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GnRH Antagonist Effects on Follicle Number and Size in Rat Neonates and Infants

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ABSTRACT—Increased levels of gonadotropins in the perinatal and prepubertal period may be responsible for the rapid phase of concurrent follicular atresia. This study tests the hypothesis that follicular atresia during this period can be reduced by suppressing gonadotropin release with a GnRH antagonist. Female rat litter mates were randomized to receive daily injections of GnRHi (100 μg Detirelix® [Syntex, Palo Alto, CA] from the day of birth and were sacrificed at 5, 15, or 26 days of life. Follicular atresia was assessed by measuring number and size distribution of ovarian follicles. Serum FSH levels were assayed. GnRHi treatment significantly depressed serum FSH and decreased the number of large antral follicles in 26 day rats, while body weight, reproductive tract weight and total follicle number per representative section were not significantly altered. Age-related changes were significant for all variables. The loss of primordial follicles is likely the result of another mechanism or combination of mechanisms. Gonadotropins do not appear to play a major role in follicular atresia in the neonatal and infant rat.

INTRODUCTION

In Mammals, the number of oocytes declines throughout life as a result of recruitment of primordial follicles and atresia. When the quantity of oocytes reaches a number that is no longer responsive to gonadotropins, ovarian cycling stops and, in menstruating species, menopause begins (Speroff *et al.*, 1989). The cycle of follicular recruitment, selection, and atresia begins in fetal life. In fetal and prepubertal stages, full follicular maturity, expressed by ovulation, does not occur. However, the decline in oocyte number by atresia is dramatic.

Atresia may occur at any point on a continuum of follicular development, from the initial recruitment of the primordial follicle to the development of the large, preovulatory follicle (Speroff *et al.*, 1989). Interest has been directed towards understanding oocyte atresia in an attempt to control follicular function for protection from toxic entities (such as chemotherapeutics) and for the prolongation of reproductive potential (Ataya *et al.*, 1985; Jarrell, *et al.*, 1987; Ataya *et al.*, 1989).

Pulsatile secretion of GnRH from the hypothalamus leads to LH and FSH secretion from the pituitary. In the adult ovary, the number of follicles that undergo maturation is dependent upon the amount of FSH and LH that is available to the ovary and the sensitivity of the ovary to the gonadotropins. GnRH acts directly in the rat ovary to trigger meiotic maturation of the ova within preanthral follicles (Banka and Erickson, 1985). GnRH therefore, plays an important role in the regulation of follicular maturation and ovulation.

GnRH antagonists act by direct occupation of GnRH receptors on the pituitary, blocking the release of gonadotropins. The GnRH agonists act by depleting hormone stores (resulting in an initial serum elevation of gonadotropins) followed by rapid internalization of the receptors and depletion of available GnRH surface receptors (Wierman *et al.*, 1989). GnRH antagonists have been found to effectively suppress LH and FSH serum levels (Wierman *et al.*, 1989; Dvorshak-Harvey *et al.*, 1985; Zeleznik and Kubik, 1986; van den Dungen *et al.*, 1989; Wierman and Wang, 1990; Sharpe *et al.* 1990; Meijs-Roelofs *et al.*, 1990; Andreyko *et al.*, 1992), while avoiding the initial elevation in gonadotropin levels before desensi-

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tization, which is characteristic of the GnRH agonist.

The rate of atresia of primordial follicles in the rat is greatest during the fetal through infant periods and continues at a slower rate during the adult life (Lintern-Moore, 1977). In the neonatal period of the female rat, a high serum FSH level and premature, preovulatory type LH peaks are present during days 9–21 of life (van den Dungen *et al.*, 1989; Goldman *et al.*, 1971; Ojeda and Ramirez, 1972; Dohter and Wuttke, 1976). These peaks occur during a period of substantial loss of follicles – between birth and 20 days of life (Lintern-Moore, 1977). Treatment of young cycling rats with a GnRH agonist inhibited the process of follicular recruitment, resulting in the preservation of a large number of small follicles which would have been lost in the physiologic process of recruitment and atresia (Ataya *et al.*, 1989).

A GnRH agonist was examined for effects on follicular atresia in neonatal and infant rats (Ory *et al.*, 1990). While FSH levels were found to be significantly decreased by day 15 of life, no significant difference in follicular size or number between treated and untreated animals was present at day 25. The early agonist release of gonadotropins may have obscured the effects of GnRH suppression in this study.

To test the hypothesis that the rate of follicular atresia could be slowed during the neonatal period by suppression of serum gonadotropins, administration of a GnRH antagonist to rat pups and its effects on the number and size of follicles were examined.

MATERIALS AND METHODS

Pregnant Sprague-Dawley rats were fed rat Chow® and water ad libitum and housed in an environmentally controlled room in a 14 hr light, 10 hr dark cycle (20–22°C). The afternoon following delivery, female pups were randomly assigned to either control or treatment groups. The pups were identified with an ear punch and remained with their littermates. Male pups were removed from each litter. Rat pups in the treatment group were injected with a GnRH antagonist (100 μg Detirelix®, Syntex, Palo Alto, CA, in 0.1 ml of vehicle consisting of 50% propylene glycol and 50% normal saline) subcutaneously every 24 hr (Ory *et al.*, 1990) commencing within 24 hours of birth. The control rats were injected with vehicle only.

To examine the effects of GnRH antagonist on follicular atresia, rat pups were sacrificed by cervical dislocation 5, 15, or 26 days after birth. The upper reproductive tracts (oviducts and ovaries) were identified, rinsed with normal saline, weighed, fixed in 10% buffered formalin, set in paraffin, and serial step-sectioned (6 μm thickness). Sections from both ovaries were mounted on slides. The slides were stained with hematoxylin and eosin. The sections were viewed and a section representing the mid-longitudinal axis of the ovary was selected from one ovary of each animal. The follicles with a germinal vesicle visible in the oocyte were counted and classified according to grades of maturation using a Zeiss microscope. Grade I follicles were defined as primordial follicles with a small oocyte in prophase of the first meiotic division and a single layer of granulosa cells. Grade II follicles displayed an oocyte of increased diameter surrounded by a single layer of granulosa cells. Grade III follicles were defined as those with multiple layers of granulosa cells and an increased oocyte diameter. Grade IV follicles displayed an antrum. In addition, every serial section was morphologically evaluated using a previously published method (Stachecki *et al.*, 1994). Ovary sections were visualized on an Olympus BH-2 microscope at 25X magnification. Each follicle was digitized as a video image using a VSP SC series high resolution camera Model SC505 (Applied Intelligence Systems, Ann Arbor, MI) which connected to a Dell 486 SX computer and was analyzed using MCID M1 image analysis software (Version 4.12; Imaging Research, St. Catherine's, Ontario, Canada). The image field was calibrated in micrometers with a stage micrometer at the same magnification prior to any measurements. Area, perimeter and maximum chord diameter were measured for each follicle. Measurements were obtained by tracing the outer edge of a follicle using a computer mouse (assuming spherical geometry). Areas were recorded in square microns.

To document FSH suppression in the GnRH antagonist treated rats, blood was collected by cardiac aspiration from sacrificed rats and stored at –70°C. The FSH levels on Day 5, 15, and 26 samples were determined by NIH radioimmunoassay (FSH RIA kit NIHDDK, NIH Bethsda, MD).

Data were analyzed using two-way ANOVA with treatment and age of assessment as the factors. Student-Newman-Keuls post-hoc testing was used to compare group means. Nonparametric methods, such as Friedman's analysis of ranked data, were used when necessary to meet assumptions of normality and equal variance. $P < 0.05$ was taken as significant.

RESULTS

Measuring circulating FSH levels assessed the effect of GnRH antagonist treatment on pituitary release of gonadotropins. Treatment with GnRH antagonist significantly ($P < 0.001$) decreased serum FSH levels at all age points (5, 15, 26 days) as compared to those of control animals (Fig. 1). The effect was not related to an adverse effect of GnRH antagonist on animal health and growth as assessed by comparing animal

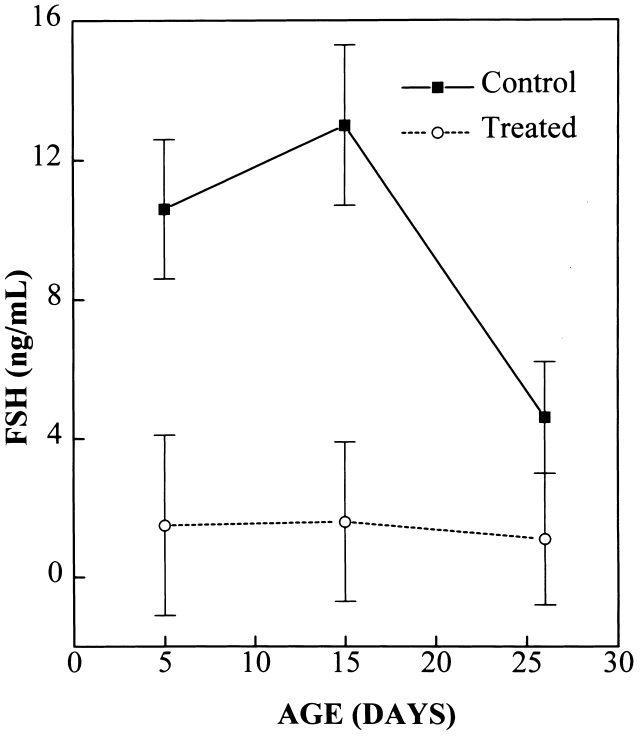

Fig. 1. FSH blood levels (means ± SE) in control (N = 5,4,8) and GnRH antagonist treated (N = 3, 4, 6) rats at 5, 15, and 26 days of life, respectively.

body and organ weights. There were no significant differences in upper reproductive tract weights and total body weights between the control and treated animals (P = 0.41 and 0.38, respectively). Age related differences in weights are apparent between the 5, 15, and 26 day groups. A discrepancy in sample sizes occurred between the data set for FSH levels (Fig. 1). Fewer animals at 5 and 15 days of age were assayed for FSH level due to difficulty in obtaining sufficient amounts of blood from the smaller animals.

There was not a significant difference between the control and treated animals (P = 0.77) in the total number of ovarian follicles in representative 6 micron sections. There were age-related changes in the number of ovarian follicles (P < 0.001), confirming the significance of atresia in the infant and juvenile period (Fig. 2). There were no significant differences in the number of grade I, II, and III follicles between control and treated animals (Fig. 3), The control animals exhibited more grade IV follicles (P < 0.05) than the treated animals (Fig. 3), likely in response to the availability of gonadotropins (Fig. 1). There is a trend towards an increase in the mean follicular area in the control animals (Fig. 4). However, due to the small number of antral follicles in comparison to primordial follicles, there is no statistical difference between the two groups.

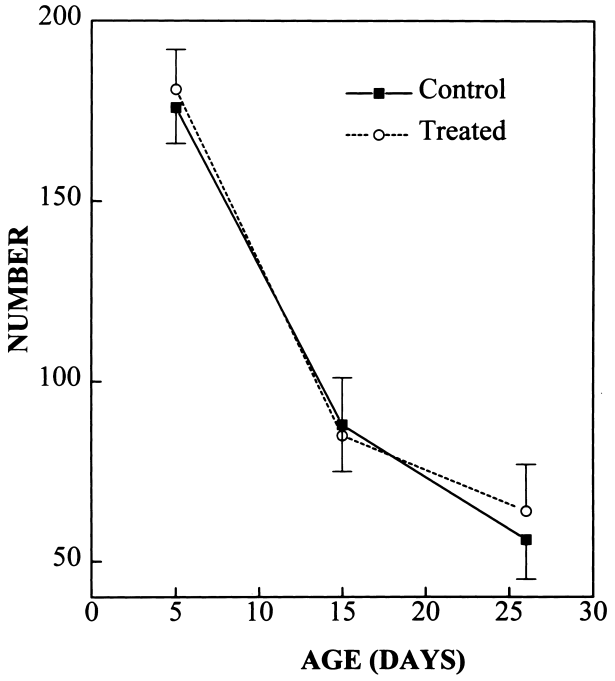

Fig. 2. Numbers (means ± SE) of follicles in a representative ovarian section in control and GnRH antagonist treated rats at 5, 15, and 26 days of life.

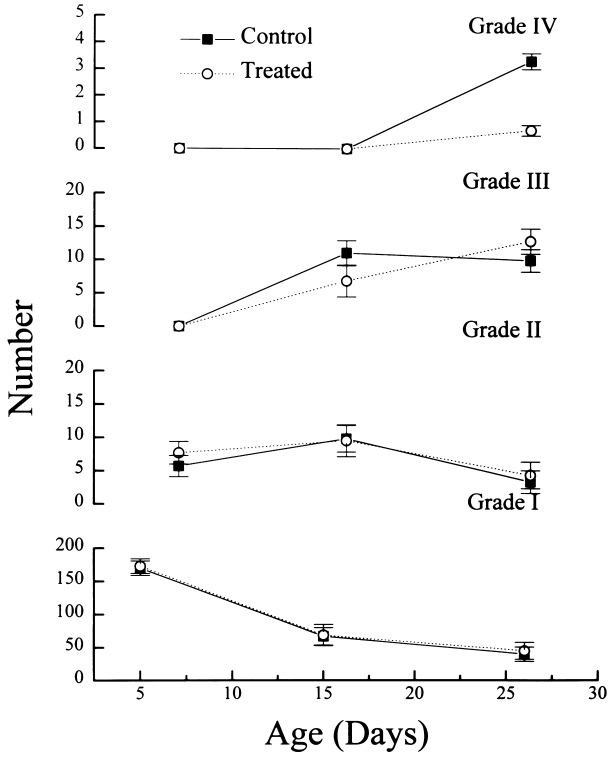

Fig. 3. Numbers (means ± SE) of grade I to IV follicles in control and GnRH antagonist treated rats at 5, 15, and 26 days of life.

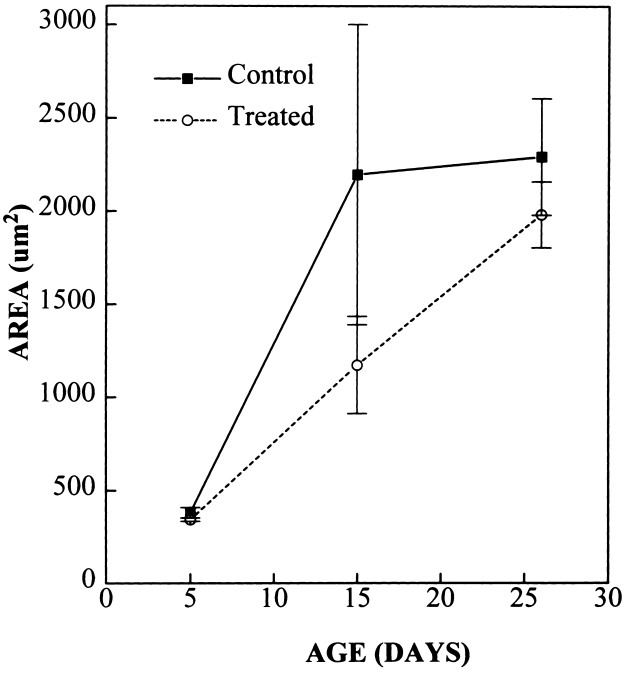

Fig. 4. Follicular area (means ± SE) in control and GnRH antagonist treated rats at 5, 15, and 26 days of life.

DISCUSSION

The orchestration of the process of follicular atresia in the prepubertal period is a mystery. Gonadotropin surges have been shown to occur during the neonatal and infant periods, coinciding with a dramatic decrease in follicle number in the rat (van den Dungen *et al.*, 1989; Lintern-Moore, 1977; Goldman *et al.*, 1971; Ojeda and Ramirez, 1972; Dohter and Wuttke, 1976). However, we observed that inhibition of gona-

dotropin secretion with a GnRH antagonist fails to alter the reduction in number of ovarian follicles in the prepubertal rat. Ataya *et al*. (1985) demonstrated that the process of follicular recruitment could be inhibited by a GnRH agonist in young cycling rats. Taken together, these observations suggest that either the ovarian response varies due to the differing nature of the GnRH analogues, or the process of follicular recruitment and atresia in the prepubertal rat is the result of a different mechanism than in the cycling rat.

Atresia in the neonatal and juvenile rat could result from a loss of oocytes directly through the surface epithelium of the ovary, degeneration of large follicles, or as a result of a preprogrammed event (Lunenfeld *et al*., 1975). These events could be dependent upon other events occurring in utero and not upon neonatal factors or the postnatal environmental factors, or perhaps, as combination of these mechanisms is responsible for atresia in the rat.

During the prepubertal period follicles may grow to a large size, but the hormonal environment does not support the development of an ovulatory follicle (Lunenfeld *et al*., 1975). There follicles likely undergo atresia through degeneration and reabsorption. Gonadotropins, therefore, may play a role in atresia as it relates to the development and degeneration of large follicles. There was a significantly greater number of antral follicles (grade IV) in the 26 day old control animals, but the number of large follicles contributes minimally to the total follicle population. Therefore, degeneration of large follicles may be reduced with suppression of gonadotropins, but this reduction is not significant in the process of atresia. The loss of primordial follicles is likely the result of another mechanism or combination of mechanisms. Gonadotropins do not appear to play a major role in follicular atresia in the neonatal and infant rat.

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