Anovulation in the prevention of cytotoxic-induced follicular attrition and ovarian failure

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BACKGROUND: Gonadal failure secondary to alkylating agents may be related to ovulatory status. The objective of this investigation was to evaluate whether anovulation protected ovarian follicles during treatment with cyclophosphamide. METHODS: Four groups (n = 20 mature female Sprague–Dawley rats per group) were studied: control (group I), 5 mg/kg/day cyclophosphamide only (group II), 5 mg/kg/day cyclophosphamide and the combination of 50 μg ethinyl estradiol/2 mg norgestrel (group III) and 5 mg/kg/day cyclophosphamide and 2.5 μg leuprolide acetate daily (group IV). Animals were sacrificed after 4 weeks of treatment. Follicles were classified as medium (300–450 μm) and large (>450 μm) per section of ovary. RESULTS: Group II developed a significantly greater number of medium and large follicles [15.1 ± 6.6 and 4.9 ± 1.9 (mean ± SD), respectively] compared with group I [7.1 ± 2.1 and 1.0 ± 0.7 (mean ± SD), respectively] (P < 0.05). Groups III and IV developed a significantly greater number of medium follicles [13.2 ± 2.5 and 10.8 ± 2.3 (mean ± SD), respectively] compared with group I (P < 0.05). There was a trend toward a greater number of large follicles in groups III and IV when they were compared with group I. There were no differences in medium follicles in groups II, III and IV. No differences were noted in the number of large follicles between groups III and IV (2.9 ± 1.2 and 2.3 ± 1.0, respectively). CONCLUSIONS: These results suggest that in the rat model, cyclophosphamide exerts a stimulatory effect on the ovary resulting in the greater development of both medium and large follicles. Anovulation conferred no protection against cyclophosphamide-induced gonadal toxicity. These data suggest that in the rat model, cyclophosphamide may result in ovarian failure by enhancing recruitment of follicles regardless of ovulatory status or hormonal milieu.

Key words: chemotherapy/cyclophosphamide/cytotoxic therapy/oocyte apoptosis/ovarian failure

Introduction

Several cytotoxic agents used to treat neoplastic or immunological diseases may alter gonadal function in humans (Janssen and Genta, 2000). Cyclophosphamide, an alkylating agent, is widely used as an antineoplastic or immunosuppressive agent. In addition to the well known complications of alopecia, leukopenia and thrombocytopenia, gonadal failure is described in 30–70% of women treated with cyclophosphamide (Meirow et al., 1999). The exact aetiology of gonadal failure in this setting is unknown. Data suggest a direct cytotoxic effect on follicles, possibly dependent on age or hormonal milieu (Marcello et al., 1990). Studies of childhood leukaemia treatment suggest that prepubertal patients who receive cyclophosphamide maintain or recover gonadal function better than similarly treated adults (Chapman and Sutcliffe, 1981). These outcomes suggest that active or quiescent germinal cells may have an enhanced or reduced sensitivity, respectively, to cyclophosphamide because of varying mitotic activity. This variable outcome of gonadal activity after treatment with alkylating agents suggests that pharmacological suppression of gonadal function may spare the ovary any toxicity associated with these agents (Rivkees and Crawford, 1988).

The purposes of the present investigation using the rat model are 3-fold: (i) to determine the maximum tolerated dose(s) and cytotoxic effects of cyclophosphamide on ovarian follicles in a dose–response group; (ii) to compare the effectiveness of two dosages of two hormonal regimens in achieving anovulation; and (iii) to determine if the induction of an anovulatory state by two different hormonal methods may spare the ovary the cytotoxic effects of cyclophosphamide. The investigation was performed in two parts as a dose–response study and as an experimental study.

Materials and methods

Animal model

Mature female Sprague–Dawley (Charles River) rats that weighed between 200 and 250 g were used for all groups. The age of the animals at the start of the study was 60–70 days. The rats were fed Standard Purina Rat Chow (Ralston-Purina Company) ad libitum and were housed in windowless rooms illuminated from 07.00 to 19.00 h. To ensure adequate adaptation, all animals were observed in this environment for 14 days prior to commencing treatment. Animals were weighed weekly and examined daily. In conducting the research
described in this report, compliance with the USDA Animal Welfare Act and the National Research Council’s Guide for the Care and Use of Laboratory Animals was maintained. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. The protocol was reviewed and approved by the Institutional Review Board and Research Advisory Committee.

Drug treatment
Cyclophosphamide was administered by daily i.p. injection. The combination of ethinyl estradiol and norgestrel was delivered using time-released, subdermally placed capsules. The capsules consisted of the active hormones embedded in a methyl cellulose matrix and were designed to deliver constant, fixed daily doses for up to 60 days per capsule. All capsules were placed s.c. through a 1 cm incision in the right paravertebral area under light general anaesthesia achieved with metofane. Leuprolide acetate was injected s.c. Control animals were injected i.p with sterile saline solution.

Treatment protocols
The investigation was performed as a dose–response study and experimental study. In the dose–response study, the maximum tolerated dose of cyclophosphamide and the anovulatory doses for the combination of ethinyl estradiol/norgestrel and the GnRH agonist, leuprolide acetate, were studied. Cyclophosphamide was chosen as the alkylating agent because of its known impact on gonadal function and its prevalence in clinical practice. The combination of ethinyl estradiol/norgestrel and leuprolide acetate were selected as anovulatory agents to study the impact of two different hormonal environments on cyclophosphamide toxicity, namely an estrogen/progestin-rich environment and a hypoestrogenic environment secondary to the ethinyl estradiol/norgestrel combination and leuprolide acetate, respectively. Toxicity was assessed by monitoring weight weekly and physical well-being daily and by measuring complete blood counts after sacrifice. Haematological function was assessed to determine whether the varying hormonal milieu induced by the two anovulatory protocols altered cyclophosphamide toxicity. The least toxic, effective dose of cyclophosphamide and lowest effective dose of the two anovulatory regimens were used in the experimental study. The experimental study sought to investigate if there was any protective effect of these two anovulatory regimens on follicular number after treatment with cyclophosphamide.

Dose–response study
To determine the maximum tolerated dose of cyclophosphamide needed to induce follicular attrition, the number of follicles as determined by ovarian histology was studied after treatment. Sixty rats were divided into three groups. All rats were given a 50 mg/kg i.p. loading dose of cyclophosphamide and maintenance doses of cyclophosphamide of either 5, 10 or 15 mg/kg/day i.p. After 6 weeks respectively of treatment, animals were sacrificed.

To compare the anovulatory doses of the combination of ethinyl estradiol/norgestrel and the GnRH agonist, leuprolide acetate, 40 rats were divided into two groups. Combinations of 100 mg of ethinyl estradiol/4 mg of norgestrel, and 50 mg of ethinyl estradiol/2 mg of norgestrel were administered s.c using time-release capsules to 20 rats in each group. In the second group, 2.5 mg of leuprolide acetate s.c. twice daily was compared with 2.5 mg s.c. daily in 20 rats in each group. Ovulatory status was assessed by examination of vaginal cytology. For analysis of estrous cycles, vaginal smears were obtained on a daily basis, stained with haematoxylin and eosin and examined for cellular content. Vaginal smears were indexed according to cellularity to determine ovulatory status. Rats were assessed as being in one of four stages of the cycle: proestrus, estrus, metestrus or diestrus. All animals were cycling for a 2 week observation period prior to capsule placement or s.c. injection. Anovulatory smears were reliably induced within 6 days in all animals after capsule placement and 3 weeks after the first injection of leuprolide acetate, and maintained for the duration of observation.

Experimental study
In the second part of the investigation, the impact of cyclophosphamide on follicular number without treatment with anovulatory agents and after induction of anovulation was studied and compared with saline-treated controls. All experimental animals with the exception of the control group were treated with i.p. cyclophosphamide alone or in combination with one of the two anovulatory agents. Four groups of 20 mature Sprague–Dawley female rats in each group were studied as follows: saline-treated control (group I), cyclophosphamide only (group II), cyclophosphamide and ethinyl estradiol/norgestrel (group III) and cyclophosphamide and GnRH analogue (group IV). Treatment with cyclophosphamide was started after an observation period in groups I and II and a treatment in groups III and IV with ethinyl estradiol/norgestrel and leuprolide of 3 weeks. Anovulation was documented using vaginal cytology prior to starting treatment and assessed weekly during treatment. Animals were sacrificed after 4 weeks of treatment and the ovaries fixed. Capsules for hormone delivery were identified in all animals in group III at time of death. If no capsule could be found, the animal was excluded from the study. All animals were sacrificed between 08.00 and 09.00 h.

Haematological studies
All animals were sacrificed by cervical dislocation. Blood was drawn by cardiac puncture for complete blood counts and analysed for total leukocytes, differential count and haematocrit. Haematological studies were performed as a secondary measure of the impact of cyclophosphamide on metabolic function, well-being and toxicity, and to assess any differential effect that the anovulatory agents may have had on cyclophosphamide toxicity.

Histological analysis of ovarian tissue
Both ovaries were removed in their entirety for processing. After removal, the ovaries were placed in formalin. The tissue was embedded in paraffin, step-sectioned and stained with haematoxylin and eosin. Among the various parameters, follicular diameter was chosen as the parameter to classify follicles and assess the impact of treatments (Pedersen and Peters, 1968; Hirshfield and Midgley, 1978). All follicles were measured in two dimensions. The maximum follicular diameter and a diameter at right angles to it were used to calculate a mean diameter for each follicle. Only those follicles that contained the nucleus of the oocyte were included in the final count as a safeguard. The ovaries were examined at 5 μm sections and studied for the number of medium (300–450 μm) and large (>450 μm) follicles per section of ovary. An average of 50 sections per ovary were studied (range 37–63). The degree of follicular atresia and primordial follicle count were not assessed.

Statistical analysis
Statistical analysis was performed using a two-way analysis of variance and Bonferroni correction. Level of significance was considered to be $P \leq 0.05$ (Instat Instant Biostatistics, Graph Pad Software, San Diego, CA).
Results

Dose–response study

The maximum tolerated dose of cyclophosphamide was 5 mg/kg i.p. after a 50 mg/kg loading dose. Doses of 10 and 15 mg/kg resulted in a mortality of 50 and 65%, respectively, after 3 weeks of treatment for both groups. Spontaneous deaths occurred as early as the second and third week of treatment for animals treated with 15 and 10 mg/kg/day. Complications were also greater in these groups. Weights dropped significantly during the treatment interval in these two groups (Table I). Doses of 15 mg/kg resulted in hind-leg paralysis in one-third of animals surviving beyond 3 weeks. Periorbital haemorrhage and loss of coat lustre were noted in 50 and 100% of animals surviving beyond 4 weeks. Because of this extreme toxicity, neither dose was considered an option for use in the experimental study.

In the group treated with 5 mg/kg, six spontaneous deaths occurred between the fourth and sixth weeks of treatment. No significant weight loss was noted over the 6 week treatment interval. Weight remained stable, with a gradual downward trend between treatment weeks 5 and 6. Because of the spontaneous deaths between weeks 5 and 6 of the treatment period, a treatment interval of 4 weeks was chosen. In a saline-treated control group (n = 20), weights increased significantly from the start to completion of the 6 week period [200.1 ± 5 to 245 ± 41 g (mean ± SD)]. All control animals were sacrificed at 6 weeks. There were no differences in total white blood counts or haematocrit between treated and control groups (Table I). At a dose of 5 mg/kg/day, a significant impact on marrow function was noted when compared with controls. A significant increase in polymorphonuclear cells and a decrease in lymphocytes were noted. This reversal of the ratio of polymorphonuclear cells to lymphocytes is characteristic of cyclophosphamide toxicity in this model (Wheeler et al., 1962).

All anovulatory regimens were effective. S.c. capsules of 50 µg ethinyl estradiol/2 mg norgestrel or leuprolide acetate 2.5 µg daily s.c. resulted in anovulation and in the shortest time period as determined by vaginal histology. There was no morbidity or mortality in these groups and no change in weights when compared with controls at the start and completion of treatment (Table III).

Experimental study

Follicle counts are presented in Table IV and Figure 1 for all groups. Treatment with cyclophosphamide resulted in a significantly greater number of medium and large follicles in group II [15.1 ± 6.1 and 4.9 ± 1.9 (mean ± SD)], respectively, when compared with group I (7.1 ± 2.1 and 1.0 ± 0.7, respectively) (P < 0.05). Treatment with cyclophosphamide and suppression with ethinyl estradiol/norgestrel and leuprolide acetate resulted in a significantly greater number of medium follicles [13.2 ± 2.5 and 10.8 ± 2.3 (mean ± SD) for groups III and IV, respectively] when compared with group I.
and no difference when compared with group II. No differences were noted in the number of large follicles between groups III and IV (2.9 ± 1.2 and 2.3 ± 1.0, respectively). There was a trend toward a greater number of large follicles when groups III and IV were compared with group I that did not achieve significance. No difference in the number of large follicles was noted between groups II, III and IV.

There was no difference in weight between groups at the start of treatment (201 ± 6 versus 201 ± 9 versus 203 ± 7 versus 200 ± 10 for groups I, II, III and IV, respectively) (Table V). There were significant weight differences in all groups treated with cyclophosphamide at the conclusion of the 4 week treatment interval when compared with controls (233 ± 5 for group I versus 197 ± 10 versus 184 ± 12 versus 167 ± 12 for groups II, III and IV, respectively (weight in g) (P < 0.05). Significant weight loss was noted for groups III and IV only when compared with weights at the start of the treatment (211 ± 7 and 216 ± 2 at the start and 184 ± 4 and 167 ± 7 at the conclusion of treatment for groups III and IV, respectively). There was a significant reduction in total white blood count when groups I and IV were compared. A reversal of the ratio of polymorphonuclear cells to lymphocytes was observed consistent with that noted in the dose–response study across all groups, suggesting a similar haematological impact regardless of hormonal milieu (Table II).

Discussion
The aetiology of follicular exhaustion and ovarian failure in association with alkylating agents is not clear. The findings of the present study suggest significant alterations in distribution of follicular sizes in response to treatment with cyclophosphamide in the rat model. Two observations are notable: a stimulatory effect of cyclophosphamide on follicle number and a failure of anovulation to protect against cyclophosphamide toxicity. These results suggest that cyclophosphamide stimulates the appearance of follicles in the pre-Graafian size class (300–450 μm). Significant increases in medium and a statistically insignificant trend for an increase in large follicles were observed in all of the groups treated with cyclophosphamide. These data suggest that cyclophosphamide may exert a stimulatory effect on the follicular dynamics, resulting in the development of a significantly greater number of both medium and large follicles when compared with controls. This trend was also noted in groups III and IV, in which two anovulatory agents were used to achieve ovarian quiescence. This finding of follicular stimulation is similar to that noted when a single oral dose of cyclophosphamide was used successfully for the induction of superovulation in rats (Russel et al., 1973).

Cyclophosphamide appears to influence the number of growing follicles. The drug may induce a discontinuous movement of follicles into the next class size (from small
Cytotoxic-induced ovarian failure

Table V. Weights (g) for the experimental group in the cyclophosphamide-treated groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial weight</th>
<th>Week of treatment</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Group I</td>
<td>201 ± 6</td>
<td>207 ± 7</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Group II</td>
<td>201 ± 9</td>
<td>211 ± 5</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Group III</td>
<td>203 ± 7</td>
<td>211 ± 7</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Group IV</td>
<td>200 ± 10</td>
<td>216 ± 2</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
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</tbody>
</table>

<sup>a</sup>Significant when compared with controls ($P < 0.05$).
<sup>b</sup>Significant when compared with start of treatment ($P < 0.05$).
Data are the mean ± SD.

<200 to medium 300–450 μm). These unique observations suggest that the alkylating agent, cyclophosphamide, and/or its metabolites, in addition to gonadotropins and anti-estrogens, are unique among drugs in their ability to increase the maturation of follicles and movement toward ovulation. Cyclophosphamide may force smaller follicles beyond a critical stage into the next pool of growing and developing follicles. The observations of ovarian stimulation noted in the present study are restricted to medium and large follicles. It is unclear whether primordial follicles were affected. By study design, any possible impact on primordial follicles by and/or differential sensitivity to cyclophosphamide were not evaluated. Given the fixed, limited store of follicles in an ovary, it is tempting to infer, oversimplistically, that movement of follicles into a larger size category occurs at the expense of the pool of primordial follicles.

Prior studies together with the data of the present study suggest that follicle attrition and gonadal failure secondary to cyclophosphamide may be due to a marked acceleration in follicular maturation, depletion and eventual exhaustion. The observation interval in the present study was long enough to induce movement of follicles to larger size classes. However, it may have been too brief to observe follicular exhaustion noted in prior studies (Meirow et al., 1999). Morbidity and mortality observed during the dose–response study precluded a longer duration of treatment. This impact on follicular activity may be due to an effect of cyclophosphamide or, after hepatic oxidation, its aldehyde derivatives on the follicle itself, the granulosa cell or the basement membrane. Two laboratory models support this concept. Cumulus-enclosed germinal vesicle stage porcine oocytes demonstrate a dose–response inhibition of nuclear maturation when cultured with cyclophosphamide (Chen et al., 1998). Data also suggest that the 4-hydroperoxycyclophosphamide-activated metabolite of cyclophosphamide directly decreases granulosa cell survival and function (Ramahi-Ataya et al., 1988). Electron microscopy of human ovarian cortical tissue cultured with cyclophosphamide suggests toxicity to follicles possibly mediated through damage to both granulosa cells and basement membrane (Raz et al., 2002).

Age and hormonal status appear to correlate with the effect of cyclophosphamide on follicles (Marcello et al., 1990; Familiari et al., 1993). Observational studies suggest that prepubertal patients administered alkylating agents may be spared gonadal damage. In this age group, protection may be afforded secondary to a larger reservoir of primordial oocytes (Faddy et al., 1992). These observations suggest a possible role for hormonal suppression of gonadal function to minimize the impact alkylating agents may have on gonadal function (Chapman and Sutcliffe, 1981; Horning et al., 2001). In the present study, cyclophosphamide induced ovarian damage regardless of ovulatory or hormonal status. The impact of cyclophosphamide was observed in spite of varying the hormonal milieu by inducing an estrogen/progestin steady state with the combination ethinyl estradiol/norgestrel or a hypoestrogenic environment using the GnRH analogue leuprolide acetate.

Previous studies of the impact of hormonal suppressive therapy in males and females of different species on gonadal protection during treatment with cytotoxic agents have been conflicting (Fossa et al., 1988; Blumenfeld et al., 1996, 2000). Testicular suppression with the GnRH analogue triptorelin was protective in rats (Karashima et al., 1988). In contrast, nafarelin potentiated testicular damage in dogs (Goodpasture et al., 1988). In humans, results after treatment with both oral contraceptives and a variety of GnRH analogues have been inconclusive, in part due to study design and low numbers of patients studied (Johnson et al., 1985; Thibaud et al., 1998). In the present study, no protective effect of anovulation against follicular attrition using two protocols was observed in the rat model. Pathways of oocyte loss are complex and may involve factors other than the degree of follicular stimulation and the hormonal environment of the follicle (Gougeon, 1996). In a limited comparative study in humans, ovarian suppression with GnRH analogues was not an effective method to prevent ovarian failure (Waxman et al., 1987). The hypothesis that any reduction in endogenous follicular stimulation may spare the ovary the impact of cyclophosphamide may be an oversimplification.

Results of the present study suggest that induction of anovulation in the rat model, whether by combination ethinyl estradiol/norgestrel or a GnRH analogue, may not provide a reliable method of maintaining ovarian function and reproductive potential for patients using cyclophosphamide regard-
less of indication. Assisted reproductive technologies such as IVF, reimplantation of cryopreserved ovarian tissue, or cryopreservation of oocytes may be more effective for this purpose. Several reports describe favourable outcomes with the second of these techniques, first harvesting, cryopreserving and then reimplanting ovarian cortical strips (Gosden, 1990; Oktay and Karlikaya, 2000; Radford et al., 2001). In these cases, estradiol secretion and folliculogenesis were resumed after transplantation. Although cryopreservation may induce damage on chromosomes X, 16 and 18, functional ovarian tissue and a cohort of chromosomally competent oocytes remain within the ovarian substance, offering the possibility of continued secretion of estradiol and progesterone and reproduction (Poiriot et al., 2002). A second option for patients confronting cytotoxic chemotherapy is cryopreservation of oocytes. In this circumstance, immature oocytes may be retrieved, cryopreserved and stored until needed (Yoon et al., 2000). Early studies with vitrified oocytes have resulted in live births (Hong et al., 1999). Both techniques offer viable options but require further study before considered acceptable clinically.

Recent data suggest that interference with the steps of apoptosis may reduce follicular attrition induced by anticancer therapy. This approach may be more effective than ovulation prevention in reducing follicular loss in this setting. Apoptosis has been identified recently as a possible mechanism for oocyte depletion associated with both ageing and cytotoxic therapies (Morita and Tilly, 1999). Sphingomyelin-derived second messengers may be key in the programmed loss of oocytes (Kolesnick and Kronke, 1998). Inhibition of the enzyme sphingomyelin phosphodiesterase results in disruption of ceramide, a sphingolipid-based second messenger. Manipulation of these intracellular events may offer an opportunity to alter the rate of oocyte attrition. Data from animal studies suggest that normal apoptotic oocyte loss may be suppressed by disruption of the gene for acid sphingomyelinase (Morita et al., 2000). Furthermore, radiation-induced oocyte loss may be prevented by treatment with the small lipid molecule sphingo-1-phosphate. These studies suggest that manipulation of second messengers and treatment with small lipid molecules may be an effective and efficient treatment to prevent oocyte loss associated with cytotoxic therapy.

In conclusion, cyclophosphamide may result in ovarian failure by rapid development and attrition of medium follicles in the rat model. These data support previous studies suggesting that cyclophosphamide may have a unique effect of superovulation in this species. No clearly protective effect is noted on either medium or large follicles in this study in the rat model by an induction of anovulation using either the combination of ethynyl estradiol and norgestrel or the GnRH analogue leuprolide acetate. These results further suggest that this impact may occur whether the hormonal milieu is estrogen/progesterin dominant or hypoestrogenic. Cryopreservation of either ovarian tissue or immature oocytes and, in the future, manipulation of molecular events critical to oocyte depletion may be more effective and reliable techniques for preservation of gonadal function during cytotoxic therapy.

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