Acute depletion of murine primordial follicle reserve by gonadotropin-releasing hormone antagonists

Douglas R. Danforth, Ph.D., Laura K. Arbogast, M.S., and Chad I. Friedman, M.D.

Department of Obstetrics and Gynecology, The Ohio State University, Columbus, Ohio

Objective: To examine the effects of GnRH antagonists on preantral follicle survival in vivo and to investigate whether GnRH antagonist use during cyclophosphamide treatment would protect the ovary and preserve primordial follicle survival in a murine model.
Design: Prospective basic research study.
Setting: Research laboratory in an academic medical center.
Animal(s): Adult C57Bl/6 mice (5 to 6 weeks old).
Intervention(s): Mice received either a single injection of GnRH agonist (leuprolide acetate) on study day −10 or injections of the GnRH antagonist (antide or cetrorelix) on study days −3 and 0. Some animals also received the chemotherapeutic agent cyclophosphamide on day 0. All animals were killed by CO_2 asphyxiation on day 7. To examine direct vs. indirect effects, some mice received GnRH antagonist under the bursa of one ovary, with the contralateral ovary receiving vehicle. Ovaries were fixed in Kahle's solution; 7-μm tissue sections were stained with Lillie's allochrome, and preantral follicles were counted on every fifth section.
Main Outcome Measure(s): Numbers of primordial, primary, and secondary follicles.
Result(s): Systemic administration of both GnRH antagonists caused a significant destruction of primordial follicles compared with control mice. Similar results were obtained whether the antagonists were administered systemically or directly to the ovary. Gonadotropin-releasing hormone agonist had no effect on primordial follicle numbers by itself but reduced the follicular depletion caused by cyclophosphamide.
Conclusion(s): In contrast to the effects of GnRH agonists to reduce chemotherapeutic destruction of primordial follicles, GnRH antagonists do not protect the ovary from the damaging effects of cyclophosphamide. More importantly, GnRH antagonists alone deplete primordial follicles in this murine model, likely through a direct effect on the ovary. Whether these observations apply to other species requires further study. (Fertil Steril® 2005; 83:1333–8. ©2005 by American Society for Reproductive Medicine.)

Key Words: GnRH antagonist, antide, primordial follicles, GnRH analog, ovarian reserve

Gonadotropin-releasing hormone analogues have been used for more than 2 decades to indirectly manipulate ovarian function by stimulating or inhibiting the hypothalamic–pituitary axis. Agonists, which transiently stimulate then suppress gonadotropin secretion through desensitization and receptor down-regulation, have been used clinically since the mid-1970s and are now a staple of assisted reproductive technology programs worldwide. Gonadotropin-releasing hormone agonists have also been used in clinical practice with some success to lower the incidence of ovarian failure after chemotherapy (1–5). Pereyra Pachecio et al. (3) reported that adolescent girls treated with GnRH agonist resumed cyclic ovarian function after chemotherapy, whereas all patients treated with chemotherapy alone remained amenorrheic and experienced premature ovarian failure as demonstrated by increased gonadotropin secretion. Similar results have been reported in young women by Blumenfeld et al. (4, 5) and in Rhesus monkeys by Ataya et al. (1). However, the benefit of gonadotropin suppression during chemotherapy is not universally accepted (2), and other investigators have failed to demonstrate a protective effect of agonists on ovarian reserve (6). The mechanism(s) whereby GnRH agonists could prevent ovarian failure is not clear but presumably involves the suppression of endogenous gonadotropin secretion. However, the early stages of follicular growth (primordial through preantral) are gonadotropin-insensitive, if not completely gonadotropin-independent (reviewed by McGee and Hsueh [7]). This "gonadotropin insensitivity" might account for the relatively limited success of GnRH agonists in protecting the ovary, because the agonists do not completely suppress gonadotropin secretion, even at high doses.

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Reprint requests: Douglas R. Danforth, Ph.D., Department of Obstetrics and Gynecology, The Ohio State University, 5th Floor Means Hall, 1654 Upham Drive, Columbus, Ohio 43210 (FAX: 614-688-3391; E-mail: danforth.2@osu.edu).

Recently, GnRH antagonists have entered into clinical use, primarily as an adjunct to FSH treatment in assisted reproductive technology programs. Gonadotropin-releasing hormone antagonists have the advantage of providing immediate and more complete suppression of gonadotropins than GnRH agonists and seem to be equally effective as agonists in preventing unwanted LH surges while maintaining adequate pregnancy rates (8, 9). Other clinical uses for the antagonists have been proposed (e.g., as chemotherapeutic agents [10]), but none have yet entered routine clinical practice.

Apart from inhibiting pituitary gonadotropin secretion, GnRH analogues have the potential to modulate ovarian function through a direct effect on the ovary. Numerous studies have documented a functional ovarian GnRH and GnRH receptor system in the rat ovary (reviewed by Kang et al. [11]), and the direct effects of GnRH agonists on the rat ovary are well established. Although newer, GnRH antagonists have also been demonstrated to directly modulate ovarian function in the rat (12). The situation in other species is less clear. Although GnRH receptor message has been identified in the human ovary (11), definitive demonstration of receptor protein expression is lacking. There are even fewer data for other species.

The purpose of the present study was to examine the effects of GnRH antagonists on preantral follicle survival in the murine ovary. We hypothesized that GnRH antagonist treatment would abate the chemotherapy-induced depletion of primordial follicles in our rodent model.

MATERIALS AND METHODS

Animals

All studies were approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University and were in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Adult C57Bl/6 mice (5 to 6 weeks old) received either a single injection of GnRH agonist (leuprolide acetate, Lupron Depot; Tap Pharmaceuticals, Deerfield, IL) on study day −10 or injections of the GnRH antagonist antide (Contraceptive Development Branch, NIH, Bethesda, MD) or cetrorelix (Cetrotide; Serono Laboratories, Waltham, MA) on study days −3 and 0. Some animals also received the chemotherapeutic agent cyclophosphamide on day 0. All animals were killed by CO_2 asphyxiation on day 7.

For some experiments, we administered GnRH antagonists directly to the ovary. Prepubertal female C57Bl/6 mice (21 days old) were anesthetized with ketamine HCl (80 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (4 mg/kg; The Butler Company, Columbus, OH) and the dorsal lumbar region prepared for surgery. A 1.0-cm midline incision was made, and the opening was moved to the flank for entrance into the abdominal cavity through the muscle and peritoneum. The uterine horn was grasped with forceps and the ovary exteriorized. Mice then received a 1-μL injection of cetrorelix in saline under the bursa of one ovary. The contralateral ovary was then exteriorized in the same manner and injected with a corresponding volume of vehicle.

Injections were performed under an operating microscope with pulled glass micropipettes (PB-7 micropipette puller; Narishige, Tokyo, Japan) attached to an injector (Narishige IM-5B) and a micromanipulator (Narishige MO-108). Injection solutions were prepared with trypan blue dye to visualize accuracy of injection. Any injection resulting in a leakage of dye was considered a failure, and that animal was not included in the study. The side of treatment (left or right) and the order of injection (control vs. antagonist) were chosen randomly. After injection, ovaries were replaced and the incision closed with sutures. At 1 week after surgery, the mice were killed by CO_2 asphyxiation.

Tissue Processing

Ovaries were removed and fixed in Kahle's solution (4% formalin, 28% ethanol, and 0.34 N glacial acetic acid) for at least 24 hours. After fixation, the ovaries were dehydrated, embedded in Paraplast (VWR Scientific, West Chester, PA), serially sectioned at 7 μm, and mounted on glass microscope slides. Slides were stained with Lille's allochrome. Primordial, primary, and secondary follicles were counted on every fifth section. Only follicles containing an oocyte were counted to avoid counting any follicle twice. Primordial follicles were described as those having a small oocyte with a single layer of squamous granulosa cells. Primary follicles had an intact enlarged oocyte with a visible nucleus and a single layer of cuboidal granulosa cells. Secondary follicles had two or more layers of cuboidal granulosa cells. Secondary follicles were further classified as small if they contained fewer than four layers of granulosa cells and large if they contained four or more layers of granulosa cells.

Statistical Analysis

Results are depicted as mean + SEM. Potential differences in follicle numbers among groups were analyzed by analysis of variance (ANOVA) followed by a Fisher's least significant difference comparison. For the intrabursal experiments, potential differences in the number of follicles between control and treated ovaries were analyzed by paired *t*-test followed by a Fisher's least significant difference comparison. A P value of <0.05 was considered significant for all analyses.

RESULTS

Our initial experiments were designed to test the hypothesis that GnRH antagonist would protect the ovaries from the damaging effects of chemotherapy. As such, we compared the effects of short-term GnRH antagonist (antide) administration with those of longer-term gonadotropin suppression