Effects of Ionizing Radiation and Pretreatment with \([D-\text{Leu}^6,\text{des-Gly}^{10}]\) Luteinizing Hormone-releasing Hormone Ethylamide on Developing Rat Ovarian Follicles

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ABSTRACT

To assess the effects of a gonadotropin-releasing hormone agonist, \([D-\text{Leu}^6,\text{des-Gly}^{10}]\) luteinizing hormone-releasing hormone ethylamide, in ameliorating the damage caused by ionizing radiation, gonadotropin-releasing hormone agonist was administered to rats from day 22 to 37 of age in doses of 0.1, 0.4, and 1.0 \(\mu\)g/day or vehicle and the rats were sacrificed on day 44 of age. There were no effects on estradiol, progesterone, luteinizing, or follicle-stimulating hormone, nor an effect on ovarian follicle numbers or development. In separate experiments, rats treated with gonadotropin-releasing hormone agonist in doses of 0.04, 0.1, 0.4, or 1.0 \(\mu\)g/day were either irradiated or sham irradiated on day 30 and all groups sacrificed on day 44 of age. Irradiation produced a reduction in ovarian weight and an increase in ovarian follicular atresia. Pretreatment with the agonist prevented the reduction in ovarian weight and numbers of primordial and preantral follicles but not healthy or atretic antral follicles. Such putative radioprotection should be tested on actual reproductive performance.

INTRODUCTION

Gonadal failure is an adverse outcome in the treatment of a variety of benign and malignant conditions due either to the disease itself, single or multiple agent chemotherapy, or ionizing radiation (1). In young men such treatments have been shown to induce defects in spermatogenesis, while in young women the outcome is often sterility and hypergonadotropic hypogonadism (2, 3). Recent reviews have suggested that the outcome in women is largely related to age and reproductive status (4). With improved survival afforded by aggressive chemotherapy and irradiation, attempts have been made to prevent gonadal toxicity associated with these treatments.

The analogue \([D-\text{Leu}^6,\text{des-Gly}^{10}]\) luteinizing hormone-releasing hormone ethylamide (GnRHa) has been suggested to protect ovarian follicles in rats treated with cyclophosphamide (5). The possibility of protection from effects of this alkylating agent were initially suggested in a murine testes model where the histological appearance of the seminiferous tubules was the outcome measure (6). Similar treatment was associated with a partial sparing of sperm counts in the canine model (7). Such findings of gonadal tissue protection are not, however, universal (8). Investigations in humans with the GnRH analogue are inconclusive (9).

Based on the above, we hypothesized that GnRHa might be a possible agent to protect the ovary from ionizing radiation. The mechanism by which this is brought about could be through a cessation of follicular development. Since treatment of younger individuals is becoming more frequent and preservation of fertility is more acute, we felt that the peripubertal age should be the appropriate one to study initially. If GnRHa is indeed protective, we would expect the maintenance of healthy follicles, and the preservation of pituitary and ovarian hormone secretion. These parameters were therefore measured.

MATERIALS AND METHODS

Animals. Sprague Dawley rats were purchased from Charles River and were placed on a 14-h light/10-h dark cycle and were given food and water ad libitum. These animals were treated with GnRHa from days 22 to 37 of age by daily s.c. injection and were sacrificed on day 44. On day 30 of age, the animals were anesthetized with pentobarbital, and both ovarian arteries were ligated through a midline incision while uterine collateral circulation was maintained. The ovaries were exteriorized and positioned on the side of a lead box (1-cm thickness). A similar lead cover was placed over the box and ovaries irradiated as previously reported (10, 11). A General Electric Maxitron 250 was used to deliver a dose of 10 Gy/min to the ovary. Vertical distance from the collimator was 17 cm. Time was calculated as so as to deliver 30 Gy because this dose was previously found to be the median effective dose with respect to ovarian follicular atresia.

On day 44 the rats were weighed, killed, and ovarian and uterine weights recorded. Trunk blood was collected for the measurement of serum estradiol, FSH, and LH. One ovary was placed in Davidson's fixative, sectioned at 7-μm thickness, and stained with hematoxylin and eosin. For experiments relating to the effects of GnRHa alone on ovarian function, every 40th section of the entire ovary was assessed for the number and mean maximal diameter of primordial oocyte and preantral, healthy, and atretic antral follicles. In order to determine if the GnRHa analogue demonstrated radioprotective activity, every third and fifth section was evaluated for the number of primordial, preantral, and antral follicles and diameters, respectively.

Radioimmunoassay. Serum LH and FSH were measured using a homologous double antibody radioimmunoassay technique with reagents provided by National Institute of Diabetes, Digestive and Kidney Diseases (12). Estradiol was extracted from serum with diethyl ether and the dried extracts subjected to radioimmunoassay using specific antisera (13).

Treatment. To determine if GnRHa had an effect on ovarian function in this model, 0.1, 0.4, and 1.0 \(\mu\)g/day GnRHa or vehicle were administered daily from days 22 to 37 of age, and then these animals were sacrificed on day 44.

To determine whether or not GnRHa has radioprotective properties, two experiments were done. In the first, the groups included surgical and radiation controls and treatment groups which received 0.1, 0.4, and 1.0 \(\mu\)g GnRHa or vehicle daily from days 22 to 37 of age. In the second experiment, the controls were similar and treatment groups included doses of 0.04, 0.1, and 0.4 \(\mu\)g GnRHa daily from days 22 to 37 of age. In both experiments the animals were irradiated or sham irradiated on day 30.

There were five animals/group in each experiment for a total assessment of 90 rats.

Histological Analysis. For each experiment a single observer, blinded to groups, recorded numbers and maximal diameters using a Bioquant Digitizing System with a Sakata High Resolution Display Monitor connected to a Leitz microscope and a digitizer. Measurements were calibrated with a standard micrometer slide for each magnification. Two ensure that duplicate counting of primordial follicles was not a possibility, the diameter of the primordial oocyte was assessed in the first experiment. Only follicles containing the nucleus of an oocyte were
assessed and in larger follicles, tracking of follicles was done again to ensure that follicles were not counted more than once. Such tracking also enabled the appropriate allocation of a follicle to preantral or antral categories. All antral follicles were also assessed for the presence of atresia (15). Statistical Analysis. The differences due to treatments were analyzed for significance by the analysis of variance and two-way t test (16).

RESULTS
The effects of GnRHa on ovarian function in terms of weight, serum estradiol, LH, and FSH and detailed follicle analysis are presented in Tables 1 and 2. There are no changes in these parameters. The results of the radiation experiments were combined as there was no difference between the two. All animals survived, but in one case the ovary was found to contain a dense polymorphonuclear leukocyte infiltrate, presumably due to ischemic necrosis, and follicle analysis was not possible. While there was no significant change in rat or uterine weight after ovarian irradiation, there was a marked decline in ovarian weight (Table 3). With radiation there was a significant reduction in the number of primordial, preantral, and healthy antral follicles (Table 4). Pretreatment with GnRHa was associated with greater numbers of primordial and preantral follicles at the 1.0- and 0.4-μg/day dose, respectively, than with radiated control ovaries. The increase in healthy antral follicles above control values was not statistically significant. The number of atretic antral follicles remained the same with radiation combined with GnRHa treatment (Table 4). Pretreatment with all doses of the agonist followed by radiation produced a significant increase in the number of corpora lutea.

Table 1 Effects of GnRHa on ovarian and uterine weights and serum hormone levels

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.1</th>
<th>0.4</th>
<th>1.0</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian wt (mg)</td>
<td>207.5 ± 26.8</td>
<td>263.2 ± 15.3</td>
<td>355.0 ± 23.9</td>
<td>380.5 ± 28.8</td>
<td>NS</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>13.9 ± 2.2</td>
<td>29.4 ± 20.5</td>
<td>10.4 ± 4.2</td>
<td>22.3 ± 5.2</td>
<td>NS</td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>61.4 ± 5.3</td>
<td>76.6 ± 24.9</td>
<td>50.6 ± 2.5</td>
<td>53.0 ± 2.8</td>
<td>NS</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>416.0 ± 40.9</td>
<td>378.1 ± 44.4</td>
<td>321.5 ± 34.8</td>
<td>305.8 ± 38.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

When these data are combined to provide a global view of the ovary as a whole, irradiation resulted in a significant number of follicles, and GnRHa appears to have a dose-related radioprotective action which is significant at 0.4- and 1.0-μg/day doses.

Table 3 shows that pretreatment with the analogue provided similar results on serum hormonal values with or without radiation and agonist pretreatment.

DISCUSSION
Results of effects of GnRHa indicate that the analogue does not have an appreciable effect on follicular development within an experimental model that permits a 7-day period of recovery. Specifically, there was no evidence that the agonist stimulates growth of the primordial follicle either in terms of diameter or movement into the growing pool of follicles. Although GnRHa administration has been reported to cause premature antrum formation, there was no significant variance in healthy or atretic follicle diameters (17).

Effects of radiation on the exteriorized ovary were consistent with initial reports of Mandl and Zuckerman (18) and previous experiments reported from this laboratory (10). Radiation caused a reduction in ovarian weight while uterine weight was maintained. This finding is consistent with the maintenance of serum estradiol and LH levels despite the destruction of many healthy antral follicles. The continued production of estrogen from irradiated ovaries was first noted by Mandl and Zuckerman (18), but the site of production has not yet been identified.

This model of ovarian irradiation requires ligation of the ovarian artery which should not alter the vascular integrity of oxygenated blood flow as there is substantial collateral circulation from the uterine artery, although the measurement of tissue PO2 would be an important determinant. It is noteworthy, however, that rates of follicular atresia, a direct consequence of ischemia, were consistent with control rates of atresia in sham-irradiated animals (10). The one animal with apparent ischemic necrosis could be identified by a marked purulent infiltrate.

Concurrent administration of GnRHa partially ameliorated effects of radiation in this model. There was a sparing of paired ovarian weights which may reflect the influence of corpora lutea more than any follicle compartment. There was a sparing of primordial and preantral follicles at the high doses of agonist. It is of interest that follicles appeared to be spared in proportion to radiosensitivity in absolute but not proportional numbers.

The nature of such a putative radioprotective action of GnRHa is not understood at present, although important observations have been made with respect to the interaction of the agonist with cyclophosphamide (5). Coadministration of the agonist with cyclophosphamide appeared to protect the reduction in medium to large size follicles by inhibiting the process of follicle recruitment into the growing pool of follicles. GnRHa might have acted at an earlier stage of development, preventing growth into a more chemotherapy-sensitive stage.

The present experimental model differs in several respects from the cyclophosphamide model. While radiation was directed to the exteriorized ovary, parenteral cyclophosphamide may alter the reproductive axis by generally altering hypothalamic-pituitary ovarian relationships. Additionally, the lesion induced by cyclophosphamide appears to involve medium to large follicles (16) while it is the primordial and preantral follicles that are most sensitive to radiation. In these current studies, the greatest apparent protection was found in primordial, preantral, and small antral groups. Because these animals
were sacrificed 14 days after radiation and 7 days following GnRHa withdrawal, it is possible that protected follicles may have been uncommitted to growth at the time of radiation (19). This possibility would suggest that in this model, protection does not occur by arresting follicular development alone, although it may occur.

Other possible mechanisms of the apparent protection may include direct or indirect actions of GnRHa on the ovary. Recently, a physiological role of native GnRH in the ovary has been proposed because the administration of a GnRH agonist with FSH resulted in increased numbers of ovulations in response to human chorionic gonadotropin (20). The dramatic increase in corpora lutea in agonist-pretreated animals suggests a response to human chorionic gonadotropin (20). The dramatic increase in corpora lutea in agonist-pretreated animals suggests the functional capacity of these follicles in terms of reproductive capacity and hormonal production.

ACKNOWLEDGMENTS

The authors wish to recognize the assistance of Dr. D. R. Mattison in the preparation of this manuscript. The support of J. Bennett is appreciated.

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