

Reproductive ageing and ovarian function: is the early follicular phase FSH rise necessary to maintain adequate secretory function in older ovulatory women?

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BACKGROUND: Serum FSH elevations and decreases in inhibin B have been consistently demonstrated in the early follicular phase of cycles in women of advanced reproductive age. However, secretory products of the dominant follicle (estradiol and inhibin A) in the serum of older ovulatory women are maintained at levels similar to those of their younger counterparts. The goal of this investigation was to determine if ovarian secretory capacity is dependent on relative FSH levels and if basal measures of ovarian reserve reflect ovarian secretory capacity. **METHODS:** We administered equivalent low, but effective doses of recombinant FSH for 5 days to a group of older subjects (40–45 years, $n = 9$) and younger controls (20–25 years, $n = 10$) after pituitary suppression with a GnRH agonist. Outcome measures included follicular development as determined by serial transvaginal ultrasound examinations and serum levels of estradiol, inhibin A and inhibin B. **RESULTS:** Serum levels of estradiol and inhibin A were not statistically different between the two groups, while the number of large follicles formed was greater in the younger subjects. Basal parameters of ovarian reserve were not significantly correlated with ovarian secretory capacity, but did correlate with the number of follicles recruited in response to low-dose FSH. **CONCLUSIONS:** By providing equivalent serum levels of FSH in older and younger reproductive aged women, this study demonstrates that the secretory capacity of recruited follicles is maintained in older reproductive aged women.

Key words: antral follicles/inhibin/ovarian ageing/ovarian reserve

Introduction

Studies investigating age-related changes in the hypothalamic–pituitary–ovarian axis have consistently demonstrated physiological and endocrine changes associated with reproductive ageing. One of the earliest signs of reproductive ageing is the FSH rise observed throughout the menstrual cycle, but most prominently noted in the early follicular phase of older ovulatory women (Sherman and Korenman, 1975; Reyes *et al.*, 1977; Lee *et al.*, 1988; Rannevik *et al.*, 1995; Klein *et al.*, 1996a; Burger *et al.*, 1998, 1999; van Zonneveld *et al.*, 2003). More recent investigations have demonstrated decreases in serum inhibin B levels in the early follicular phase in women of advanced reproductive age (Klein *et al.*, 1996b, 2004; Danforth *et al.*, 1998; Reame *et al.*, 1998; Burger *et al.*, 1998, 1999; Santoro *et al.*, 1999; Welt *et al.*, 1999; Muttukrishna *et al.*, 2000). Furthermore, older reproductive aged women experience an earlier onset of the follicular phase FSH rise associated with earlier recruitment and

ovulation of the dominant follicle (Lee *et al.*, 1988; Klein *et al.*, 1996a, 2002; van Zonneveld *et al.*, 2003). The physiological mechanisms behind the FSH rise are not completely understood, but probably involve decreased negative feedback (lower inhibin B secretion) from the diminishing number of pre-antral and early antral follicles in older reproductive aged women (Burger *et al.*, 1998; Roseff *et al.*, 1989; Hall *et al.*, 1992; Reame *et al.*, 1998; Santoro *et al.*, 1999).

Ageing in women is associated with depletion of the primordial follicle pool (Gougeon, 1979; Richardson *et al.*, 1987; Faddy *et al.*, 1992), disorganization of the oocyte meiotic spindle (Battaglia *et al.*, 1996, 1997) and a marked decrease in fertility (Menken *et al.*, 1986; van Noord Zaadstra *et al.*, 1991). In spite of these early changes in the reproductive ageing process, ovarian secretory capacity remains equivalent to (or surpasses) that of younger reproductive aged women (Burger *et al.*, 1998; Lee *et al.*, 1988;

Klein *et al.*, 1996a; Reame *et al.*, 1998; Welt *et al.*, 1999; van Zonneveld *et al.*, 2003). Furthermore, women in these early stages of reproductive ageing [equivalent to stage -3 to -2 according to the Stages of Reproductive Ageing Workshop (STRAW), Soules *et al.*, 2001] continue to be ovulatory, and can be indistinguishable from their younger counterparts in the absence of endocrinological testing. Women in these early stages of reproductive ageing were selected as subjects for this study, as these are the women who frequently experience age-related infertility after intentionally delaying childbearing, often for career and other social reasons.

One mechanism whereby ovarian secretion of hormones and growth factors may remain adequate despite age-related decreases in oocyte quality and number (Gougeon, 1979; Menken *et al.*, 1986; Richardson *et al.*, 1987; Faddy *et al.*, 1992; Battaglia *et al.*, 1996, 1997) is through a compensatory effect of the early follicular phase FSH rise. In this model, early follicular phase FSH elevations observed in older reproductive aged women may not only be due to a decrease in the number of recruitable follicles (and the resulting decrease in negative feedback), but may also be due to a relative resistance of the follicles to gonadotrophins. To investigate the relative role of FSH elevations in ovarian follicular development and secretion, we administered equal doses of recombinant FSH to a group of older (40–45, $n = 9$) and younger (20–25, $n = 10$) reproductive aged subjects after suppression of endogenous FSH secretion. A relatively low (but effective) dose of FSH (150 IU) was chosen specifically to lessen the possibility that decreased ovarian reserve would be the limiting factor in the secretory response of the older subjects. Outcome measures included serum estradiol, inhibin A, inhibin B, and follicular development as determined by serial transvaginal ultrasound examinations. We also investigated the association between ovarian secretory capacity and basal parameters of ovarian reserve, including cycle day 3 FSH, estradiol, inhibin B and the antral follicle count (AFC).

Materials and methods

Subjects

As a continuation of a series of clinical studies of normal reproductive ageing, we recruited healthy, ovulatory women aged 40–45 years ($n = 9$) and 20–25 years ($n = 10$) for participation. To qualify, participants were required to have regular 21–35 day cycles with a luteal phase progesterone level of >10 nmol/l in a recent cycle. Subjects had normal levels of prolactin (<20 ng/ml) and testosterone (<3 nmol/l), a normal body mass index (BMI 18–24 kg/m²), and were not on other medications or exogenous hormones for 6 weeks prior to participation. Subjects also had no history of infertility or reproductive endocrine problems (e.g. hirsutism or galactorrhoea). Women who performed ≥ 5 h/week of aerobic exercise were excluded from the study. For the duration of the study, all participants were either sexually abstinent or utilized non-hormonal forms of contraception. Written consent was obtained and monetary compensation was provided to all volunteers. The protocol was reviewed and approved by the University of Washington Human Subjects Review Committee.

Protocol and procedures

Baseline blood samples and ultrasound examinations for AFC were obtained on day 3 of a spontaneous menstrual cycle. Seven days after detection of a urinary LH surge, subjects were suppressed with a GnRH agonist, leuprolide acetate (Lupron, Tap Pharmaceuticals) 0.5 mg s.c. daily. Beginning with the first day of the subsequent menses, daily venipuncture was performed and serum estradiol levels were determined. Once suppression was confirmed (estradiol <73 pmol/l), the subjects continued on Lupron for an additional 5 days prior to beginning FSH. Both groups were then treated with 150 IU of recombinant FSH (Gonal F, Serono) s.c. per day for 5 days. Blood samples were obtained on a daily basis throughout the treatment interval and for 4 days after FSH was discontinued. Daily Lupron injections were continued throughout the FSH treatment interval and for 2 days after treatment was completed. All blood samples were stored at -20°C for subsequent analysis of FSH, estradiol, and inhibin A and B.

Transvaginal ultrasound

Ultrasound examinations for baseline AFC and follicle growth during the treatment interval were performed by one of following investigators: M.R.S., N.A.K., A.C.T. or K.R.H. We recently have demonstrated that inter-observer differences in AFC are minimal (Hansen *et al.*, 2003). Sonolucent structures visualized within the ovary and having regular contour were considered to be follicles. All follicles 2–10 mm in size were considered antral follicles. AFC refers to the total antral follicle count for both ovaries, and this number was used for calculations. Ultrasound examinations for AFC and determination of the number of follicles >10 mm bilaterally were performed following suppression with Lupron and daily beginning after 3 days of FSH treatment. Transvaginal ultrasound examinations were performed with an ATL Ultramark 400C with a 6.5 MHz vaginal transducer.

Hormone assays

Serum FSH levels were determined using a solid-phase two-site monoclonal enzyme-linked immunosorbent assay (ELISA; DELFIA, Wallace Inc., Gathersburg, MD). The intra- and inter-assay coefficients of variation (CVs) were 1.9 and 8.9%, respectively.

Serum estradiol measurements were performed with a radioimmunoassay using reagents supplied by ICN Biomedicals, Inc. (Costa Mesa, CA). The intra- and inter-assay CVs were 9 and 18%, respectively.

Inhibin B assays were performed with a solid-phase sandwich ELISA (Serotec, Oxford, UK) based on the use of plates coated with a monoclonal antibody specific for the inhibin B β -subunit with a second monoclonal antibody specific for the α -subunit for detection. The sensitivity of the assay was 15.6 pg/ml, and inhibin A had a 0.5% cross-reaction in the inhibin B ELISA. The assay was controlled in triplicate using samples with mean concentrations of 155.3, 316.3 and 919.3 pg/ml, with inter-assay CVs of 11.6, 7.6 and 9.7%, respectively.

A solid-phase sandwich ELISA (DSL, Webster, TX) was also used to measure inhibin A. The first monoclonal antibody is specific for the β -subunit of inhibin A, with a second monoclonal antibody specific for the α -subunit and labelled with horseradish peroxidase for detection. The assay sensitivity was 0.1 IU/ml. The assay standard provided by the manufacturer was calibrated using the World Health Organization's First International Standard for Inhibin (recombinant human inhibin, Lot 91/624). The assay was controlled in duplicate using aliquots of specimens containing 2.00 or 9.56 IU/ml, with inter-assay CVs of 8.7 and 3.3%, respectively.

Data analysis

Power analysis

This report represents an interim analysis of what was intended to be a larger study (30 older subjects and 30 younger controls) designed to investigate the relative role of FSH elevations in ovarian follicular development and function. Based upon earlier work, we anticipated a peak estradiol of 1100 pmol/l in the younger group and 880 pmol/l in the older group with an equivalent SD of 290 pmol/l. With this sample size, we would have had an 80% power to detect a difference of ~ 200 pmol/l between groups. The interim analysis demonstrated significantly smaller differences in peak estradiol levels between the groups (812 pmol/l, younger, and 749 pmol/l, older) and greater SD (540 pmol/l) than anticipated. These recent calculations would indicate the need for a study population that would amount to >200 subjects in order to demonstrate a statistical difference in estradiol levels between groups. These large subject numbers would be impractical considering the intensity of this study. As a result, the decision was made to discontinue enrolment.

Statistics

Differences between groups for hormone levels and follicle numbers were evaluated with a two-factor repeated measures ANOVA. Single point measurements were compared with the Mann–Whitney (rank sum) test. Spearman correlation coefficients were used to determine correlations. Statistical evaluations were performed with StatView software, version 5.0.1 (Abacus Concepts, Inc., Berkeley, CA). Statistical significance was defined as $P < 0.05$.

Results

Study subject characteristics are shown in Table I. Consistent with previous reports, older subjects had higher basal (cycle day 3) serum levels of FSH (8.1 ± 1.2 versus 4.8 ± 0.4 IU/ml, $P < 0.01$) and lower basal AFCs (9.7 ± 1.6 versus 18.5 ± 1.8 , $P < 0.01$) compared with the younger controls. Older subjects also had higher basal estradiol levels and lower levels of inhibin B than the younger controls, although these differences did not reach statistical significance.

Serum levels of estradiol, inhibin A, inhibin B, and the number of large follicles (>10 mm) developing during FSH stimulation for both the older subjects and younger controls are illustrated in Figure 1. Serum levels of estradiol, inhibin A and inhibin B tended to be greater in the younger than in the older women; however, these differences were not statistically significant. The number of large follicles developing during the study period was significantly greater in the younger controls compared with that of the older group (group effect $P < 0.05$). Serum FSH levels were equivalent between groups during the treatment interval (Figure 1). At the completion of

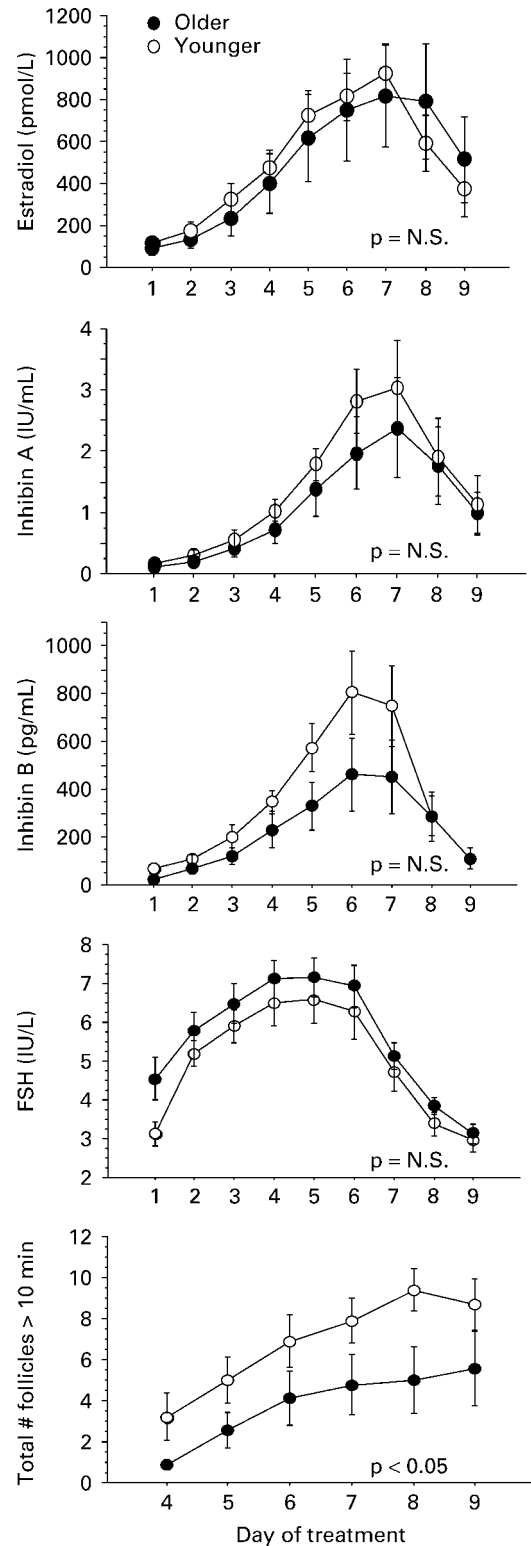


Figure 1. Serum levels of estradiol, inhibin A, inhibin B and FSH, and total number of large (>10 mm) follicles formed during and after stimulation with recombinant FSH. Older subjects (40–45 years, $n = 9$, ●) and younger controls (20–25 years, $n = 10$, ○) were treated with FSH for 5 days (day of treatment 1–5) following pituitary suppression with leuprolide acetate. Values are shown as the mean \pm SEM. The presence or absence of a significant difference is noted under each graph.

Table I. Study subject basal (cycle day 3) characteristics (means \pm SEM)

	Younger ($n = 9$)	Older ($n = 10$)	P
Age (years)	22.6 ± 1.8	42.1 ± 0.7	<0.01
Antral FC (total)	18.5 ± 1.8	9.7 ± 1.6	<0.01
FSH (IU/l)	4.8 ± 0.4	8.1 ± 1.2	0.02
Inhibin B (pg/ml)	61.0 ± 11.4	54.8 ± 12.8	NS
Inhibin A (IU/ml)	0.1 ± 0.02	0.2 ± 0.05	NS
Estradiol (pmol/l)	87.2 ± 16.4	128.2 ± 22.5	NS

the stimulation interval, there was a trend toward increased estradiol and inhibin A secretion per large (> 10 mm) follicle in the older compared with the younger participants, but these differences were not statistically significant (data not shown).

We also evaluated the correlation between basal parameters of ovarian reserve (day 3 FSH and AFC) and the major secretory products of growing follicles (estradiol and inhibin A, Figure 2.). Both basal FSH and AFC were poorly

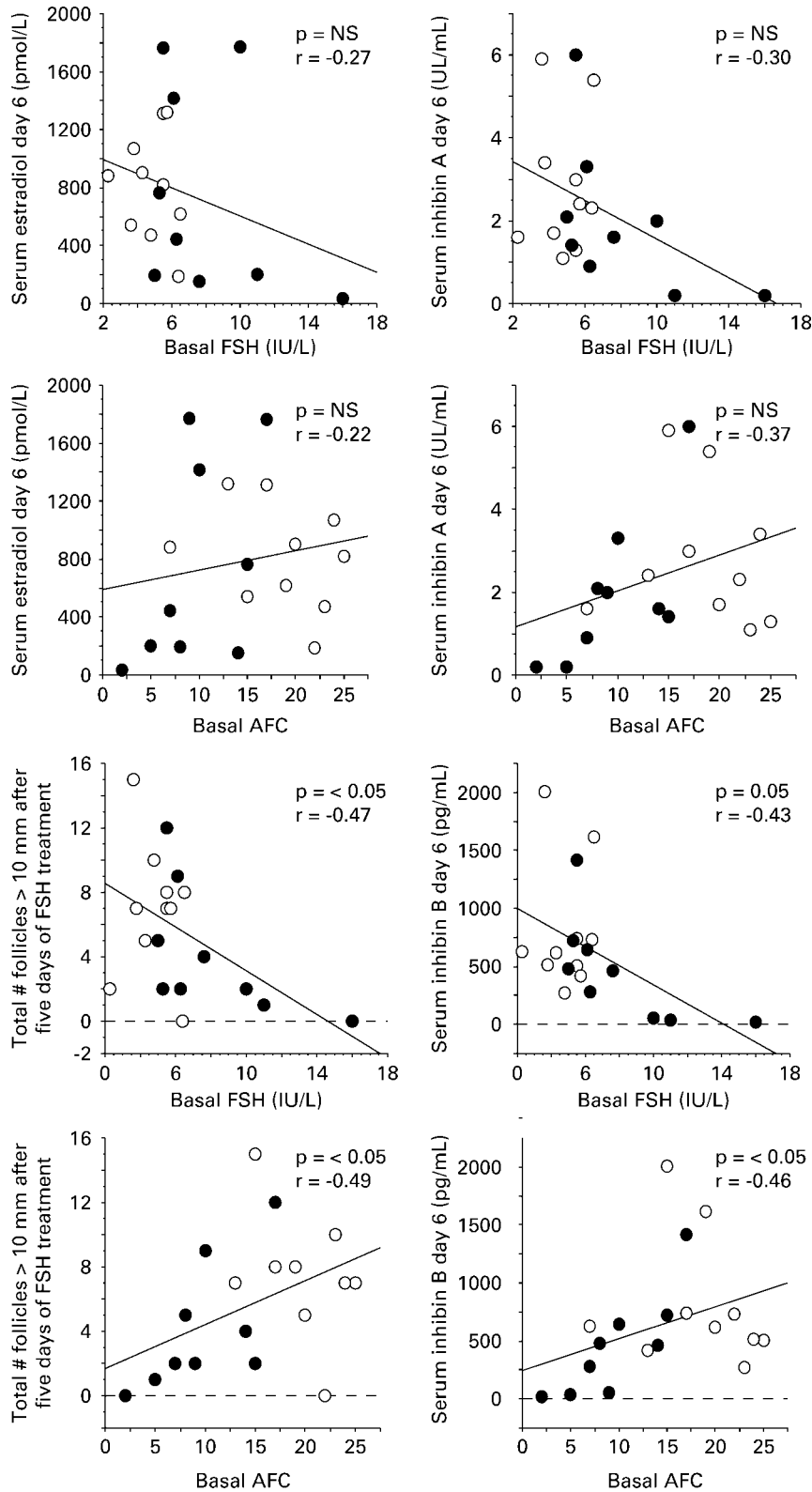


Figure 2. Scattergrams with regression lines for basal parameters of ovarian reserve versus serum estradiol, inhibin A, inhibin B, and total number of large (> 10 mm) follicles formed after 5 days of stimulation with recombinant FSH in older subjects (40–45 years, $n = 9$, ●) and younger controls (20–25 years, $n = 10$, ○). Spearman correlation coefficients and statistical significance are indicated in each graph.

correlated with serum estradiol and inhibin A levels at the end of the stimulation interval. However, both basal FSH and AFC were significantly correlated with parameters reflecting the size of the pool of recruitable follicles (the number of large follicles that developed and the serum inhibin B levels obtained following FSH treatment). Basal inhibin B levels were not significantly correlated with serum estradiol, inhibin A or the number of follicles that developed during stimulation (data not shown).

Discussion

Reproductive ageing is a continuous process that begins many years before menopause. In the context of clinical relevance for the large number of women experiencing infertility due to relatively advanced reproductive age, we designed this study to examine ovarian function in normal women in the late reproductive (STRAW stage -3) and early menopausal transition (STRAW stage -2) stages (Soules *et al.*, 2001). Individuals in these early stages of reproductive ageing experience regular menstrual cycles and are largely indistinguishable from their younger counterparts in the absence of endocrine testing.

Numerous investigations have determined that one of the earliest detectable changes in reproductive ageing is the early follicular phase rise in serum FSH (Sherman and Korenman, 1975; Reyes *et al.*, 1977; Lee *et al.*, 1988; Rannevik *et al.*, 1995; Klein *et al.*, 1996a; Burger *et al.*, 1998, 1999; van Zonneveld *et al.*, 2003). Although the exact cause of this FSH rise remains to be fully elucidated, a key component of this mechanism appears to be a decrease in negative feedback as a result of lower early follicular phase inhibin B levels as observed in older reproductive aged women (Burger *et al.*, 1998, 1999; Roseff *et al.*, 1989; Hall *et al.*, 1992; Reame *et al.*, 1998; Santoro *et al.*, 1999; Klein *et al.*, 2004). Lower serum inhibin B levels, in turn, are likely to be due to the smaller pool of pre-antral and early antral follicles remaining in the ovaries of older reproductive aged women (Faddy *et al.*, 1992; Scheffer *et al.*, 1999).

Although women at these stages of reproductive ageing have decreased natural fecundity (Menken *et al.*, 1986; van Noord Zaadstra *et al.*, 1991) and experience earlier recruitment and ovulation of the dominant follicle (Klein *et al.*, 2002; van Zonneveld *et al.*, 2003), serum levels of inhibin A and estradiol remain at levels similar to or greater than those observed in younger women (Burger *et al.*, 1998; Lee *et al.*, 1988; Klein *et al.*, 1996a; Reame *et al.*, 1998; Welt *et al.*, 1999; van Zonneveld *et al.*, 2003). Furthermore, follicular fluid levels of steroids and inhibins have also been determined to be equivalent to those seen in younger reproductive aged women (Klein *et al.*, 1996c, 2000). These observations suggest that FSH elevations may serve a compensatory mechanism to maintain dominant follicle development and secretion in the presence of a diminishing primordial follicle pool. If true, then at equivalent FSH levels, the secretory capacity of the older ovary would be less than that of the younger ovary. To test this hypothesis, we treated a group of older (40–45 years) and younger (20–25 years) women with

equivalent doses of FSH following pituitary suppression. To limit the influence of differences in ovarian reserve impacting the ovarian secretory response, we specifically chose a relatively low dose of exogenous FSH (150 IU).

At equivalent induced levels of FSH, we were unable to detect a difference in serum levels of the major secretory products of large follicles (estradiol and inhibin A), although both tended to be greater in the younger than in the older group. Similarly, when evaluated on a per large (>10 mm) follicle basis, secretion of estradiol and inhibin A was not significantly different between the groups. These findings would suggest that at equivalent serum FSH levels, the secretory capacity of the 'older' and 'younger' follicle is roughly equivalent. Furthermore, it would suggest that FSH elevations seen in the early follicular phase of older reproductive aged women are a reflection of the diminishing size of the primordial follicle pool and are not necessary to maintain adequate follicular secretion. The higher levels of estradiol and inhibin A observed in some natural cycles of older reproductive aged women may result from increased FSH levels in the presence of normally functioning (but fewer) follicles.

Although we detected no statistically significant differences in the secretion of estradiol and inhibin A between the groups, we did detect significant differences in the number of large pre-ovulatory size follicles. Additionally, inhibin B levels were also greater in the younger than in the older subjects, although this difference did not quite reach statistical significance ($P = 0.08$). Both of these parameters are indicative of ovarian reserve (Eldar-Geva *et al.*, 2000) and would be anticipated to be greater in the younger than in the older subjects.

The complexity and expense of recruiting and treating large numbers of women with this study design precluded a large number of subjects. With small prospective studies such as ours, the possibility of a type II error exists. In particular this seems likely in the case of inhibin B differences between the groups. However, even with this small sample size, we were able to detect a difference in follicular recruitment between the groups. Although a larger sample size might have detected statistical differences in estradiol and inhibin A levels between groups, in absolute terms, these differences would probably be small and of uncertain clinical significance.

It is also important to note that elevated serum FSH levels and decreased AFC were not part of the entry criteria for our older subjects. Although the study and control groups were statistically different in terms of these basal parameters, several of the older subjects had basal FSH levels and AFC that overlapped those of the younger control group. It is possible that an investigation of the ovarian secretory capacity of a more highly selected group of older reproductive aged subjects (those clearly more advanced in the reproductive ageing process) would have given different results.

Consistent with previous investigations, we determined that the AFC and basal FSH (Scott and Hofmann, 1995; Tomás *et al.*, 1997; Bancsi *et al.*, 2002; Yong *et al.*, 2003) were significantly correlated with parameters reflecting

ovarian reserve (the number of large follicles developing and inhibin B levels obtained during stimulation). Nevertheless, neither of these basal parameters accurately reflected ovarian secretory capacity. Given the lack of statistical differences in secretory capacity between the two age groups, these findings are not surprising. We also detected no significant correlations between basal inhibin B and ovarian stimulatory or secretory capacity. These findings are consistent with recent investigations suggesting that both basal FSH and AFC are better markers of ovarian reserve than is basal inhibin B (Scott and Hofmann, 1995; Bancsi *et al.*, 2002; Yong *et al.*, 2003).

In summary, we detected no significant differences in ovarian secretion of estradiol and inhibin A in older reproductive aged women compared with younger controls at equivalent serum FSH levels. Per large follicle, estradiol and inhibin A secretion also tended to be similar between the two groups. These observations suggest that in older reproductive aged women, higher FSH levels are not necessary to maintain adequate follicular secretion, but more probably are due to the diminished size of the primordial follicle pool and the resulting decreased negative feedback. While the AFC and basal FSH measurements reflect ovarian reserve, they are not significantly correlated with ovarian secretory capacity.

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