < 0.01 \) than in the control \( [71 ± 2] \). Moreover, the percentage of small follicles was higher in the CTX group compared to control \( P < 0.05 \), again suggesting that CTX affected mainly medium to large follicles. The administration of LHRHa alone (Group IV) was associated with a higher number of total follicles compared to control (Table 3), but this difference was not statistically significant. There appeared to be a reduction in the recruitment of small follicles \( [2280 ± 310 \text{ for LHRHa versus } 1000 ± 90 \text{ for control, } P < 0.01] \) into the pool of follicles undergoing further development and atresia \( [140 ± 30 \text{ for LHRHa versus } 810 ± 80 \text{ for control, } P < 0.01] \). Consistent with this interpretation, the resultant mean follicular diameter of the LHRHa group \( [24 ± 1] \) was markedly reduced \( P < 0.01 \) compared to control \( [71 ± 2] \). Moreover, the percentage of small follicles in the LHRHa group was much higher \( P < 0.01 \) than in the control group, which is again consistent with the above interpretation.

LHRHa, administered with CTX (Group III) appeared to prevent the CTX-induced reduction in the total number of follicles. This effect, however, was not statistically significant \( P = 0.2 \). The number of large follicles is significantly lower \( P < 0.01 \) in the CTX + LHRHa group than in CTX alone group, while the number of small follicles is significantly higher \( P = 0.05 \). The mean follicular diameter was significantly lower \( P < 0.01 \) in the CTX + LHRHa compared to CTX alone. Furthermore, the percentage of small follicles was higher \( P < 0.01 \) in the CTX + LHRHa group compared to the CTX alone group.

**Follicular Atresia.** Although no attempt at quantitating follicular atresia per se was made, qualitative findings suggestive of accelerated atresia of the large follicles were observed in the ovaries of rats treated with either CTX or LHRHa alone or in combination. The most prominent finding not usually seen in physiological atresia is the dark spherical extracellular globules.

### Table 2

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>No. of rats</th>
<th>Body wt. change (g)</th>
<th>Ovarian wt. (mg)</th>
<th>Uterine wt. (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Control</td>
<td>10</td>
<td>+2 ± 4</td>
<td>62 ± 5</td>
<td>378 ± 24</td>
</tr>
<tr>
<td>II CTX only</td>
<td>8</td>
<td>-65 ± 11</td>
<td>43 ± 2</td>
<td>185 ± 17</td>
</tr>
<tr>
<td>III CTX + LHRHa</td>
<td>5</td>
<td>-49 ± 17</td>
<td>41 ± 6</td>
<td>134 ± 8</td>
</tr>
<tr>
<td>IV LHRHa only</td>
<td>10</td>
<td>+32 ± 5</td>
<td>42 ± 4</td>
<td>118 ± 4</td>
</tr>
</tbody>
</table>

*Animals which died during the course of the experiment were excluded for weight measurements. Numbers in parentheses, initial body weight ± SE. *These numbers represent the mean wet weight at the end of the experiment ± SE.

### Table 3

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>No. of follicles/ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Control</td>
<td>1810 ± 120*</td>
</tr>
<tr>
<td>II CTX only</td>
<td>1390 ± 140</td>
</tr>
<tr>
<td>III CTX + LHRHa</td>
<td>1740 ± 240</td>
</tr>
<tr>
<td>IV LHRHa only</td>
<td>2420 ± 310</td>
</tr>
</tbody>
</table>

*The ovaries were serially sectioned and stained with hematoxylin and eosin. Every 10th section was examined for the number and size of all follicles found to have an intact nuclear membrane. The total number of follicles was estimated by multiplying the number of follicles counted by 10. *Represents the mean diameter of all follicles counted. *Medium to large follicles, >30 μm. Small follicles, <30 μm. Numbers in parentheses, mean percentage of each subgroup compared to the total. *Mean ± SE.
of various sizes within the antrum of the follicles (Fig. 1). These presumably represent nucleoproteins and debris of the disintegrated follicular elements.

DISCUSSION

In the first experiment, we confirmed that CTX treatment of mature cycling rats resulted in a significant reduction in the number of follicles in both treatment regimens (5 and 10 mg/kg/day for 21 days following a loading dose of 50 mg/kg). Although the duration and dose used in clinical CTX therapy is very variable, we selected a regimen of treatment similar to one of the regimens used clinically. We selected the method of i.p. injection because of its convenience. After establishing our model in Experiment 1, we elected to use 5 mg/kg/day for Experiment 2, as this maintenance dose did produce a significant reduction in the total number of follicles. Measurement of follicular diameter was selected as the parameter to classify follicles, because it can be rapidly and accurately determined, without imposing arbitrary class limits on the continuum of follicular development. Hirshfield and Midgley (18) showed that follicular diameter correlated well with granulosa cell numbers, which is an important index of follicular growth. Analysis of the number of small and medium to large follicles in Experiment 2 indicated that the loss of follicles produced by CTX alone was mainly due to the destruction of the medium to large follicles without an apparent effect on the number of the small follicles during the rather short course of CTX treatment. The mechanism of CTX ovarian toxicity may be conceptualized as a dynamic process whereby the loss of large follicles leads to a relative decrease in their hormone secretion. The pituitary gland, in the absence of exogenous LHRHa, responds through a feedback mechanism by increasing gonadotropin secretion and thus enhancing the recruitment of small follicles into the pool of proliferative and cytoxan sensitive large follicles. These follicles are again destroyed by CTX, thereby establishing a vicious cycle that leads to the diminution of small follicles. Thus, it seems likely that a longer duration of therapy with CTX might reduce the number of small follicles and eventually produce ovarian failure.

Treatment with LHRHa alone appears to inhibit the process of recruitment from the pool of small follicles into the pool of the larger follicles undergoing further development and atresia. This latter pool of follicles seems to be the one which is sensitive to the effects of chemotherapy, as we have demonstrated. Thus, it appears that LHRHa acts at an earlier stage of follicular development than CTX, hence preventing the follicles from reaching the chemotherapy-sensitive stage. The mechanism of action of LHRHa may involve direct suppression of gonadotropin receptors in the rat ovary (29), resulting in the functional deprivation of follicles from gonadotropin, leading to atresia of the already developed follicles as well as inhibition of recruitment of small follicles into the proliferating pool. In addition, the plasma levels of follicle-stimulating hormone and luteinizing hormone in rats at the end of chronic treatment with LHRH agonists have been found to be elevated, thus possibly down-regulating the gonadotropin receptors in the ovary (8). A third possible mechanism is that the gonadotropins released following chronic LHRH agonist’s treatment may have reduced biological activity (24). It has also been shown that the response of the pituitary gonadotropins to exogenous releasing hormone agonist decreases following prior treatment with the agonist (30), suggesting down-regulation of LHRH receptors in the pituitary. The end result is the functional deprivation of follicles from gonadotropins. A similar situation is encountered in hypophysectomy, which has been reported to decrease the normal process of progressive loss of oocytes from the ovary in mice (20). In the human, Kallman’s syndrome is associated with low levels of gonadotropins due to a hypothalamic abnormality. Follicular development rarely progresses beyond the primordial stage in the ovaries of those women (16).

The results obtained from the prior and concomitant treatment with LHRHa demonstrate that follicular loss caused by CTX might indeed be preventable. This conclusion is based on the following observations. First, the ovarian follicular reserve, as represented by the number of small follicles, was significantly greater in the CTX + LHRHa group compared to the CTX alone group. Second, the lower number of large follicles in the CTX + LHRHa group as compared to CTX alone further suggests that there might be an inhibition of the process of recruitment of small follicles into the pool of proliferative and cytoxan sensitive large follicles. Although the total number of follicles in the CTX + LHRHa group was larger than the CTX alone group, which is consistent with our concept, this difference did not reach significance. This may be related to the duration and dose of CTX therapy. A longer duration and possibly higher dose of CTX may cause a more marked loss of follicles.

Prevention of gonadal cytotoxic effects induced by chemotherapy has been attempted before. Nelson et al. (28) reported that recovery from spermatogenic arrest induced by nitrofuran in the rat was enhanced by the administration of estrogen and testosterone. Glode et al. (15) using a LHRH agonist were able to partially prevent testicular damage induced by CTX in male rats. In the human female, Chapman and Sutcliffe (6) suggested that contraceptive pills may protect the ovary from follicular destruction by a combination chemotherapeutic regimen. However, the number of patients in this study was too small to be conclusive. In addition to the known side effects of the birth control pills, the use of estrogen and progesterin in patients on chemotherapy may be hazardous. These steroids are known to affect liver enzymes (e.g., the cytochrome P450 enzyme system) which may be involved in the metabolism of chemotherapeutic agents such as CTX (1, 5). Therefore, the use of LHRHa analogues as possible protective agents during chemotherapy may be preferable. As one of the aims of the study was to demonstrate that LHRHa treatment could preserve fertility potential after chemotherapy, it is important to note that the antifertility effects of long-term therapy with LHRHa were reversible and that the offspring of rats treated earlier with LHRHa were apparently normal (19). Furthermore, chemotherapy per se, including CTX, does not appear to affect subsequent pregnancy outcome when administered prior to pregnancy in males or females (3). Further studies are required to firmly establish that treatment with LHRHa can prevent chemotherapy-induced ovarian failure and to elucidate the mechanism of this protective effect. The potential clinical application of such a protective effect is evident.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. A histological section through a follicle in the ovary of a rat treated with CTX + LHRHa. Note the dark spherical extracellular globules (arrows) suggestive of accelerated atresia (x 250).
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