

Article

Initial analysis of variability among basal hormone biomarkers of ovarian reserve



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Abstract

The most commonly used biomarker tests of ovarian reserve are basal hormone measurements during the early follicular phase, including mainly FSH but also oestradiol, FSH:LH ratio, and inhibin B. This study was designed to assess prospectively the intra- and inter-cycle variability of serum values of those hormone biomarkers in the early follicular phase of consecutive cycles in a group of women candidates for assisted reproduction. Fifty eumenorrhoeic women underwent blood sampling for hormone measurement on cycle day 3 for three consecutive cycles, and during the first study cycle, daily samples were obtained on cycle days 2, 3, 4 and 5. No significant difference was detected among FSH concentrations and FSH:LH ratios during cycle days 2–5; in contrast, oestradiol and inhibin B were not constant through the early follicular phase. No difference in FSH or inhibin B serum concentrations and FSH:LH ratio on cycle day 3 during three consecutive cycles was noted; however, significant inter-cycle variability for oestradiol serum concentration on cycle day 3 was detected. FSH and inhibin serum concentrations, and FSH:LH ratio varied significantly less than oestradiol on cycle day 3, but inter-cycle variability was similar for the first three hormonal biomarkers of ovarian reserve. There was significantly less intra-cycle variability of FSH serum concentration and FSH:LH ratio than oestradiol and inhibin B serum concentrations. Basal FSH serum concentrations (or FSH:LH ratio) during the early follicular phase showed neither significant inter-cycle nor intra-cycle variability when measured during 3 consecutive months in an assisted reproduction patient population, thus offering greater flexibility of pretreatment sampling.

Keywords: FSH, FSH:LH ratio, inhibin B, oestradiol, ovarian reserve, variability

Introduction

Recruitment and development of multiple ovarian follicles in response to gonadotrophin stimulation is a basic goal in assisted reproduction technologies. The ability of the ovaries to respond to gonadotrophins with adequate follicular development has been referred to as ovarian reserve (Scott, 2001). Although it declines with age, ovarian reserve is a biological and not just a chronological function and importantly, the timing of its onset is highly variable (Scott and Hofmann, 1995; Scott, 2001).

Identifying potential low responders is considered of critical clinical importance because these patients require specialized

management to optimize the number and quality of oocytes that may be available for assisted reproduction procedures (Lisi *et al.*, 2001; Scott, 2001; Ulug *et al.*, 2003). This has led to the recommendation that screening tests should be performed on women attending an assisted reproduction programme in an attempt to offer these patients an accurate prognosis for treatment success (Scott and Hofmann, 1995; Cohen, 2002).

The most commonly used biomarker tests of ovarian reserve are basal hormone measurements during the early follicular phase including mainly FSH but also oestradiol, FSH:LH ratio, and inhibin B (Sharara and Scott, 1997; Fasoulitis *et al.*, 2000; Bukman and Heineman, 2001; Cohen, 2002; van der

Spuy and Alberts, 2002; Loverro *et al.*, 2003). Traditionally, basal hormone testing has been performed on cycle day 3. Cycle day 3 testing emerged initially as a dictum from early studies where ovarian stimulation protocols without pituitary desensitization were initiated on cycle day 3, 4 or 5 (Jones *et al.*, 1984; Marrs *et al.*, 1984). However, more recent studies investigating FSH and oestradiol variability, suggested that rigid adherence to cycle day 3 collections no longer seems necessary with respect to FSH (but not oestradiol), values which are equivalent on cycle days 2–5 (Brown *et al.*, 1995; Hansen *et al.*, 1996). On the other hand, to be useful in practice, it would be necessary to define prognostic criteria before treatment started. Thus, in clinical practice patients are usually sampled for basal hormone values within 3 months of the assisted reproduction cycle (Cahill *et al.*, 1994; Balasch *et al.*, 1996; Creus *et al.*, 2000). This is supported by a previous study showing no significant inter-cycle variability for basal FSH and oestradiol when hormone concentrations for two cycles were considered (Hansen *et al.*, 1996). However, others reported intercycle variability of day 3 FSH [coefficient of variation (CV), 25.6%; 95% confidence intervals (CI), 21.4–29.9%] and oestradiol (CV, 44.1%; 95% CI, 37.0–51.2%) (Brown *et al.*, 1995). In those previous studies (Brown *et al.*, 1995; Hansen *et al.*, 1996), however, serum samples from different menstrual cycles were obtained over a 1-year period in healthy women and thus subjects were not necessarily sampled on consecutive cycles. In addition, FSH:LH ratio and inhibin B were not investigated.

On the above evidence, this study was designed to assess prospectively the intra- and inter-cycle variability (but not the clinical value as ovarian reserve tests) of serum concentrations of FSH, oestradiol, FSH:LH ratio, and inhibin B in the early follicular phase of consecutive cycles in a group of women candidates for assisted reproduction which are the population of patients usually undergoing ovarian reserve tests in daily clinical practice.

Materials and methods

A total of 50 infertile women between 24 and 40 years of age (mean $\pm$ SD, 34.5 ± 3.84 years) were included in the present study. All of them were candidates for assisted reproduction due to male factor (40%), tubal factor (30%), minimal to mild endometriosis (16%), or unexplained infertility (14%). All patients had regular menstrual cycles (length 26–34 days) and normal ovulatory function as evidenced by basal body temperature and serum progesterone and prolactin measurement. No patient had received any hormone therapy, including gonadotrophins, for at least 6 months preceding the study.

In all patients, serum samples were drawn on menstrual cycle day 3 for three consecutive cycles, and during the first study cycle daily samples were obtained on cycle days 2, 3, 4 and 5. All samples were obtained in the fasting state between 0800 and 0900 hours. The sera were stored frozen at -20°C , and at the conclusion of the study samples were batched and assayed for FSH, oestradiol, LH, and inhibin B. Frozen blood samples from each patient were examined in one run.

Hormones were measured using commercially available kits as reported previously (Creus *et al.*, 2000; Peñarrubia *et al.*,

2000; Balasch *et al.*, 2001). FSH and LH serum concentrations were measured by an immunoenzymatic assay with two monoclonal antibodies (Immuno 1, Technicon; Bayer, Tarrytown, NY, USA). Data are expressed in terms of IRP 78/549 and 68/40 for FSH and LH respectively. The sensitivity of the assays was 0.1 IU/l for FSH and 0.3 IU/l for LH, and the interassay CV were 2.7 and 3.1% respectively. Oestradiol concentrations in serum were estimated by a competitive immunoenzymatic assay (Immuno 1, Technicon). The sensitivity was 10 pg/ml and the interassay CV 5%. Inhibin B measurements were performed by a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) carried out in microtitre plates (Serotec Ltd, Oxford, UK). The assays sensitivity was 15 pg/ml and the intraassay CV 5.5%. The interassay CV at low (36 pg/ml) and high (246 pg/ml) concentrations were 12 and 7% respectively.

Data were analysed by Statistics Package for Social Sciences statistical software. Means between groups were compared with one-way analysis of variance (ANOVA). The Bonferroni test was used for post-hoc comparisons. Statistical significance was set up at a *P*-value of <0.05 . CV were calculated to quantitate the month-to-month variability of day 3 FSH, oestradiol, FSH:LH ratio, and inhibin B. CV were calculated for each subject, and then the mean CV were determined for each individual hormone marker. Similarly, consecutive hormone values on days 2, 3, 4 and 5 in the first study cycle were used to calculate CV for each subject in order to quantitate the day-to-day variability.

Results

Results are presented in **Tables 1, 2** and **3**. Mean FSH, oestradiol, FSH:LH ratio, and inhibin B serum values on cycle days 2–5 during the first study menstrual cycle are given in **Table 1**. No significant difference was detected among FSH concentrations and FSH:LH ratios during this time period. In contrast, oestradiol and inhibin B were not constant through the early follicular phase. Serum oestradiol concentrations commenced to increase by cycle day 4 such that the mean serum values on cycle days 4 and 5 were significantly higher than on cycle days 2 and 3. The same was true for inhibin B serum concentrations for which, in addition, the mean value on cycle day 5 was significantly greater than that on cycle day 4.

As shown in **Table 2**, when the inter-cycle variability of FSH, oestradiol, FSH:LH ratio, and inhibin B serum concentrations for three consecutive cycles was considered, no difference in FSH or inhibin B serum concentrations and FSH:LH ratio on cycle day 3 was noted. However, significant inter-cycle variability for oestradiol serum concentration on cycle day 3 was detected.

CV with CI were used to quantitate the month-to-month and day-to-day variability of FSH, oestradiol, FSH:LH ratio, and inhibin B in the early follicular phase (**Table 3**). FSH and inhibin serum concentrations, and FSH:LH ratio varied significantly less than oestradiol on cycle day 3, but inter-cycle variability was similar for the first three hormonal biomarkers of ovarian reserve. On the other hand, there was significantly less intra-cycle (days 2–5) variability of FSH serum concentration and FSH:LH ratio than oestradiol and inhibin B serum concentrations.

Table 1. Mean hormonal serum concentrations between cycle days 2 and 5 in the 50 women studied. Values are mean $\pm$ SD.

<i>Hormone</i>	<i>Cycle day</i>				<i>P-value^a</i>
	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	
FSH (IU/l)	8.37 $\pm$ 2.73	8.7 $\pm$ 2.30	8.35 $\pm$ 2.92	8.13 $\pm$ 2.54	NS
Oestradiol (pg/ml)	38.71 $\pm$ 16.21	38.61 $\pm$ 16.59	44.55 $\pm$ 21.10 ^b	46.35 $\pm$ 27.89 ^b	<0.05
FSH:LH ratio	1.65 $\pm$ 0.63	1.58 $\pm$ 0.64	1.61 $\pm$ 0.66	1.50 $\pm$ 0.61	NS
Inhibin B (pg/ml)	58.58 $\pm$ 26.48	61.39 $\pm$ 25.18	69.72 $\pm$ 21.49 ^b	75.00 $\pm$ 25.47 ^{b,c}	<0.05

^aANOVA test.^bSignificantly different from cycle days 2 and 3 ($P < 0.05$; post-hoc Bonferroni test).^cSignificantly different from cycle day 4 ($P < 0.05$; post-hoc Bonferroni test).

NS = not significant.

Table 2. Hormone serum concentrations measured on cycle day 3 during three consecutive cycles in the 50 women studied. Values are mean $\pm$ SD.

<i>Hormone</i>	<i>Cycle number</i>			<i>P-value^a</i>
	<i>1</i>	<i>2</i>	<i>3</i>	
FSH (IU/l)	8.7 $\pm$ 2.30	8.18 $\pm$ 2.52	9.24 $\pm$ 2.87	NS
Oestradiol (pg/ml)	38.61 $\pm$ 16.59	37.41 $\pm$ 3.22	35.10 $\pm$ 3.39	<0.05
FSH:LH ratio	1.58 $\pm$ 0.64	1.49 $\pm$ 0.53	1.66 $\pm$ 0.73	NS
Inhibin B (pg/ml)	61.39 $\pm$ 25.18	55.80 $\pm$ 23.25	55.16 $\pm$ 19.77	NS

^aANOVA test.**Table 3.** Inter-cycle variability of cycle day 3 hormone serum concentrations and day-to-day variability of hormonal biomarkers in the early follicular phase as assessed by coefficient of variation (CV) and confidence intervals (CI).

<i>Hormone</i>	<i>Inter-cycle variability: CV (95% CI)</i>	<i>Intra-cycle variability: CV (95% CI)</i>
FSH (IU/l)	10.4 (8.6–12.2)	9.3 (7.1–11.5) ^b
Oestradiol (pg/ml)	19.7 (13.8–25.6) ^a	16.6 (12.0–21.2)
FSH:LH ratio	10.0 (8.2–11.8)	10.3 (9.2–11.6) ^c
Inhibin B (pg/ml)	9.0 (7.2–10.8)	18.2 (12.8–23.6)

^aSignificantly higher than FSH, FSH:LH ratio, and inhibin B.^{b,c}Significantly lower than oestradiol and inhibin B.

Discussion

As the oocyte population declines in the decade before the menopause, subtle endocrine changes occur which impact on reproductive performance (van der Spuy and Alberts, 2002). Initially, there is a transient rise of FSH in the early follicular phase, with continued normal secretion of oestradiol from the dominant follicle. Cycle length shortens, mainly due to enhanced early follicular stimulation by the higher FSH concentrations and consequent accelerated follicular development. Elevation of LH concentrations only follows later. The inhibins are dimeric peptides that selectively inhibit FSH secretion. Inhibin A appears to be a marker of follicle maturity and rises in the late follicular phase after the increase in oestradiol secretion. In contrast, inhibin B is derived mainly from small antral follicles and may indicate baseline follicular number. A reduced inter-cycle rise in circulating concentrations of inhibin B is associated with elevated early follicular phase FSH concentrations (van der Spuy and Alberts, 2002). These are the earliest measurable endocrine manifestations of ovarian ageing and they constitute the basis to use biomarkers other than age to predict ovarian reserve.

High concentrations of serum FSH or an elevated FSH to LH ratio (Scott and Hofmann, 1995; Sharara and Scott, 1997), elevated concentrations of serum oestradiol (Licciardi *et al.*, 1995), and decreased concentrations of immunoreactive α -inhibin (Balasch *et al.*, 1996) or inhibin B (Seifer *et al.*, 1997; Creus *et al.*, 2000) have been reported to be associated with low response to ovarian stimulation for assisted reproduction. However, currently there is no accurate and 100% predictive test to assess ovarian response, and thus the clinician's dilemma is to decide which investigation would be most cost-effective and informative (Tartlatzis *et al.*, 2003).

It has been stressed that a parameter that is easily measurable, minimally invasive, inexpensive, and has acceptable predictive value is needed for counselling in assisted reproduction (Scott and Hofmann, 1995). Determination of basal FSH concentrations on cycle day 3 has been described by some authors as meeting these criteria (Scott and Hofmann, 1995; Barnhart and Osheroff, 1998; Fasouliotis *et al.*, 2000). Cycle day 3 serum concentration is an indirect estimate of ovarian reserve, being a measure of the amount of inhibin B/oestradiol produced by a cohort of follicles, and the feedback effects at the level of the pituitary (Bukman and Heineman, 2001). According to previous studies, basal FSH concentration was a better predictor of cancellation rate in IVF than basal oestradiol or inhibin B (Creus *et al.*, 2000).

On the other hand, for a screening ovarian reserve test to be useful in clinical practice, it would be necessary to define prognostic criteria before standard treatment is started. This would allow clinical strategies to be planned depending on basal FSH concentrations from a pretreatment assisted reproduction cycle. In this regard, the present results are reassuring when showing that no significant inter-cycle variability for FSH serum concentration (or FSH:LH ratio) on cycle day 3 was detected during three consecutive cycles. This is in contrast with previous studies investigating women having reduced ovarian reserve (either because of their age or their previous poor response to ovulation induction) where wide variations in basal serum FSH concentrations were reported (Lass *et al.*, 2000).

The choice of testing FSH on day 3 of the menstrual cycle may have been serendipitous, as most ovarian stimulation protocols traditionally began on days 3–5 of the menstrual cycle. On the other hand, the rationale behind testing an FSH concentration at cycle day 3, may lie in the belief that there is a 'basal state' of FSH before suppression of the pituitary from increasing ovarian feedback later in the follicular phase. Because FSH concentrations are thought to be at or near a maximum at this time, a day 3 FSH may provide a reproducible glimpse into how well the ovarian–hypothalamic–pituitary axis is functioning (Barnhart and Osheroff, 1998). In this regard, the present study showed that FSH serum concentrations (or FSH:LH ratio) are equivalent on cycle days 2–5, thus adding greater flexibility for both patients and physicians in evaluation of the infertile patient.

As discussed above, the question of the inter-cycle and intra-cycle variability of FSH has been raised before in two previous studies using healthy eumenorrhoeic women (Brown *et al.*, 1995; Hansen *et al.*, 1996). In contrast, this study included only patients awaiting assisted reproduction treatment. This is to be noted considering that the predictive value of basal FSH concentrations seems to decrease for patients who are not being treated in assisted reproduction programmes (Broekmans *et al.*, 1998; van Montfrans *et al.*, 2000). On the other hand, the intercycle variability in basal FSH concentrations was evaluated in 81 women undergoing multiple cycles of IVF. It was found that the inter-cycle variability in basal FSH concentrations generally did not affect the patient's prognostic category and therefore should have minimal impact on clinical decision making (Scott *et al.*, 1990; Martin *et al.*, 1996).

In conclusion, basal FSH serum concentrations (or FSH:LH ratio) during the early follicular phase show neither significant inter-cycle nor intra-cycle variability when measured during 3 consecutive months in an assisted reproduction patient population, thus offering greater flexibility of pretreatment sampling. In contrast, oestradiol varies significantly from one cycle to another, and intra-cycle variability was also significant. Similarly, inhibin B varied significantly between cycle days 2 and 5. However, the potential clinical significance of oestradiol and inhibin B variability remains to be shown.

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