The GnRH antagonist cetrorelix reduces cyclophosphamide-induced ovarian follicular destruction in mice

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BACKGROUND: It has remained controversial whether and in what way suppression of the pituitary–gonadal axis using GnRH analogues can reduce the destructive effects of chemotherapy on ovarian primordial follicles and thus prevent ovarian failure. GnRH antagonists suppress gonadotrophin levels immediately after administration. In this study we determined whether administration of the GnRH antagonist cetrorelix before exposure to increasing doses of cyclophosphamide (Cy) affected the number of surviving primordial follicles (PMF) in the mice ovary.

METHODS: Highly inbred young Balb/c mice (114 females) were injected with 0, 50 and 75 mg/kg of Cy. In each treatment group, half of the females were injected daily with cetrorelix starting 9 days before and 7 days post-administration of Cy. In serial sequential ovarian sections the total number of PMF in both ovaries was counted.

RESULTS: Ovaries exposed to Cy at doses of 50 and 75 mg/kg had significantly fewer PMF than those in the control group (P < 0.01). In each of the Cy groups used, pretreatment with cetrorelix resulted in significantly higher numbers of PMF: in the 50 mg/kg Cy group only 14% were destroyed (cetrorelix group) versus 53% (P < 0.001), while in the 75 mg/kg Cy group only 35% of PMF were destroyed versus 54% in animals treated only with Cy (P < 0.004). The interaction between the effect of cetrorelix and the different doses of Cy did not reach statistical significance (P = 0.089, two-way ANOVA). CONCLUSIONS: Administration of the GnRH antagonist cetrorelix to mice significantly decreases the extent of ovarian damage induced by the chemotherapeutic agent Cy. The use of different substerilizing doses of Cy suggested that the extent of protection achieved by the antagonist is dose-dependent and decreases with increasing Cy doses. The results of this study may suggest a possible similar beneficial effect in women undergoing chemotherapy, can explain the discrepancy in results of existing clinical studies and indicate possible pathways for ovarian GnRH agonist protection. Further research and clinical studies are needed in order to confirm these results.

Key words: chemotherapy/GnRH antagonist/mice/premature ovarian failure

Introduction

Over the last decades attempts have been made to minimize the toxic effects of chemotherapy on the ovaries and to reduce the well-known risk of premature ovarian failure (Warne et al., 1973; Whitehead et al., 1983; Meirow and Nugent, 2001). It has been suggested that administration of GnRH agonists during chemotherapy treatment might reduce the damage to the ovaries, apparently by reducing the number of primordial follicles (PMF) destroyed by toxic agents. While studies in animals suggest that pituitary gonadotrophic suppression by GnRH agonists can reduce the ovarian damage caused by chemotherapy (Bokser et al., 1990; Ataya et al., 1995b), there is a lack in prospective randomized human trials assessing the efficacy of GnRH agonists. A few studies have shown that GnRH agonists can reduce ovarian toxicity caused by chemotherapy (Blumenfeld et al., 1996; 1999), while in another study the ovaries of women who underwent bone marrow transplantation were not protected by the drug (Meirow, 1999). However, these studies were either retrospective or lacked adequate control groups.

The alkylating drug cyclophosphamide (Cy) is a widely used chemotherapeutic and immunosuppressive agent with a potent ovarian toxic effect (Warne et al., 1973; Jarrell et al., 1991). In rat ovaries, Cy causes widespread ovarian follicular atresia (Jarrell et al., 1991), which leads to a significant reduction in the total number of ovarian follicles (Bokser et al., 1990). Concomitant use of a GnRH agonist can reduce these Cy-induced toxic ovarian changes in rats (Bokser et al., 1990). In another study, GnRH agonist administration improved the reproductive performance of female rats as assessed by pregnancy and implantation rate (Ataya and Ramahi-Ataya, 1993).
Whether GnRH agonists protect the ovaries from the toxic effects of chemotherapy and which mechanisms mediate such putative ovarian protection have remained unclear. Ovarian primordial follicles, which constitute the vast majority of ovarian follicles and represent the ‘ovarian follicular reserve’, are not under gonadotrophic control. Therefore, a decrease in pituitary gonadotrophins probably does not directly protect the PMF. Alternatively, since GnRH receptors were characterized in ovaries of different species (Janssens et al., 2000), a protective effect of GnRH agonists on murine ovaries exposed to chemotherapy could also be mediated by direct ovarian effects. It is also possible that the decline in ovarian blood flow that occurs during ovarian quiescence, caused by GnRH agonist, may decrease ovarian levels of the chemotherapeutic agent and could lead to reduced ovarian toxicity.

Several studies have demonstrated the differential effects of GnRH agonists and antagonists on gonadotrophin mRNA expression in rats (Clayton, 1993) and on serum levels of immunoreactive and bioactive LH, FSH and gonadotrophin α-subunit in humans (Couzinet et al., 1991; Matikainen et al., 1992). GnRH agonists reduce pituitary gonadotrophin secretion through receptor down-regulation. They cause an initial flare-up effect on pituitary FSH and LH secretion, which results in a parallel transient ovarian response followed by a maximal decrease of about 93% in FSH and 93% in LH immunoreactive levels, respectively, and ovarian quiescence (Couzinet et al., 1991; Matikainen et al., 1992). In contrast, GnRH antagonist preparations act on the GnRH receptor through competitive inhibition, and significantly decrease gonadotrophin secretion (Couzinet et al., 1991; Matikainen et al., 1992; Rabinovici et al., 1992).

Studies in rodents have indicated that the inhibitory effect of cetrorelix on serum gonadotropins and pituitary GnRH receptors was greater than that of GnRH agonist (Horvath et al., 2002). In addition, the direct effects of GnRH agonists and antagonists on the murine ovarian follicle are different (Yano et al., 1997). Thus, concomitant administration of a GnRH antagonist and Cy might elicit a different response that might shed light on whether and by what mechanisms the ovaries are protected with GnRH treatment.

Therefore, the aims of the present study were to examine the effectiveness of a GnRH antagonist in the preservation of the ovarian follicular reserve during administration of increasing non-sterilizing doses of Cy. To this end, we determined the efficacy of a GnRH antagonist in the preservation of ovarian primordial follicles in mice ovaries after Cy treatment with and without pretreatment with a potent, third-generation GnRH antagonist, cetrorelix.

Materials and methods

The study was performed using highly inbred Balb/c adult female mice (Harland Laboratories, Oxon UK), kept under specific pathogen-free conditions and with the ethical approval of institutional ethics committee. Female mice 8–9 weeks of age (114 animals in total), mean weight 15–25 g, were divided randomly into six groups (Figure 1). Groups 1 (n = 18) and 2 (n = 18) did not receive Cy, but were treated by single intraperitoneal dose of 0.1 ml of saline. Groups

3 (n = 20) and 4 (n = 19) received a single intraperitoneal dose of 50 mg/kg Cy. Groups 5 (n = 20) and 6 (n = 19) received a single intraperitoneal dose of 75 mg/kg Cy. Cy was administrated on day 9 of the study. The doses of Cy were chosen based on previous studies that showed significant dose–response ovarian toxicity but not sterilization (Meirow, 1999). Groups 2, 4 and 6 were pretreated from day 1 until day 15 of the study with a daily subcutaneous injection of 0.5 mg/kg cetrorelix (ASTA Medica, Frankfurt, Germany), while the other groups were observed. Previous dose studies in rodents demonstrated inhibition of gonadotrophin secretion when cetrorelix was administered at this dose range (Trimino et al., 1993). All mice were sacrificed on day 16 of the study and both ovaries were removed and fixed in 4% paraformaldehyde in phosphate-buffered saline. The ovaries were embedded in paraplast and serially sectioned to 5 μm slices. Care was taken to ensure that both ovaries were removed from each mouse in their entirety for histological processing. The sections were stained with haematoxylin–eosin and the ovarian PMF number was counted in every tenth section by one examiner who was unaware of the treatments. To reach a value representative of the total number of PMF in both ovaries, the numbers of PMF were then multiplied by 10. PMF were counted when the nucleus was clearly identified surrounded by a single layer of squamous pregranulosa cells without a theca layer (Meirow et al., 1999). In this species, PMF are located almost exclusively in the ovarian cortex, they are very small (~15 μm diameter), with a single layer of squamous pregranulosa cells without a theca layer.

Statistical analysis

One- and two-way ANOVAs were used for statistical analysis of the different treatment groups. t-tests with Bonferroni correction were performed to assess the differences between the group pairs with and without the antagonist. Statistical significance was determined at P ≤
NS = not significant.

0.05. Two-way ANOVA was used to evaluate the degree of PMF survival using the antagonist in correlation with the different doses of Cy. Statistical significance was determined at \( P \leq 0.05 \).

Results

Of the 114 female mice that started the experiment, all survived and were equally active, and all gained weight. The mean (± SD) number of PMF in the control group was 673 ± 286 and in the group treated with cetrorelix was 740 ± 264. Administration of Cy at doses of 50 and 75 mg/kg reduced about half of the PMF reserve (53% and 54%, respectively) compared with untreated controls (\( P < 0.01 \)), but there was no significant difference between the two doses of Cy used (Table I). Pretreatment with cetrorelix resulted in a significantly higher mean (± SD) number of PMF in both the 50 mg/kg group, 580 ± 104 versus 317 ± 222 for pretreated versus untreated, respectively (\( P < 0.001 \)), and in the 75 mg/kg group, 430 ± 85 versus 307 ± 153 for pretreated versus untreated, respectively (\( P < 0.004 \)). In the present study we could not show any significant difference between the two Cy doses used (Table I).

While exposure to 50 mg/kg of Cy resulted in a mean decrease of 53% in PMF compared with untreated controls, pretreatment with cetrorelix before administration of 50 mg/kg of Cy led to a mean decrease in PMF of only 14%. Exposure to higher dose of Cy (75 mg/kg) resulted in a mean decrease of 54% in PMF, pretreatment with cetrorelix before Cy administration (75 mg/kg) led to a mean decrease in PMF of 36%. As expected, addition of cetrorelix to the untreated control animals did not have any effect on the number of PMF (Table I). Based on these results, the relative protection (dividing the mean number of PMF in the group pretreated with cetrorelix and the mean number of PMF in the group treated with Cy only protocol) of cetrorelix was 1.83 for the group treated with 50 mg/kg and 1.4 for the group treated with 75 mg/kg of Cy. Using the two-way ANOVA that evaluated the categories antagonist treatment (exposure versus non-exposure) and the three groups of Cy used (0, 50 and 75 mg/kg), the observed differences did not reach statistical significance (\( P = 0.089 \)).

Discussion

The results of this study in highly inbred young mature mice of the same age demonstrate that administration of Cy at doses of 50 and 75 mg/kg causes significant ovarian damage and a reduction in the number of PMF. However, pretreatment with the potent, third-generation GnRH antagonist cetrorelix significantly reduced the number of PMF destroyed by the toxic effects of Cy (Table I). Administration of Cy at doses of 50 and 75 mg/kg led to a loss of more than 50% of PMF. These findings are in agreement with previous reports (Mattison et al., 1981; Meirow et al., 1999), although in this group of mice (8–9 weeks of age) we did not observe a different dose-effect for the two doses used. When the administration of Cy at doses of 50 and 75 mg/kg was preceded by daily subcutaneous injection of cetrorelix at a dose of 0.5 mg/kg, significantly fewer follicles were lost compared with Cy only groups: just 14% versus 53% and 36% versus 54% of PMF for the two doses of Cy, respectively. These findings indicate a highly protective effect of the antagonist with a significant protective ratio of 1.83 for the 50 mg/kg Cy dose, and a lower but still significant protective ratio of 1.4 for the 75 mg/kg Cy dose. As expected, the addition of cetrorelix to untreated control animals did not have any effect on the number of PMF.

Although the protective effects of cetrorelix were dependent on the dose of Cy and were higher with 50 mg of Cy than with 75 mg of Cy, this difference did not reach statistical significance (\( P = 0.083 \)). This can probably be explained by the fact that in this study we did not show a significant difference in ovarian toxicity of the two Cy doses used. However, the difference between a loss of 14% and 36% with the two doses of Cy can affect the PMF stockpile left, and might have long-term biological significance such as infertility and age of menopause. Previous data that indicated a dose-dependent toxic effect of chemotherapeutic agents on ovarian reserve have supported such biological significance (Wang et al., 1995; Sanders et al., 1996; Meirow et al., 1999). In-vitro studies have shown that chemotherapy destroys PMF within hours (Meirow, 1998). However, the quality of the surviving PMF 1 week post-chemotherapy cannot be assessed directly. We cannot be sure that all surviving follicles that were counted are in good quality.
and are capable of maturation, ovulation and fertilization. However, it was shown previously, in a similar setting, that Cy which reduced the PMF population by 50% did not effect ovulation fertilization or pregnancy rate of follicles surviving long-term after chemotherapy (Meirow et al., 2001). In addition, clinical studies have indicated that patients who are not sterile post-chemotherapy exhibit normal reproductive outcome (Arnon et al., 2001).

A larger PMF reserve might have clinical significance. Previous studies that examined the effect of chemotherapy on ovarian function, especially in young women, indicated that even when the ovaries function after high-dose chemotherapy treatment, ovarian failure can occur at a relatively early age (Wallace et al., 1993; Chiarelli et al., 1999).

Since clinical studies do not necessarily mirror the actual degree of ovarian damage, use of animal studies for the investigation of the effects of chemotherapy and various protective measures such as hormonal manipulations seems to us to be of importance. Such an approach allows the direct quantitation of ovarian damage as assessed by the total number of ovarian PMF. However, because of the different activities and regulations of murine and human ovarian follicular function, prospective randomized clinical human trials are necessary to corroborate the animal data.

What are the possible explanations for such protection? A few, but consistent, animal and human in-vitro studies have indicated that the direct mechanisms of chemotherapy-induced PMF injury and loss are related to pathways of apoptosis signalling. Among the first steps of PMF damage are caspase activity and internucleosomal DNA cleavage seen in pregr-nulosa cells and oocytes (Perez et al., 1997; 2000; Meirow et al., 1998). Since murine ovaries contain GnRH ligand sites (Adashi et al., 1991; Kaiser et al., 1992), the protective ovarian effects observed in the past with GnRH agonists could have been mediated by the direct effects of the GnRH agonist on the ovarian follicles. In the ovary, a differential effect of GnRH agonists and antagonists was described by Yano et al. (1997). Their results demonstrated that GnRH agonists and antagonists inhibited directly proliferation of granulosa cells through apoptosis, whereas only GnRH agonists stimulated cell differentiation in the preovulatory follicle (Yano et al., 1997). Moreover, the direct effects of GnRH analogs on the ovary are variable, and large physiological variations in GnRH receptor abundance exist that depend on the species and/or the timing in the cycle (Janssens et al., 2000). Thus, although the effects of the GnRH agonist and antagonist on the ovary are not identical, the similar effects shown by our study do not preclude a direct effect of the GnRH analogues on the ovary.

In the pituitary, both GnRH agonist and antagonist reduce the secretion of gonadotrophins. The antagonists do not elicit a receptor response on the GnRH receptor but act through competitive inhibition. However, low gonadotrophin levels cannot directly alter the toxic effects of Cy on PMF population and ovarian reserve. A large body of evidence indicates that PMF are not under gonadotrophic control (Rabinovici and Jaffe, 1990).

Alternatively, changes in ovarian blood flow that are influenced by gonadotrophin secretion and ovarian activity could contribute to the protective effect of GnRH analogues. Human studies have demonstrated that ovarian blood flow throughout the menstrual cycle is regulated by follicular development, which in turn is determined by gonadotrophic stimulation (Lunenfeld et al., 1996; Tan et al., 1996). Intraovarian blood flow increases throughout follicular development and peaks during the luteal phase (Lunenfeld et al., 1996). In the ovary that contains the dominant follicle, follicular and ovarian stromal peak systolic blood flow velocity rises significantly during the menstrual cycle, and the changes in blood flow velocity correlate significantly with changes in FSH, LH and progesterone concentrations (Tan et al., 1996). In contrast, there are no significant changes in either blood flow velocity or pulsatility index in the contralateral, quiescent ovary (Tan et al., 1996). During pituitary desensitization with GnRH agonists there is a good correlation between the decrease in ovarian blood flow and the decrease in circulating estradiol levels (Dada et al., 2001). Thus, the protective effect seen in this study with a GnRH antagonist in mice exposed to the toxic activity of Cy, as well as the protective effects observed previously with GnRH agonists, could be mediated by a decline in ovarian blood flow and a resulting decrease in the exposure of the ovaries to the chemotherapeutic agent. This hypothesis suggests an effect on the entire organ, the ovary, and not directly at the level of the follicle. It can explain the decline in ovarian protection with increasing Cy doses, and would also explain the lack of protective effect of GnRH antagonists on radiotherapeutic ovarian damage (Gosden et al., 1997) and the relatively better ovarian outcome seen after chemotherapy in young, pre-menarcheal girls (Matsumoto et al., 1999; Papadakis et al., 1999).

Similarly, Ataya et al. demonstrated in Rhesus monkeys an ovarian protective effect of GnRH agonists after Cy administration (Ataya et al., 1995b), but not after irradiation (Ataya et al., 1995a). Indeed, chemotherapy effects on the ovary showed narrowing of the lumen with diffuse hyalinization of the blood vessel walls, and clear signs of either focal or diffuse cortical fibrosis and a significant decrease in the number of follicles (Meirow, 2002). These potential mechanisms of ovarian damage induced by chemotherapy do not directly act on PMF, and are influenced by gonadotrophins.

When chemotherapy treatments are expected to be highly toxic to the ovaries, other strategies should be offered to preserve fertility. These include IVF and cryopreservation of embryos or oocytes, or the still experimental cryopreservation of ovarian cortical tissue (Meirow and Nugent, 2001).

In conclusion, the protective effect of GnRH agonists on human ovaries in women undergoing chemotherapy was suggested previously (Blumenfeld et al., 1996; 1999). This murine study demonstrates that administration of a GnRH antagonist protects the ovaries from the toxic effect of the chemotherapeutic agent Cy. The protection seems to depend on the dose of the chemotherapeutic agent, and is therefore not absolute, thus explaining the diverse results of clinical studies. Owing to the complex nature of chemotherapeutic effects on gonadal function, it is possible that with other groups of chemotherapeutic agents other interactions exist, at least if protection is achieved by direct effects on the ovary. The
choice of the chemotherapeutic agent as well as the dosage will determine in clinical practice the need and ability of ovarian protection. With the availability of long-acting GnRH agonists, the continuous administration of this drug to women who need ovarian protection is readily available. Until the development of long-acting GnRH antagonists, GnRH agonist preparations seem to have a clinical advantage. Antagonists might be clinically indicated in cases where immediate gonadotrophic suppression is warranted and the initial gonadotrophic flare-up seen with GnRH agonists is undesirable. Well-prepared, large clinical trials are needed to elucidate the indications and effectiveness of hormonal treatments to protect the ovaries from damage caused by chemotherapy. More basic studies are needed to better understand the mechanisms of PMF destruction, the effects of chemotherapy on the entire ovary and the process by which inhibiting the pituitary–gonadal axis may protect the ovaries from chemotherapeutic damage.

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