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Gonadal Protection and Fecundity Rates in Cyclophosphamide-treated Rats

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ABSTRACT

Premature ovarian failure and reduced fecundity are well-documented consequences of cytotoxic chemotherapy used to treat patients with malignant diseases. To investigate the ability of different hormonal agents to block the effects of cyclophosphamide (CTX) on reproductive function, sexually mature female Long-Evans rats were studied. Model development demonstrated that CTX, 6 mg/kg/day, 5 days/week for 3 weeks, was successful at inducing acyclicity and significantly reducing fertility and fecundity, with acceptable mortality, when compared to higher/lower dosages. Utilizing this model, animals were treated with CTX in combination with an inert vehicle, Lupron, 80 μg/kg every 24 h, Lupron, 40 μg/kg every 12 h, or s.c. progesterone capsules obtaining serum progesterone levels of 20–30 ng/ml. We concluded that progesterone was able to protect the gonad from the negative effects of CTX, maintaining fertility and fecundity rates not significantly different from those of untreated control animals. Lupron given every 12 h had a similar effect on fertility, but failed to protect fecundity ($P < 0.001$).

INTRODUCTION

Cytotoxic chemotherapy used to treat nongynecological malignancies and some nonmalignant diseases commonly produces menstrual irregularities, immediate or subsequent ovarian failure, and associated infertility (1–11). The potential for chemotherapy-induced gonadal dysfunction and diminished fertility is related to the cytotoxic agent used, its dosage, dose schedule, and patient age (12–19).

Other investigators have proposed several strategies to protect gonadal tissue from chemotherapeutic agent-induced destruction, (20, 21) with these studies demonstrating variable success (22, 23). Because of these mixed results further studies are needed to demonstrate the effects of protective strategies. Such studies should include: (*a*) an objective determination of any toxic effects on gonadal tissue with or without the agent, and (*b*) an assessment of fecundity which is the most important reflection of the status of reproductive function.

To that end, we decided to study the protective effect of a gonadotropin-releasing hormone analogue (Lupron) or progestin therapy in a standard rat model, using fecundity as the measure of protective effect.

MATERIALS AND METHODS

Model Development. Sixty sexually mature virginal female, Long-Evans rats were used. Animal care procedures were performed in accordance with the standards described in the National Institute of Health Guide for Care and Use of Laboratory Animals. Mean age (12 weeks) and weight (43.3 g) were documented. Prior to utilization of any animal, normal estrous cyclicity was confirmed by vaginal cytological smear quantitating the nuclear cytoplasmic ratio and the cellular characteristics. At 90 days of age, regular cycling animals with 4- or 5-day estrous cycles were enrolled in our studies. The animals were divided into 5 groups of 12 animals each. Daily i.p. injections of cyclophosphamide were administered in an aseptic manner 5 days a week for 4 weeks. Using sterile technique, cyclophosphamide was prepared every other day in 0.9% NaCl solution at a concentration of 1 mg/ml. Dosages of cyclophosphamide utilized were 4, 6, 8, and 12 mg/kg of body weight. Control animals received only the liquid vehicle at a volume of 8 ml/kg of body weight. Estrous cycle patterns were monitored during and after treatment. When cyclicity persisted despite treatment or cyclicity returned after cyclophosphamide therapy was discontinued, the rats were mated. The interval between completion of cyclophosphamide therapy and institution of mating ranged from immediately to 2 weeks, depending on when cyclicity returned, if at all. Mating was confirmed by the presence of sperm on vaginal smears. Those animals with sperm present in their vaginal lavage the morning after mating were placed in individual cages for the duration of any resulting pregnancy. Animals were considered fertile only if a pregnancy occurred that was documented by the birth of at least one pup. Pregnancy rates and litter size (including both dead and alive pups) were determined for animals that successfully mated and conceived. Two days after delivery, the mothers were placed back into the group cages. One month later, the regularly cycling females were mated again. After delivery of the second litter, or in rats that did not reestablish cyclicity within 2 weeks of discontinuation of cyclophosphamide chemotherapy, the animals were sacrificed and the ovaries were processed for histological evaluation, using standard hematoxylin and eosin preparations. Follicle counts were performed and compared to control ovaries. The amount of follicular destruction was classified as mild (10–30% decrease in follicles), moderate (40–70% decrease in follicles), or severe (80% or more).

Gonadal Protection by Hormonal Agents. In order to determine whether hypothalamic pituitary gonadal axis suppression with two different hormonal agents [Leuprolide acetate (Lupron) at two different dosage schedules and progesterone] was effective in blocking the negative effects of cyclophosphamide on fertility and fecundity, further investigations were undertaken. Four groups of regularly cycling rats were treated with 6 mg/kg cyclophosphamide for 3 weeks as described above. Animals were treated with cyclophosphamide alone ($n = 20$) or cyclophosphamide simultaneously with Lupron, 80 μg/kg s.c. each day ($n = 20$), Lupron, 40 μg/kg/s.c. every 12 h ($n = 20$), or s.c. progesterone capsules obtaining serum progesterone levels of 20–30 ng/ml ($n = 20$) (24). Two groups of animals received liquid vehicle at a volume of 6 ml/kg of body weight (control for cyclophosphamide) plus either s.c. inert vehicle each day (control for Lupron) ($n = 10$), or blank Silastic capsules (control for progesterone) ($n = 10$).

To ensure steady state levels and prechemotherapy gonadal suppression, treatment with the hormonal agents or vehicle was instituted 1 week prior to beginning cyclophosphamide therapy. In those animals receiving pellets, the implants were removed and repositioned every 2 weeks to ensure that proper serum progesterone levels were maintained. The day following completion of cyclophosphamide injections, Lupron and vehicle injections were stopped and the progesterone and blank Silastic capsules were removed. The estrous cyclicity of the animals was followed, mating was allowed, and the tests of fertility and fecundity were carried out as described in the model development experiment.

RESULTS

Model Development. Weight loss and mortality rates are presented in Table 1. Both were excessive (>20% and >50%, respectively) in the 8- and 12-mg/kg groups. At the completion of the third week of cyclophosphamide treatment, chemotherapy was withheld in the 12-mg/kg group. Animals received only

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the inert vehicle for the final week of i.p. injections, in an attempt to limit any further toxicity from the active agent. Cyclophosphamide-induced acyclicity occurred in 100% of the 12-mg/kg and 8-mg/kg groups, 84% of the 6-mg/kg group, and 32% of the 4-mg/kg group. None of the control animals had persistent diestrus.

After the completion of therapy, 75% (3 of 4 animals) of the 12-mg/kg group, 80% (4 of 5 animals) of the 8-mg/kg group, 89% (8 of 9 animals) of the 6-mg/kg group, 91% (10 of 11 animals) of the 4-mg/kg group, and 100% (12 of 12 animals) of control animals had return or persistence of cyclicity. Subsequent mating behavior was observed only in those rats with persistence or reestablished cyclicity. Not all females that mated were fertile. Fertile animals had varying litter sizes (Table 2) with the 6-, 8-, and 12-mg/kg groups having significant decreases when compared to the control animals. Those animals that were mated a second time demonstrated a significant reduction in fertility rates and litter sizes when compared to similar data from the initial mating.

In an attempt to decrease mortality rates while maintaining the negative effects on fertility and fecundity, a group of 18 animals were treated with 6 mg/kg cyclophosphamide for a period of 3 weeks. This dose reduction decreased the mortality rate from 50 to 24% while decreasing fertility (71%) and fecundity (6.2 ± 1.7 pups/litter), similar to the animals receiving 4 weeks of cyclophosphamide.

Table 1 *Dosage-dependent weight change and mortality rates*

Stepwise increases in cyclophosphamide dose increased weight loss and mortality rates when compared to animals receiving inert vehicle only.

Dosage (kg/mg)	Weight change (%)	Mortality (% of total)
0	+2	0
4	−2	8
6	+1	60
8	−23	75
12	−22	75

Table 2 *Dose-dependent fertility rates and litter size*

Stepwise increase in cyclophosphamide dose decreased both fertility rates and litter size when compared to animals receiving inert vehicle only.

Dosage (kg/mg)	% fertile	Litter size
0	83	6.7 ± 1.3[a]
4	70	5.9 ± 1.6
6	50	2.9 ± 1.5
8	33	2.3 ± 1.4
12	33	0.7 ± 0.7

[a] Mean ± SD.

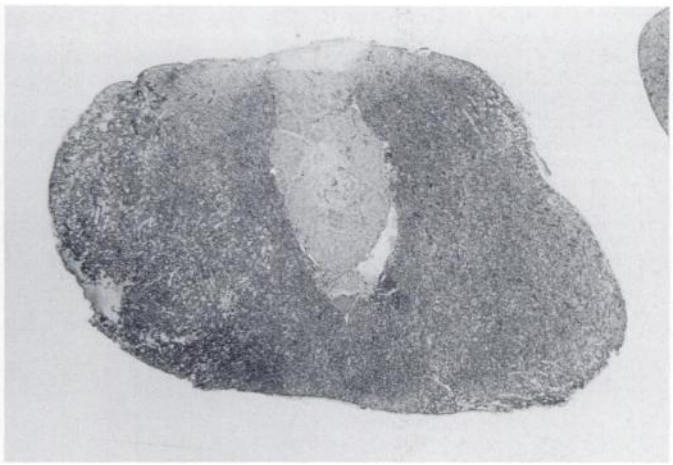

Fig. 1. Extensive destruction of follicular units in the ovary of a rat receiving a high dose (12 mg/kg) of cyclophosphamide. H & E, × 400.

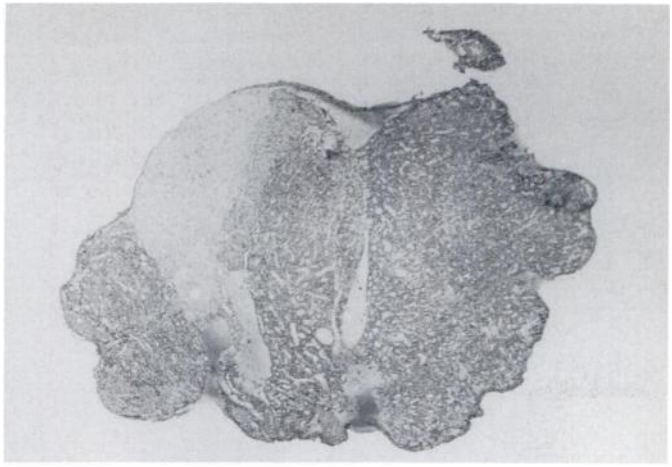

Fig. 2. Moderate amount of follicular unit destruction in ovary of rat receiving moderate dose (6 mg/kg) of cyclophosphamide. H & E, × 400.

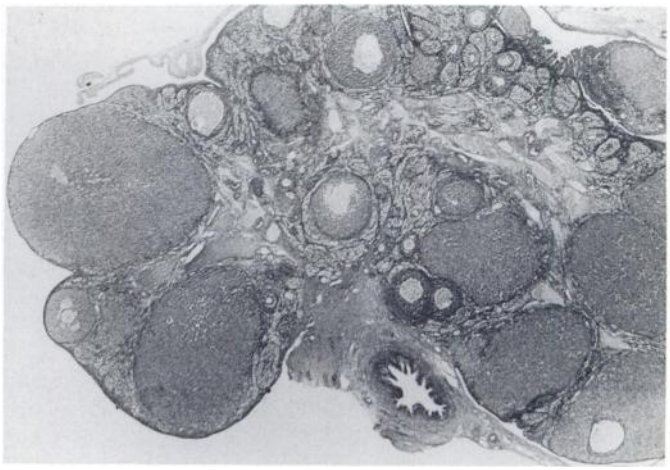

Fig. 3. Minimal amount of follicular unit destruction in ovary of rat receiving low dose (4 mg/kg) of cyclophosphamide. H & E, × 400.

Histological evaluation demonstrated that the higher dosage of cyclophosphamide (8 and 12 mg/kg) destroyed 80% or more of ovarian follicular units when compared to control (Fig. 1). The 6-mg/kg dosage had an intermittent amount of destruction (Fig. 2), with the 4-mg/kg dosage having a minimal effect when compared to controls (Fig. 3).

Gonadal Protection by Hormonal Agents. Findings of the investigation of both Lupron and progesterone abilities to protect fertility and fecundity are summarized in Table 3. There was no significant difference in the two control groups, (s.c. injection of inert vehicle or blank Silastic capsules) and therefore these groups are combined. As was the experience in our initial model development studies, after delivery of the first litter of pups, fertility and fecundity rates declined proportionately for each of the groups.

Progesterone was able to protect the gonad from the negative effects of cyclophosphamide, maintaining fertility and fecundity rates not significantly different from those of control animals. Lupron when given on a once a day basis failed to demonstrate any protective effect. However when given at the same total dose but on a split every 12-h basis, Lupron was able to protect fertility but failed to protect fecundity when compared to controls ($P < 0.001$) or progesterone-treated animals ($P < 0.001$).

Table 3 *Agent-dependent mortality rates, fertility rates, and litter size*

Progesterone implants were able to protect the gonad from the negative effects of chemotherapy, maintaining fertility/fecundity rates similar to those of control animals. Split dose Lupron was able to protect fertility, but failed to protect fecundity when compared to controls ($P < 0.001$) or progesterone-treated animals ($P < 0.001$).

Group	% mortality	% cyclicity	% fertile	Litter size	No. of animals
Control	5	100	93	13.3 ± 0.4[a]	20
CTX[b]	25	60	80	6.3 ± 1.3	20
CTX + L.24h	45	55	71	0.7 ± 0.7	20
CTX + L.12h	25	73	100	8.0 ± 1.5	20
CTX + P	10	83	100	11.4 ± 0.8	20

[a] Mean ± SD.

[b] CTX, cyclophosphamide (6 mg/kg every day); L.24h, Lupron (80 μg/kg every day); L.12h, Lupron (40 μg/kg every 12 h); P, progesterone capsules.

DISCUSSION

Advances in chemotherapy technology and supportive care techniques have led to effective treatment and cure in many malignant diseases that were previously assumed to be universally fatal (25–29). Unfortunately, up to 70% of females who have received prolonged chemotherapy are infertile/subfertile secondary to ovarian dysfunction, or suffer from premature ovarian failure (1–22). Many of these patients have been treated for nonmalignant, nongynecological diseases (*e.g.*, systemic lupus erythematosus, minimal lesion nephrotic syndrome, or rheumatoid arthritis) or nongynecological malignancies. Cyclophosphamide, a commonly utilized alkylating agent when given to ovulating women in a continuous manner for greater than 6 months appears to be the most damaging of the commonly used chemotherapeutic agents (3, 5).

A number of hormonal manipulations have been proposed to protect the ovarian follicular unit from the disruptive effects of cytotoxic agents. The use of oral contraceptives has most commonly been proposed, with the recent thought that gonadotropin-releasing hormone analogues may be more effective. Previous investigators have not used a standard model of cytotoxic agent-induced ovarian dysfunction that uses fecundity as the final measure of reproductive function. Similarly, no investigator has utilized such a model to compare agents that might have a protective effect on the follicular unit. Utilizing this type of model, we were able to demonstrate, in this preliminary study, that progesterone was successful at protecting fecundity while Lupron failed to demonstrate such a protective effect.

These findings, if confirmed by larger studies, may be applicable to the clinical management of females of reproductive age who are receiving cytotoxic chemotherapy. Using agents that have an effect on the human follicular unit which mimic the effect of continuous progesterone in the rat, medium dosage OCP,[3] ovarian function should be adequately protected. It is important that a medium dose (35 μg or greater) OCP be utilized, as investigators have demonstrated that the variable dose OCP often fails in suppressing follicular maturation and subsequent ovulation (30). Failure to suppress follicular recruitment may also fail to protect the gonad. So as to induce ovarian quiescence prior to institution of chemotherapy, OCPs should be started as soon as the decision to utilize cytotoxic agents is made. The OCPs should be continued uninterrupted until therapy is completed. If further chemotherapy is deemed necessary, the OCPs could then be reinstituted.

If our preliminary animal findings are confirmed, studies should be performed in the human model to support or refute our hypothesis.

[3] The abbreviation used is: OCP, oral contraceptive.

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