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Is anti-mullerian hormone a marker of acute cyclophosphamide-induced ovarian follicular destruction in mice pretreated with cetorelix?

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Abstract

Objective—To define whether anti-mullerian hormone may be a marker of acute cyclophosphamide-induced germ cell destruction in mice pretreated with the GnRH antagonist, cetorelix.

Design—Controlled, experimental study.

Setting—Research laboratory in a federal research facility.

Animals—Balb/c female mice (6 weeks old).

Interventions—Mice were treated with GnRH antagonist (cetorelix) or saline for 15 days followed by 75 mg/kg or 100 mg/kg of cyclophosphamide or saline control on day 9.

Main Outcome Measure(s)—Number of primordial follicles (PMF), DNA damage, AMH protein expression, and AMH serum levels.

Results—Ovaries in mice pre-treated with cetorelix had significantly more PMF and reduced DNA damage compared to those exposed to cyclophosphamide alone. Immunohistochemical staining for AMH expression and serum AMH levels did not differ significantly between treatment groups.

Conclusions—Cetorelix protected primordial follicles and reduced DNA damage in follicles of mice treated with cyclophosphamide, but AMH levels in tissue and serum did not correlate with germ cell destruction. Further research is needed to determine the mechanism responsible for the protective effects on PMF counts observed with cetorelix.

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Keywords

GnRH antagonist; cyclophosphamide; primordial follicles; DNA damage; anti-mullerian hormone

INTRODUCTION

The majority of female cancer patients are expected to be cured and become long-term survivors (1, 2). For this reason, methods to preserve fertility in women are of the utmost importance. Options for fertility preservation include the use of less toxic regimens, which may compromise survival, assisted reproductive technologies with cryopreservation of oocytes or embryos, or administration of hormonal agents to protect gonadal function during chemotherapy. Both animal and human studies have shown beneficial results with the use of GnRH analogues in preserving ovarian function during exposure to cytotoxic therapy (3–8).

Most studies have used GnRH agonists, which are associated with an initial stimulatory effect of gonadotropin secretion, known as a flare. In contrast, immediate down-regulation of the hypothalamic-pituitary-ovarian (HPO) axis and inhibition of follicular recruitment and development occurs with GnRH antagonists, which may be beneficial when immediate initiation of cytotoxic therapy is recommended. However, only a few studies address the efficacy of GnRH antagonists in preserving ovarian function and follicle number after chemotherapy. Meirow et al. (7) showed that mice given a GnRH antagonist prior to treatment with cyclophosphamide had more surviving primordial follicles (PMF) compared to controls. In a limited case series of four patients who received the GnRH antagonist cetrorelix prior to chemotherapy, all patients remained eumenorrheic and one patient spontaneously conceived post-treatment (8).

Although GnRH analogues have been shown to be efficacious in preserving ovarian function during cytotoxic therapy, the mechanism that mediates this protective effect remains unclear. The gonadotropin deprivation induced by GnRH analogues is thought to halt follicular recruitment and decrease the size of the chemotherapy-sensitive pool (7). However, initial recruitment and early development of ovarian primordial follicles is gonadotropin independent (9, 10), therefore, suppression of pituitary gonadotropins does not likely directly protect the resting pool of oocytes (7). Several alternative hypotheses have been proposed including a reduction in ovarian blood flow, a direct influence of the GnRH analogue on the ovary, or an indirect effect mediated by growth factors involved in follicle recruitment and development.

Estrogen has a known vasodilatory effect on smooth muscle cells in the pelvic vasculature (11). Logically then, the gonadotropin desensitization and subsequent hypoestrogenism induced by GnRH analogues might reduce blood flow to the ovaries and protect ovarian function by decreasing the delivery of toxic chemotherapeutic agents. Supporting this argument, Dada et al. (12) measured ovarian artery resistance indices before and after GnRH agonist treatment and confirmed that GnRH agonists significantly reduced ovarian artery blood flow. In contrast, Yu et al. (13) examined the change in ovarian stromal blood flow by power Doppler ultrasound after short term GnRH agonist treatment and found no difference.

A second possible mechanism is that GnRH analogues may have a direct ovarian effect at the level of the follicle. Messenger RNA transcripts for GnRH receptors have been demonstrated in both primate and non-primate ovaries (14), and receptors have been localized to granulosa and luteal cells of developing follicles and interstitial ovarian cells (15). Furthermore, GnRH antagonists have been shown to exert a direct inhibitory effect on the growth and proliferation of granulosa cells of pre-ovulatory follicles (16) as well as on

ovarian steroidogenesis (17, 18). These findings suggest that GnRH analogues may play an autocrine or paracrine regulatory role within the ovary (16) but again, primordial follicles might not be directly affected.

Finally, a third hypothesis is that GnRH analogues may have an indirect effect on the resting follicle pool (7). Regulation of early folliculogenesis, which is not gonadotropin-dependent, may be controlled by growth factors originating in growing follicles, which are under gonadotropin control. Gonadotropin suppression could up-regulate inhibitory factors or down-regulate promoting factors. Growth factors from the transforming growth factor- β (TGF β) superfamily, such as GDF9, BMP15, and anti-mullerian hormone (AMH), play a central role in the primordial to growing follicle transition (19) and are therefore prime candidates. The current study was undertaken to investigate whether the protective effect of GnRH analogues on primordial follicle counts might be mediated, at least in part, through regulation of AMH expression. It is even possible that the damaging effects of cyclophosphamide may be mediated through AMH with a secondary impact on the follicles themselves.

AMH is expressed postnatally in females in granulosa cells of growing follicles (20–22) and declines during reproductive life. Serum levels of AMH may reflect the size of the primordial follicle pool (23, 24). In the absence of AMH, more primordial follicles were recruited into developing follicles, resulting in accelerated depletion of the ovarian follicular pool (25). Conversely, over-expression of AMH resulted in infertile mice with ovaries devoid of germ cells (26). This suggests that AMH may have an inhibitory role in the recruitment and development of primordial follicles into growing follicles. Here we tested the hypothesis that AMH is a marker of the reduced cyclophosphamide-induced primordial follicular destruction in mice pretreated with the GnRH antagonist, cetrorelix. Higher AMH levels in GnRH antagonist pretreated mice compared to cyclophosphamide treatment alone would suggest a protection of the ovarian reserve and may warrant further studies examining AMH as a mediator of this protective effect.

MATERIALS AND METHODS

Animals

All experiments were approved by the local Animal Ethics Committee. Sixty inbred Balb/c young female mice, aged 6 weeks and mean weight 15–20 g, were randomly divided into six groups (Supplementary Figure 1). Groups 1 (n= 10) and 2 (n= 10) were treated with a single intraperitoneal dose of 0.1 ml of saline on day 9. Groups 3 (n= 10) and 4 (n= 10) and Groups 5 (n= 10) and 6 (n= 10) received a single intraperitoneal dose of 75 mg/kg or 100 mg/kg of cyclophosphamide on day 9, respectively. The dosages of cyclophosphamide used were based on a previous study (27) that demonstrated a significant dose-dependent ovarian toxicity but not sterilization. Groups 2, 4, and 6 received a subcutaneous injection of Cetrorelix 0.5 mg/kg (Serono, Inc. Rockland, MA) from day 1, when the mice reached 6 weeks of age, until day 15 of the study (Supplementary Figure 1). All mice were sacrificed on day 16 of the study and ovaries were removed in their entirety. One ovary was fixed in 4% paraformaldehyde in phosphate-buffered saline for histological processing, and blood was collected and serum isolated for hormonal measurements.

Primordial follicle counts

The ovaries were serially sliced into 4 μ m sections. Five sections per treatment group were analyzed. Tissues were stained with hematoxylin-eosin. PMF were defined by a clearly identified oocyte surrounded by a single layer of squamous granulosa cells without a thecal layer (28). Primordial follicle counts per unit area (1000 micron²) were determined from

each section. Ovarian section areas (in μm^2) were determined using ImageJ software, a public domain Java image processing and analysis program capable of displaying and measuring histologic images (<http://rsbweb.nih.gov/ij/>).

Analysis of DNA damage

DNA damage was assessed by TUNEL assay using the in situ cell death detection kit (Roche). A positive control was created by incubating one section in DNASE 1 (Ambion) for 10 minutes at room temperature. Slides were then incubated at 37 C for an hour in a solution including TdT and fluorescein-labeled dUTP. After 3 washes with PBS, Vectashield with Dapi (Vector Laboratories) was used as a fade-resistant mounting medium to visualize the nuclei in each tissue section. The apoptotic index was calculated as the percentage of granulosa cells that underwent apoptosis out of 1000 granulosa cells per high power field (29). Fluorescent images were acquired on a Zeiss Axioplan 2 Imaging microscope.

Immunohistochemistry

Immunohistochemical staining was performed as previously described (27). Sections were mounted on paraffin-coated slides. After deparaffinization, slides were quenched for 12 minutes in 3% H_2O_2 /methanol solution and washed with PBS. Sections were microwaved for 3×5 minutes at 700 W in 0.01 M citric acid buffer, pH 6.0, cooled down to room temperature (RT), and rinsed in PBS. Normal rabbit serum in 5% BSA was added for an hour at RT followed by incubation at 4°C overnight with goat polyclonal antibody against AMH (C-20; Santa Cruz Biotechnology, Inc.), diluted 1:500 in 5% BSA in PBS. After incubation, sections were rinsed in PBS-T and incubated for 30 minutes at RT with biotinylated rabbit anti-goat antibody (dilution 1:200; Vectastain Elite Goat IgG kit, PK-6105). Streptavidin-biotin-peroxidase complex (diluted 1:200 in PBS, VECTASTAIN Elite ABC) for 30 minutes at RT was used, and peroxidase activity was developed with 0.07% 3,3'-diaminobenzidine tetrahydrochloride for 5 minutes. The negative control slide was processed similarly in the absence of primary antibody.

Serum AMH assay

AMH serum levels were measured by an AMH ELISA assay (Diagnostic Systems Laboratories, Inc.). All serum samples were assayed in duplicate. Mean inter-assay and intra-assay coefficients of variation were 6.5% and 3.4%, respectively.

Statistical analysis

Repeated measures two-way ANOVA were used to assess the effects of cyclophosphamide and cetrorelix across the different treatment groups for PMF count, ovarian area, apoptotic index, H-scores, and serum AMH levels. T-tests with Bonferroni adjusted comparisons were performed to assess the differences between the group pairs with and without the antagonist. Statistical significance was determined at the $P < 0.05$ level. All analyses were carried out using SAS version 9.1 (SAS Institute, Cary, NC). Cytoplasmic expression and localization of AMH in the granulosa cells of developing ovarian follicles in each treatment group were compared using the H score. The H score was calculated with the following equation: $H \text{ score} = \sum P_i (i + 1)$, where i is the intensity score, and P_i is the percentage of stained cells for each intensity (30). For each tissue, an H score value was derived by summing the percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining (30). Each slide was scored by 3 independent observers. Differences were considered significant at $P < 0.05$.

RESULTS

Reduced cyclophosphamide-induced primordial follicular destruction in mice pretreated with a GnRH antagonist

Histologic findings revealed fewer PMF in the ovaries of mice exposed to cyclophosphamide compared to those treated with GnRH antagonist (Figure 1A–F). The mean PMF count per unit ovarian area (mean $\pm$ SEM) in mice treated with cyclophosphamide at doses of 75 mg/kg (0.84 ± 0.30) and 100 mg/kg (0.53 ± 0.33) was reduced compared with control (1.39 ± 0.30 , $p=0.0115$ and $p=0.0002$, respectively). Pretreatment with cetrorelix resulted in a significantly higher number of PMF (mean $\pm$ SEM) in both 75 mg/kg (1.78 ± 0.33) and 100 mg/kg groups (1.60 ± 0.33) compared to the cyclophosphamide-only treated animals ($p<0.0001$). In addition, the cetrorelix alone group had a higher mean number of PMF than control (2.45 ± 0.33 and 1.39 ± 0.30 , $p<0.0001$ respectively) (Figure 1G).

GnRH antagonist reduced cyclophosphamide-induced ovarian follicular DNA damage

DNA damage was largely confined to the granulosa cells of developing follicles (Figure 2A–F). When cetrorelix was administered prior to cyclophosphamide, there was a significant reduction in DNA damage. DNA damage was greater in the ovaries exposed to 100 mg/kg cyclophosphamide alone compared to those pretreated with antagonist (Figure 2E–F), with the apoptotic index (AI) reaching 19% vs 6%, $p=0.0033$ respectively (Figure 2I). The difference in apoptotic index was not observed at the lower dosage of cyclophosphamide. Treatment with cetrorelix alone also reduced the amount of DNA damage in developing follicles compared to control (12% vs 24%, $p=0.0047$) (Figure 2I).

AMH as a marker of cyclophosphamide-induced primordial follicular destruction

Immunohistochemistry was performed to determine whether pretreatment with a GnRH antagonist and cyclophosphamide induced changes in the levels of ovarian AMH protein expression (Supplementary Figure 2A–F). As expected, strong uniform positive immunohistochemical staining was observed in developing granulosa cells of primary to antral stage follicles, but not in primordial follicles. AMH staining was also absent in the largest ovarian follicles. Oocytes and stroma, in all groups, had similarly low or absent AMH expression. Staining for AMH in follicles of ovaries from mice pretreated with cetrorelix was reduced compared to the control group (H-score 2.6 vs 3.0, $p=0.0243$) but there was no change in staining for AMH among treatment groups 3–6 (Figure 3A). Consistent with the immunohistochemical results, there was no significant difference in serum AMH levels between those treated with cyclophosphamide alone versus those pretreated with cetrorelix (control vs antagonist: 5.9 vs 10.5, $p=0.1584$; 75 mg/kg: 8.5 vs 6.3, $p=1.0$; 100 mg/kg: 8.8 vs 7.2, $p=1.0$) (Figure 3B).

DISCUSSION

We sought to determine whether the protective effect on PMF counts observed with the GnRH antagonist, cetrorelix, occurred at the level of developing follicles using AMH as a marker of cyclophosphamide-induced follicular destruction. Similar to Meirow et al. (7), we observed that pre-treatment with the GnRH antagonist, cetrorelix, exerted a protective effect on PMF counts in mice exposed to cyclophosphamide. Our results extend those findings to show that one possible mechanism involved is a reduction in DNA damage in mouse ovarian tissues exposed to cyclophosphamide. However, neither immunohistochemical staining for AMH expression nor serum AMH levels correlated with primordial follicle counts after treatment with either a GnRH antagonist and cyclophosphamide or

cyclophosphamide alone. Thus, in this experimental model, cetrorelix induced granulosa cell damage, but there were no acute changes in AMH tissue expression or serum levels.

While AMH expression does not appear to be affected by GnRH antagonist pretreatment before chemotherapy, there are a number of alternate signaling pathways that could feed back to suppress the recruitment and development of primordial follicles and protect the quiescent pool of oocytes. Nobox, KIT ligand, BMP4, and FOXO3 are just a few candidate regulatory factors involved in the complex signaling loops linking early oocyte and somatic cell development (31). Gonadotropin suppression may indirectly affect recruitment of primordial follicles through one of these pathways. More research is required to investigate this hypothesis.

Another explanation for our results is that cyclophosphamide-induced a rapid and specific decrease in primordial follicles, without short-term (15 days) effects on the number of growing or large follicles, which might not affect AMH expression. In support of this notion, Mattison et al. (32) showed that seven days after a single injection of cyclophosphamide (100 mg/kg ip), 63% of the primordial follicles and 0% of growing or large follicles and their oocytes were destroyed in four-week-old C57BL/6N mice. Since the number of growing follicles remain constant in the ovary after early cytotoxic exposure (32, 33), similar staining patterns for AMH expression and its circulating levels might be seen in all treatment groups independent of GnRH antagonist pretreatment. It is possible that sampling of AMH several weeks after treatment may have demonstrated differences in AMH expression between treatment groups. Sahambi et al. (33) showed that depletion of the primordial follicle pool in 4-vinylcyclohexene diepoxide (VCD)-treated mice over several time points resulted in subsequent depletion of the growing follicle pool and greatly reduced serum AMH levels. Mice exposed to VCD and sacrificed on day 37 or day 57 had a significantly lower number of primordial and growing follicles and serum AMH levels than those sacrificed on day 16 after treatment. However, circulating AMH levels and the number of growing follicles were not significantly different between VCD-treated and control mice on day 16. These findings suggest that circulating AMH levels are highly correlated with the number of growing follicles but that AMH only correlated with the number of primordial follicles at later time points after ovarian insult at which their loss led to decreased growing follicle numbers.

The mechanism by which cyclophosphamide causes ovarian follicle destruction is poorly understood. Cyclophosphamide functions by DNA alkylation and subsequent disruption of normal cellular processes. While cyclophosphamide is cell-cycle non-specific, it is most cytotoxic to mitotically active cells (34). Still, Plowchalk et al. (34) demonstrated that primordial follicles were more sensitive to cyclophosphamide toxicity than growing follicles. It has been suggested that apoptotic signaling pathways underlie chemotherapy-induced germ cell destruction (35). Interestingly, we found that GnRH antagonist treatment significantly reduced the amount of DNA damage in developing ovarian follicles. In line with these findings, Huang et al. (29) showed that ovarian apoptotic indexes were reduced by cetrorelix pretreatment, and that pretreated ovaries expressed less caspase-3 and more Bcl-2 compared with chemotherapy alone-treated ovaries. Taken together, the results suggest that GnRH antagonist treatment may reduce chemotherapy-induced oocyte damage by regulating pro- and anti-apoptotic molecules at the level of the granulosa cell in growing follicles. By contrast, Yano et al. (16) reported that rat granulosa cells treated with cetrorelix exhibited an increase in apoptosis. The difference between these disparate results could be due to in vitro versus in vivo effects. In support of that interpretation, we observed a 50% reduction in DNA damage in the mice treated with a GnRH antagonist alone, suggesting the reduction in DNA damage could be mediated through gonadotropins.

Based on these data, AMH is not a marker of acute cyclophosphamide-induced primordial follicular destruction in mice pretreated with a GnRH antagonist or cyclophosphamide-alone. However, additional studies are needed to determine whether AMH may be a more useful marker of the long-term effects of cytotoxic exposure on follicular counts in mice pretreated with a GnRH antagonist.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Effects of cetrorelix on primordial follicle counts in mice treated with cyclophosphamide. (A–F) H&E stains of representative sections from 56-day-old mice from the 6 treatment groups. Mice exposed to saline (Con), antagonist (Ant), cyclophosphamide 75 mg/kg (Cyc 75 mg/kg), cyclophosphamide 75 mg/kg with antagonist (Cyc 75 mg/kg + Ant), cyclophosphamide 100 mg/kg (Cyc 100 mg/kg), and cyclophosphamide 100 mg/kg with antagonist (Cyc 100 mg/kg + Ant). Scale bar = 100 μ m, magnification= 5 $\times$. (G) Primordial follicle counts (mean $\pm$ SEM) per 1000 micron² ovarian area (y axis) were higher in the antagonist treated groups (x axis) (Cyc 75 vs Cyc 75 + Ant: 0.84 ± 0.30 vs 1.78 ± 0.33 , $p < 0.0001$; Cyc 100 vs Cyc 100 + Ant: 0.53 ± 0.33 vs 1.60 ± 0.33 , $p < 0.0001$). Each bar represents 5 mice. Error bars = $\pm$ SEM.

Figure 2. Cetrorelix reduced apoptosis in the granulosa cells of mice treated with cyclophosphamide. (A–F) Immunofluorescent detection of apoptotic cells (stained yellow) by TUNEL in ovaries exposed to saline (Con), antagonist alone (Ant), cyclophosphamide (Cyc) or pretreated with antagonist (Cyc + Ant). (G) The positive control (Positive Con) was incubated with DNASE 1, and (H) the negative control (Negative Con) was incubated with fluorescein-labeled dUTP. Scale bar = 100 μ m, magnification= 20 $\times$. (I) Quantitative comparison of mean TUNEL scores of the ovaries receiving cyclophosphamide (100mg/kg) with and without antagonist. Percentage of apoptotic cells (mean $\pm$ SEM) in the cyclophosphamide-only (Cyc100) treated group vs the cyclophosphamide and antagonist group (Cyc100 + Ant), respectively ($19\% \pm 6$ vs $6\% \pm 6$, $p=0.0033$, t-test). Each bar represents 3 mice.

Figure 3.

Analysis of AMH expression in mouse ovarian tissue and serum. (A) Quantitation of AMH H score staining (y axis) between the 6 treatment groups (x axis). There was no difference in the mean H-scores (mean $\pm$ SEM) between the cyclophosphamide (Cyc) vs cyclophosphamide + antagonist (Cyc + Ant) treatment groups (75mg/kg: 3.1 ± 0.3 vs 3.1 ± 0.3 , $p=0.6681$ and 100 mg/kg: 3.2 ± 0.3 vs 2.9 ± 0.3 , $p=0.1128$, t-test). Each bar represents a minimum of 6 mice. (B) Serum AMH levels on day 16 among 6 treatment groups. AMH levels (y axis, mean $\pm$ SEM) did not differ between the study groups (x axis). Control (Con) vs antagonist (Ant): 5.9 ± 2.4 vs 10.5 ± 2.4 , $p=0.1584$; 75 mg/kg cyclophosphamide (Cyc75) vs 75mg/kg cyclophosphamide + antagonist (Cyc75 + Ant): 8.5 ± 2.4 vs 6.3 ± 2.4 , $p=1.0$; 100 mg/kg cyclophosphamide (Cyc100) vs 100 mg/kg cyclophosphamide + antagonist (Cyc100 + Ant): 8.8 ± 2.4 vs 7.2 ± 2.4 , $p=1.0$, t-test. Each bar represents a minimum of 5 mice.