Gonadal Protection and Fecundity Rates in Cyclophosphamide-treated Rats

F. J. Montz,° A. John Wolff, and Joseph C. Gambone2

Abstact

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 protective efficacy. 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 progesterin therapy in a standard rat model, using fecundity as the measure of protective effect.

Materials and Methods

Model Development. Sixty sexually mature virgin 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. Male 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 adequate 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...
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.

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).

Table 1 Dosage-dependent weight change and mortality rates

<table>
<thead>
<tr>
<th>Dosage (mg/kg)</th>
<th>Weight change (%)</th>
<th>Mortality (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+2</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>-2</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>+1</td>
<td>60</td>
</tr>
<tr>
<td>8</td>
<td>-23</td>
<td>75</td>
</tr>
<tr>
<td>12</td>
<td>-22</td>
<td>75</td>
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</table>

Table 2 Dose-dependent fertility rates and litter size

<table>
<thead>
<tr>
<th>Dosage (kg/mg)</th>
<th>% fertile</th>
<th>Litter size</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>83</td>
<td>6.7 ± 1.3*</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>5.9 ± 1.6</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>2.9 ± 1.5</td>
</tr>
<tr>
<td>8</td>
<td>33</td>
<td>2.3 ± 1.4</td>
</tr>
<tr>
<td>12</td>
<td>33</td>
<td>0.7 ± 0.7</td>
</tr>
</tbody>
</table>

* 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.

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).
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).

<table>
<thead>
<tr>
<th>Group</th>
<th>% mortality</th>
<th>% fertility</th>
<th>% cyclic</th>
<th>Litter size</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>100</td>
<td>93</td>
<td>13.3 ± 0.4</td>
<td>20</td>
</tr>
<tr>
<td>CTX</td>
<td>25</td>
<td>60</td>
<td>80</td>
<td>6.3 ± 1.3</td>
<td>20</td>
</tr>
<tr>
<td>CTX + L24h</td>
<td>45</td>
<td>55</td>
<td>71</td>
<td>0.7 ± 0.7</td>
<td>20</td>
</tr>
<tr>
<td>CTX + L12h</td>
<td>25</td>
<td>73</td>
<td>100</td>
<td>8.0 ± 1.5</td>
<td>20</td>
</tr>
<tr>
<td>CTX + P</td>
<td>10</td>
<td>83</td>
<td>100</td>
<td>11.4 ± 0.8</td>
<td>20</td>
</tr>
</tbody>
</table>

Differences shown are significant at P < 0.001.

The abbreviation used is: OCP, oral contraceptive.

**REFERENCES**


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