Influences of Age and Reproductive Status on Ovarian Ovulatory Responsiveness to Gonadotropin Stimulation (44257)

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Abstract. **Reproductive aging in the female rat is associated with the gradual loss of regular ovulatory function, decreased fertility, and smaller litter sizes. In the present study, we assessed ovarian ovulatory responsiveness to exogenous gonadotropin stimulation in young and middle-aged cyclic females and in middle-aged acyclic persistent-estrous (PE) rats.**

The ovulatory response to human chorionic gonadotropin (hCG) was dose-dependent in both young and middle-aged cyclic rats, with the percentages of rats ovulating and the numbers of ova shed per rat increasing with the dose of hCG administered. At the highest dose tested (10 mIU hCG/g bw), the range in ovulation rates among middle-aged cyclic rats (0–18 ova shed/rat) was greater than that in young animals (12–18 ova/rat). However, there were no statistically significant differences in either the percentages of females ovulating or in the mean ovulation rates between young and middle-aged cyclic groups. In contrast to the normal ovulatory responses observed in most middle-aged cyclic animals, response to hCG was markedly impaired in PE females of the same age. Middle-aged PE rats consistently failed to ovulate in response to a dose of hCG (10 mIU/g bw), which elicited high ovulation rates in young rats. At an even higher dose (20 mIU/g bw), only minimal ovulatory responses were observed (1.8 ± 0.8 ova/rat; 80% of rats ovulating). Thus, most middle-aged regularly cyclic females maintain a similar ovulatory responsiveness to hCG as young rats, suggesting that decreased ovulation rates during aging may be related to attenuated preovulatory LH surges. However, impaired ovulatory responses were observed in middle-aged PE females, indicating altered ovarian function in acyclic animals, which may contribute to their anovulatory state. [P.S.E.B.M. 1998, Vol 217]

Although advanced maternal age represents an important factor in human infertility (1–3), little is known regarding the detailed mechanisms responsible for reproductive senescence in premenopausal women. By the fourth decade of life, many women experience an increased incidence of lengthened and/or anovulatory menstrual cycles (4), as well as changes in gonadotropin, ovarian steroid, and inhibin secretion (5–8). It is also reported that there is a decrease in ovarian steroidogenic response to administration of human menopausal gonadotropins (hMG) with age (9), indicating decreased ovarian sensitivity to gonadotropin stimulation. However, potential age-related changes in ovarian ovulatory responsiveness to LH and hCG have not been examined. Since the preovulatory LH surge provides important signals for follicular rupture and luteinization, as well as oocyte maturation, changes in ovarian responsiveness to LH with age may potentially play an important role in decreased reproductive capacity during aging. In this regard, we have used the middle-aged female rat as a model to examine the effects of age and reproductive status on ovarian ovulatory responsiveness to gonadotropin stimulation.

This work was supported by PHS research grants R29 AG10620 (PSL) and R37 AG04810 (JKHL), awarded by the National Institute on Aging, NIH. Part of these data was presented at the 76th Annual Meeting of the Endocrine Society, abstract 207; June, 1994; Anaheim, CA.

[1]Predoctoral trainee supported by a research supplement award (AG04810-S1) from the National Institute on Aging.

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Received July 14, 1997. [P.S.E.B.M. 1998, Vol 217]
Accepted October 3, 1997.

0037-9727/98/2174-0455$10.50/0

Similar age-related changes in endocrine and ovarian function occur in perimenopausal women and the middle-aged female rat. As in the human, middle-aged rats experience an increased incidence of reproductive dysfunction, characterized by the onset of irregular and anovulatory cycle patterns (10), changes in the levels of FSH, estradiol (E2) and inhibin secretion (11–13), and diminished fertility and fecundity (14–17). Middle-aged cyclic rats also display a decreased magnitude of the preovulatory proestrous LH surge (17–19), associated with decreased ovulation rates (15, 20). In addition, similar age-related changes have been observed in mice and hamsters (21–23). As in the human (24–25), these age-related changes in endocrine and reproductive function are associated with a decreased ovarian follicular pool (26). Recent studies in mice indicate that ovaries transplanted from cyclic middle-aged mice into young hosts are less capable of maintaining cyclic increases in ovarian steroidogenesis, suggesting an ovarian contribution to reproductive aging in rodents (27). Although fewer small developing antral follicles are available for selection on estrous morning (13, 28), the numbers of preovulatory follicles present on proestrus and presumably available for ovulation in middle-aged rats are reportedly similar to that in young animals (28). However, large preovulatory-like follicles persist on estrous morning in middle-aged females, indicating the failure of some preovulatory follicles to ovulate in response to the LH surge (28). These findings suggest that the numbers of ovulating follicles may be influenced by the magnitude of the proestrous LH surge and/or a change in ovarian follicular responsiveness to gonadotropin stimulation in middle-aged cyclic females.

Following the loss of ovulatory function, aging rats enter a persistent-estrous (PE) state, characterized by chronic anovulation, continued follicular development, and moderately elevated plasma E2 levels (29–32). The anovulatory state of PE rats is attributed to a lack of positive feedback response of LH to ovarian steroids (30), resulting in the absence of spontaneous preovulatory gonadotropin surges (29–33). However, caging/mating of PE females with fertile males often results in an LH surge comparable to that seen in cyclic proestrous females, although there are decreased ovulation rates in these animals (33, 34). It is not known whether the decline in numbers of ova shed in response to mating in PE rats is due to fewer preovulatory follicles available for rupture, and/or decreased follicular responsiveness to gonadotropin stimulation.

The present study was designed to elucidate whether a reduced ovarian sensitivity to ovulatory gonadotropin stimulation contributes to decreased ovulatory function in middle-aged cyclic and PE females. In order to assess ovarian sensitivity and ovulatory response to gonadotropin stimulation, young and middle-aged proestrous rats and middle-aged PE females received sodium pentobarbital to block endogenous preovulatory gonadotropin surges (35–37), followed by treatment with various doses of hCG to induce ovulation. Our findings demonstrate that whereas most middle-aged cyclic rats maintain an appropriate ovulatory response to hCG, some middle-aged cyclic and all PE females exhibit significantly decreased sensitivity to ovulatory stimuli.

Materials and Methods

Animals. Young (90–100-day-old) virgin and middle-aged (8–9-month-old) retired breeder female Long-Evans rats (Charles River Laboratories, Portage, MI) were housed five per cage in standard vivarium facilities with controlled temperature (24°–25°C) and photoperiod (lights on from 0500 hr to 1900 hr daily). Food and drinking water were available *ad libitum.* Use of animals was approved by the institutional animal care and use committee at UCLA.

Daily vaginal smears were obtained from these rats to monitor their estrous cycle patterns, and only those animals that had shown at least three consecutive 4-day cycles were considered to be regularly cyclic. Middle-aged females that had displayed at least 15 consecutive days of vaginal cornification were considered to be in the anovulatory PE state. In the present study, young and middle-aged regularly cyclic rats and middle-aged PE females were studied.

Experimental Procedures. Young and middle-aged cyclic rats on diestrous Day 2 and PE females were lightly anesthetized with ether and received intrajugular catheters at 1700–1800 hr (19, 38). At 1330 hr the next day, proestrous rats were given a subcutaneous (sc) injection of sodium pentobarbital (Nembutal; 4.0 mg/100g bw) to block their endogenous preovulatory gonadotropin surges (35–37). For comparison, PE females were similarly treated with sodium pentobarbital. At 1600 hr a baseline blood sample (0.3 ml) was taken *via* the catheter into a heparinized syringe, followed by the iv administration of human chorionic gonadotropin (hCG; 2.5 to 20 mIU/g bw). Subsequently, blood samples (0.3 ml each) were obtained at 5, 15, 30, and 60 min after hCG injection, and then at every 90 min until 2300 hr to monitor plasma hCG and LH levels. Each blood sample was immediately centrifuged to separate plasma, which was stored at −20°C until LH and hCG RIAs. LH levels were measured by a rat LH double antibody RIA using reagents from the National Hormone and Pituitary Program, NIDDK, NIH, and the values were expressed in terms of the reference standard RP-1 as previously described (19). Due to minor cross-reactivity of the anti-rat LH antiserum with hCG, administration of the highest hCG dose (20 mIU/g bw) resulted in a small, transient increase in LH immunoreactivity (between 100–160 ng/ml). However, this did not affect our ability to confirm blockage of the endogenous proestrous LH surge (typically greater than 1000 ng/ml). Plasma hCG values were determined by a commercial hCG kit (Coat-a-Count, DPC, Los Angeles, CA).

The ovulation rate in each animal was determined by examining the oviductal contents at 0900 hr the morning after hCG administration (15). Female rats were euthanized,

and the oviducts were removed and flushed with saline to count the number of ova shed.

Data Analysis. Statistical analyses of potential differences in plasma hormone levels and ovulation rates were performed using two-way analysis of variance, followed by the Student-Newman-Keuls test to determine differences among groups. Correlations between hCG levels and ovulation rates in individual animals were revealed by linear regression analysis using Pearson's Product Moment test. A confidence level of $P < 0.05$ was considered statistically significant.

Results

Patterns of Circulating hCG and LH in Young and Middle-Aged Rats Following Administration of Nembutal and hCG on Proestrus. Young and middle-aged regularly cyclic female rats received Nembutal at 1330 hr on proestrus to block the endogenous preovulatory gonadotropin surges, followed by an injection of hCG at 1600 hr to assess ovulatory responsiveness to gonadotropin stimulation. The proestrous LH surges were blocked by Nembutal treatment in 92% of rats studied, and only those females with blocked LH surges were included in the data analyses. In all animals, plasma hCG increased from undetectable levels at 1600 hr to maximal values 5 min after hCG injection (Fig. 1). Subsequently, plasma hCG decreased steadily over the next 7 hr, approaching basal levels. As anticipated, there were dose-dependent increases in plasma hCG levels, with peak levels doubling as the dose of hCG given (2.5, 5.0, and 10 mIU/g bw) was doubled. There were no significant differences in the circulating concentrations of hCG between young and middle-aged rats at each dose of hCG administered, indicating that both groups received similar exposure to hCG stimulation (Fig. 1).

Dose-Dependent Ovulatory Responses to hCG in Young and Middle-Aged Regularly Cyclic Rats. To assess ovulatory responsiveness to gonadotropin stimulation, ovulation rates (numbers of ova shed/rat) were determined in these same groups of young and middle-aged regularly cyclic females after hCG injection. In both age groups, the ovulatory responses to hCG were dose-dependent, with increasing doses of hCG resulting in higher numbers of ova shed (Fig. 2). This dose-dependent effect of hCG on ovulation rates is consistent with previous reports (36, 39). Furthermore, ovulatory responses to hCG were similar between young and middle-aged groups. The lowest dose of hCG (2.5 mIU/g bw) did not induce ovulation in either the young or middle-ages rats (n = 5/group). At a dose of 5.0 mIU hCG/g bw, there was no significant difference in the percentage of animals ovulating between young (6/6 = 100%) and middle-aged (6/8 = 75%) groups, and their ovulation rates were comparable (9.2 ± 1.9 ova shed/rat vs. 8.1 ± 2.4 ova shed/rat, respectively). Administration of 10 mIU hCG/g bw resulted in ovulation rates similar to that of spontaneously ovulating young cyclic rats (15), with no statistically significant difference between the young (15.4 ± 0.9 ova/rat) and middle-aged (11.0 ± 2.0 ova/rat) groups. There was a wider range in ovulation rates found in middle-aged (0–18 ova/rat) than young animals (12–18 ova/rat) at this high dose, with two middle-aged females failing to ovulate. However, the range in ovulation rates of middle-

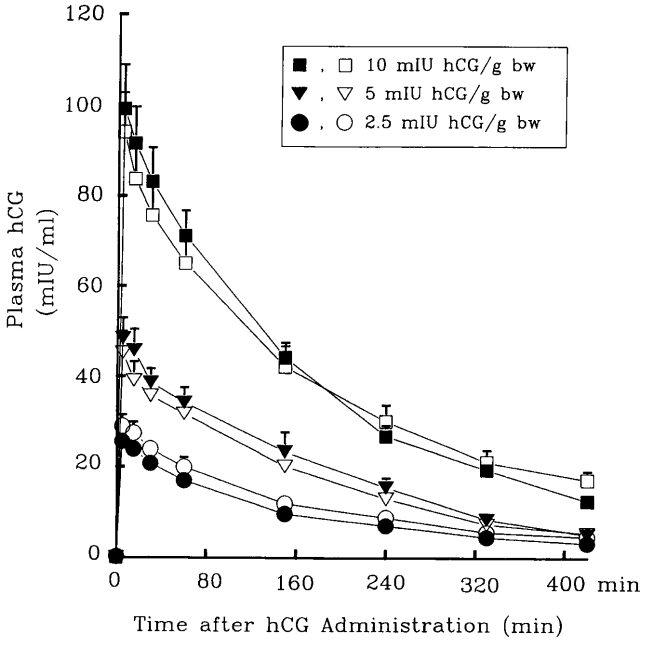

Figure 1. Levels of circulating hCG in young and middle-aged Nembutal-blocked rats treated at 1600 hr proestrus with 2.5 (circles), 5.0 (triangles), or 10 (squares) mIU hCG/g body weight. Samples were obtained before (Time 0) and at 5, 15, 30, 60, 150, 240, 330, and 420 min after hormone treatment. At each hCG dose tested, there was no significant difference in circulating hCG levels between young (closed symbols) and middle-aged (open symbols) rats.

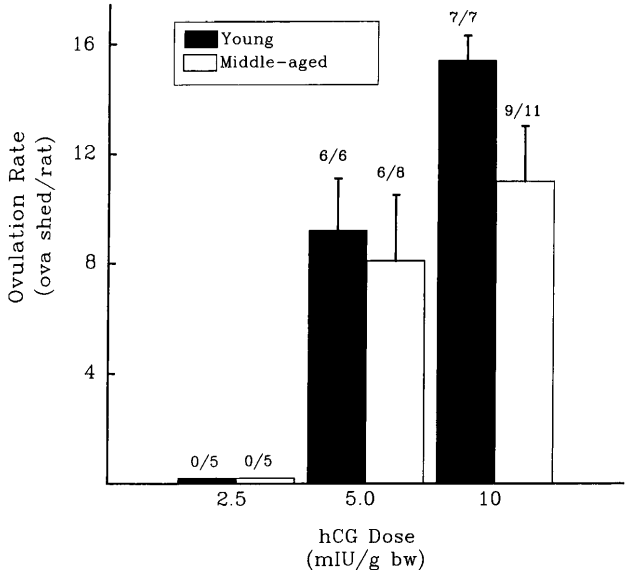

Figure 2. Dose-dependent effects of hCG on ovulation rates (ova shed/rat) in young (solid bars) and middle-aged (open bars) cyclic females. The number of animals ovulating per group at each dose is indicated above each bar. Neither ovulation rate nor the incidence of ovulation was significantly different between young and middle-aged animals at each hCG dose.

aged rats that did ovulate (11–18 ova/rat) at this dose was very similar to that of young animals.

Relationships Between Ovulation Rates and Plasma hCG Levels in Young and Middle-Aged Rats Treated with Nembutal and hCG on Proestrus. To reveal the relationship between the circulating levels of gonadotropin and ovulatory responses in individual animals, we compared the mean plasma hCG levels after hCG injection to ovulation rates in individual female rats (Fig. 3). Linear regression analysis revealed a significant correlation between mean circulating hCG levels and the numbers of ovulated oocytes in both young and middle-aged cyclic females ($r = 0.718$, $P < 0.001$). There was no significant difference in the slopes of the regression lines between young and middle-aged rats. However, two middle-aged cyclic females displayed markedly impaired hCG responsiveness, failing to ovulate despite mean hCG levels that were sufficient to induce high ovulation rates (>10 ova/rat) in young animals.

Ovulatory Response to hCG in Middle-Aged, Acyclic Persistent Estrous Rats. Previous reports have demonstrated neuroendocrine impairments that contribute to the acyclic, PE state in middle-aged rats (30, 33). However, ovulation rates are decreased even in PE females exhibiting mating-induced preovulatory LH surges (33, 34). In the present study, we assessed whether changes in ovarian ovulatory responsiveness to gonadotropin stimulation may also contribute to the acyclic PE state. As shown in Figure 4, administration of 10 mIU hCG/g bw to PE females resulted in the same levels of circulating hCG as seen in similarly treated young cyclic rats. However, this dose of hCG consistently failed to induced ovulation in PE females (Fig. 5; $n = 6$), compared with the high ovulation rates observed in young (15.4 ± 0.9 ova/rat) and middle-aged cyclic (11.0 ± 2.0 ova/rats) rats (Fig. 2). The absence of an ovulatory response in PE rats was further evidenced by the consistent absence of ovarian corpora lutea following hCG stimulation, compared with the high numbers of corpora lutea (15.8 ± 0.8 corpora lutea/rat) observed by histological examination of ovaries of young animals displaying high ovulation rates. Treatment with an even higher dose of hCG (20 mIU/g bw) resulted in a doubling of circulating hCG levels (Fig. 4), and four of six PE rats ovulated. However, the mean ovulation rate at this dose was low (1.8 ± 0.8 ova/rat), with a range of 0–5 ova/rat (Fig. 5).

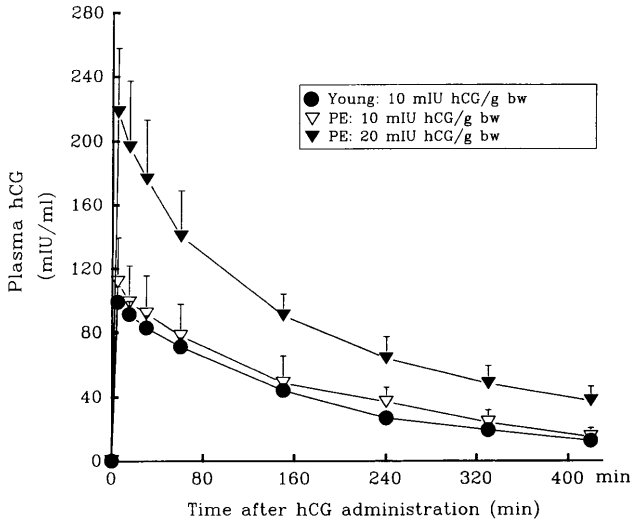

Figure 4. Levels of circulating hCG in middle-aged PE (open triangles) and young cyclic rats (closed circles) after administration of 10 mIU hCG/g bw, and in PE females after treatment with 20 mIU/g bw (closed triangles). Circulating hCG levels were similar between young cyclic and PE animals that received 10 mIU/g bw.

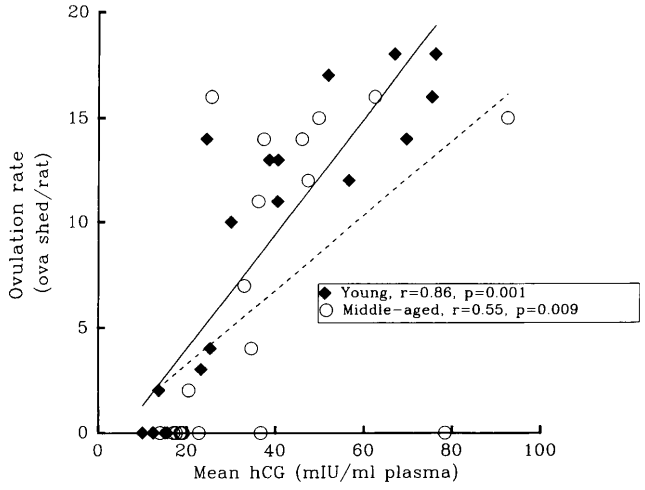

Figure 3. Relationship between mean levels of circulating hCG and ovulation rates among young (open circle) and middle-aged (closed square) cyclic rats after exogenous hormone treatment. There was no significant difference between the slopes of the regression lines of young (—) and middle-aged (----) animals.

Discussion

Reproductive aging is associated with functional changes occurring at several levels of the neuroendocrine-

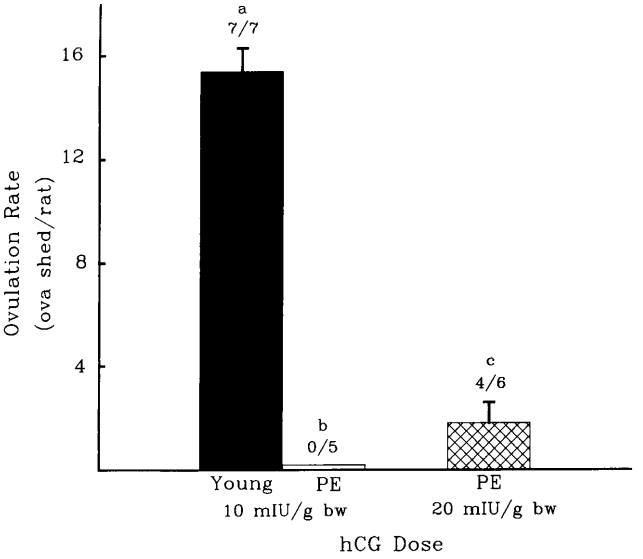

Figure 5. Ovulation rates of young cyclic and middle-aged PE rats in response to hCG treatment. The number of animals ovulating per group is indicated above each bar. Statistically significant ($P < 0.005$) differences in mean ovulation rate between groups are indicated by differing letters above each bar.

ovarian axis. Clear alterations in the neuroendocrine regulation of gonadotropin secretion take place in middle-aged rats, manifested in an attenuation of the preovulatory LH surge (17–19) and the transition from regular estrous cyclicity to irregular cycles, ultimately resulting in an anovulatory PE state (19). In addition, changes in ovarian function, such as decreased ovulation rates and altered patterns of steroidogenesis and of follicular development (13, 15, 28, 31) have also been demonstrated, and may contribute to the decline in reproductive capacity. To date, the potential ovarian contribution to impaired ovulatory function in middle-aged regularly cyclic and PE rats has not been tested directly. The current study revealed a decline in ovarian ovulatory responsiveness to gonadotropin stimulation in aging PE females, whereas most middle-aged cyclic rats displayed an ovulatory response similar to that seen in young animals.

During the PE state, aging females display chronic anovulation and a lack of spontaneous preovulatory LH surges (29). Interestingly, some of these animals experience significant gonadotropin surges in response to mating with fertile male rats although ovulation rates in these females are reduced when compared to those of young animals (33, 34). Although it is known that follicular development continues during the acyclic PE state (31), the functional capacity of these follicles in terms of their ovulatory response to gonadotropins was not known. Granulosa cells of PE rats have been shown to bind gonadotropins indicating the presence of gonadotropin receptors (40). However, it is not known whether these receptors are effective in transducing normal ovulatory responses. Despite the presence of preovulatory-like follicles in PE rats (31), none of the PE animals tested ovulated in response to 10 mIU hCG/g bw, a dose that resulted in high ovulation rates among young (15.4 ± 0.9 ova/rat) and middle-aged (11.0 ± 2.0 ova/rat) cyclic rats. Increasing the dose of hCG administered to 20 mIU hCG/g bw did elicit an ovulatory response in PE females, although the number of ova shed was low (1.8 ± 0.8 ova/rat). These findings indicate that cycle status strongly influences ovarian response to ovulatory stimulation, such that middle-aged acyclic animals show a significantly impaired ability to ovulate in response to high gonadotropin levels, compared with most cyclic rats of the same age. Whereas our data indicate that PE animals show a marked decline in ovulatory responsiveness to hCG, it is not clear whether such ovarian impairments contribute to the onset of the acyclic state, or are the result of chronic, persistent estrus. Thus, alterations in the pattern of follicular development resulting from altered basal gonadotropin levels during the PE state may result in follicles with decreased ovulatory responsiveness to gonadotropin stimulation. Further studies are required to determine whether other endpoints of gonadotropin action, such as LH-induced progesterone production, are also impaired in aging PE rats, suggesting deficits in LH receptor expression and/or signal transduction.

Middle-aged cyclic rats display decreased spontaneous ovulation rats (15), associated with alterations in neuroendocrine and ovarian function (13, 19, 20, 28, 42, 43). However, it was not clear whether decreased ovulation rates were due to an attenuated magnitude of the proestrus LH surge (17–19), and/or a decreased ovarian response to gonadotropin stimulation. A recent report from our laboratory demonstrated a significant correlation between LH surge magnitude and ovulation rate in aging females; rats with low LH surges had fewer ovulations (20). Although it has been reported that middle-aged and young cyclic females have similar numbers of preovulatory follicles on proestrus (28), the relative responsiveness of these follicles to gonadotropin stimulation is not known. A recent report indicated a decreased capacity of ovaries from middle-aged mice to support cyclic increases in estrogen production when transplanted into young animals (27), but did not address the question of ovulatory responsiveness to hCG/LH stimulation. The current study found that most middle-aged cyclic females demonstrate an equal ovulatory responsiveness to hCG as young rats, indicating that decreased ovulation rates during aging do not reflect a decline in ovarian ovulatory capacity. Since ovulation rates are lower in middle-aged rats that display attenuated LH surges (20), whereas ovulatory responsiveness remains unchanged (present findings), it is likely that such age-related declines in ovulatory function are in large part the result of decreased LH surge magnitudes. Thus, age-related changes in neuroendocrine control of LH secretion appear to have an immediate impact on ovarian ovulatory function. Whether attenuated LH surges may also impact the induction of oocyte maturation and/or luteinization of preovulatory follicles requires further investigation.

The applicability of these findings to reproductive aging in women is unknown. Some women experience an increased incidence of anovulatory cycle with age, and the onset of the LH surge may be delayed, resulting in a longer follicular phase (4–6, 44). Some reports suggest that the mean diameter of preovulatory follicles decreases with age in women (44), follicular responsiveness to hMG stimulation declines (9), and estrogen synthesis may increase, decrease, or remain the same as in younger women (5–8, 44, 45). Little data are available regarding the effects of reproductive aging on the responsiveness of the preovulatory follicle to hCG/LH stimulation in women under natural or hormone-stimulated cycles, or the potential effects of such changes on ovulation, oocyte maturation, and/or luteal function. Our data may suggest that while ovulatory responsiveness to gonadotropin stimulation is largely unaffected by age *per se,* altered patterns of follicular development occurring during anovulatory or lengthened cycles in aging individuals may have a deleterious impact upon the efficacy of ovulation induction paradigms. Further clinical investigation is required to explore this possibility.

The authors thank the National Hormone and Pituitary Program, NIDDK, NIH, for the gift of reagents used in the rat LH radioimmunoassay.

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