The endocrine and follicular growth dynamics throughout the menstrual cycle in women with consistently or variably elevated early follicular phase FSH compared with controls

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BACKGROUND: Elevated early follicular phase (EFP) FSH is frequently observed in subfertile patients. In these women, temporary normalization of FSH concentrations is known to occur. We studied the complete endocrine cycle profile of subfertile young women with elevated basal FSH compared with controls.

METHODS: Daily blood-sampling and ultrasound monitoring in the follicular phase was performed in 22 patients with elevated basal FSH levels (identified in screening) and in 16 controls during one menstrual cycle and for 5 days of the next cycle.

RESULTS: Eleven patients showed elevated basal FSH levels in the study cycle (‘High, High’; H,H group) whereas 11 had normalized basal FSH levels (‘High, Low’; H,L group). Anti-Müllerian hormone (AMH) was lower in both groups. In the H,H group, FSH was higher in all phases of the cycle and both inhibin A and B were lower during the EFP. In the H,L group, FSH was also higher than in controls in the EFP and the late luteal phase and inhibin A was higher in the periovulatory phase. ‘Normalization’ of Day 3 FSH in women with previously elevated FSH was associated with normalization of inhibin B levels in the preceding luteal phase.

CONCLUSIONS: The endocrine cycle profile in younger subfertile patients with consistently elevated basal FSH resembles that in published data from older women and also reflects a low ovarian reserve. Normalization of FSH in association with normal inhibin B suggests a temporary increase of the available cohort.

Keywords: ovarian aging; AMH; elevated FSH; intercycle variation FSH; inhibins and ageing

Introduction

Elevated early follicular phase (EFP) serum levels of FSH have been used in recent years to counsel patients in infertility treatment, because this is associated with poor response in ovarian stimulation and subsequent low pregnancy rates (Scott and Hofmann, 1995). This monotropic rise of FSH levels in the EFP has been described by a number of authors (Sherman et al., 1976; Reyes et al., 1977; Lee et al., 1988) in ageing ovulatory women. More recent investigations have shown that serum inhibin B levels are decreased in the EFP in older women (Klein et al., 1996, 2004; Danforth et al., 1998; Welt et al., 1999; Muttukrishna et al., 2000) and also in younger women with elevated FSH levels (De Koning et al., 2000). It is assumed that decreased negative feedback from the diminishing number of pre-antral and early antral follicles plays a major role with regard to this FSH rise. In a previous study (De Koning et al., 2000), higher FSH and LH pulse amplitude and response to GnRH in a group of women with elevated Day 3 FSH levels were found in combination with lower inhibin A and inhibin B levels. Apart from increased EFP FSH levels, anti-Müllerian hormone (AMH) has been suggested to be an early marker of female reproductive ageing (De Vet et al., 2002; van Rooij et al., 2002; Fanchin et al., 2003). AMH is produced by primary, secondary and early antral follicles and its concentrations do not vary throughout the menstrual cycle (Cook et al., 2000; La Marca et al., 2004; Hehenkamp et al., 2006). One question that we intended to answer is whether relatively younger women with either constant or intermittently elevated Day 3 FSH levels have diminished ovarian reserve which can be detected by persistently low AMH levels.

Often the elevated EFP FSH is not detected in every cycle of subfertile women. Many patients occasionally show normal levels (Brown et al., 1995; Jain et al., 2003). So far, no detailed knowledge is available about the cycle pattern of reproductive hormones in patients who show a temporary normalization of basal FSH. This makes understanding of this phenomenon
and its possible consequences difficult. Some authors have suggested that IVF treatment in such an occasional cycle may lead to better results than in cycles with increased FSH levels (Lass et al., 2000), although others indicate no differences in response and pregnancy outcome (Scott et al., 1990; Abdalla and Thum, 2006).

All reports detailing the menstrual cycle pattern of the reproductive hormones in relation to the issue of limited ovarian reserve commonly compare older versus younger patients rather than making a comparison between women with or without elevated FSH irrespective of age. Therefore, the aim of the present study was to evaluate in detail the endocrine hormonal profile and follicle development of relatively younger women with elevated Day 3 FSH compared with women around the same age showing normal FSH as it is not evident that the endocrine profile of these patients resembles that of published data from older women. We explicitly distinguished between patients with persistently elevated FSH and those showing a temporary normal value. The study design, which incorporated two menstrual periods, allowed us to closely evaluate hormonal determinants of the luteo-follicular transition that associate with EFP gonadotrophin levels.

Materials and Methods

Subjects

Patients, referred to our infertility clinic, were all screened on Day 3 of the menstrual cycle. All patients with Day 3 FSH values of ≥10 IU/l were asked to participate in the study. In our IVF clinic, patients with basal FSH values ≥10 IU/l show very poor outcome with respect to the number of oocytes retrieved and pregnancy rate. Controls were either patients referred to our clinic for reversal of tubal ligation or volunteers recruited by advertisement, with cycle Day 3 values of <10 IU/l ['Low, Low' (L.L) group]. All women had regular menstrual cycles (21–35 days), normal endocrine screen, no current medical illness, no current use of oral contraceptives and no pregnancy or breastfeeding in the past 6 months. The study patients were divided into two groups: 11 of the 22 patients had elevated FSH concentrations again on Day 3 of the study cycle, this was the ‘High, High’ (H.H) group, and 11 patients had FSH concentrations <10 IU/l on Day 3 of the study cycle ['High, Low’ (H.L) group]. All women were ovari- latory as determined by serial ultrasound monitoring and elevated serum progesterone levels in the luteal phase above 15 nmol/l in the study cycle and cycles were 21–35 days in duration.

Study design

Daily bloodsamples were obtained, starting from Day 1 of menstruation until Day 5 after the beginning of the next menstruation. Serum was stored at −20°C until processing. Transvaginal ultrasound was performed on Day 3, Day 8 and every other day until a follicle of 14 mm diameter was seen. Daily ultrasound was performed from then on. Follicular collapse or an increase in echogenicity of the large follicle was considered to represent ovulation. In the luteal phase, daily blood sampling was continued. FSH, LH, estradiol, inhibin B, inhibin A and progesterone were measured in all samples. AMH was measured on Day 3 of two consecutive cycles.

The study was performed in accordance with current guidelines on good practice in clinical research and the Declaration of Helsinki. The study was approved by the Institutional Review Board, and written informed consent was obtained from all participants.

Hormone measurements

LH and FSH were measured in duplicate by commercially available immunometric assays (Amerlite; Amersham, Bucks, UK). The lower limit of detection was 0.3 IU/l for LH and 0.5 IU/l for FSH. The assays were calibrated against the first International Reference Preparation (IRP) 68/40 and the second IRP 78/549 for LH and FSH, respectively. Of each individual, all samples were analysed in the same run for each hormone. The inter- and intra-assay coefficients of variation (CV) were <9% and 5% for LH and FSH.

Inhibin A and inhibin B were measured in duplicate by ultra-sensitive two-site enzyme immunoassays (SeroNet, Oxford, UK). The lower limit of detection was 3 pg/ml for inhibin A and 15 pg/ml for inhibin B. Estradiol was measured by radioimmunoassay (Sorin Biomedical, Saluggia, Italy) with a lower limit of detection of 18 pmol/l and an inter-assay CV of <11%. For progesterone, a commercially available competitive immunoassay (Delfia, Wallac Turku, Finland) was used with an intra-assay CV of 4–8% and inter-assay CV of 7–13%.

An ultra-sensitive immuno-enzymometric assay kit (Diagnostic Systems Laboratories, Webster, TX, USA) was used for estimation of AMH (Al-Qahtani et al., 2005). The limit of detection (defined as blank +3SD of blank) was 0.08 μg/l. Intra- and inter-assay CV were <5%.

Transvaginal ultrasound

All transvaginal ultrasound measurements were performed by the same observer (C.H.K.) using a 7.5 MHz transvaginal probe (Aloka SSD-1700). Only follicles >10 mm were measured by taking the mean of three perpendicular measurements. The two layers of the endometrium together were measured as endometrial thickness.

Statistical analysis

Between-group baseline data were compared using weighted least squares regression models and chi-squared tests. Weighted least squares analyses were used since the variance within groups varied significantly. To compare hormone levels between the groups, the day of LH peak was defined as Day 0. The menstrual cycle was divided into seven phases: early follicular (15–11 days before LH peak), mid-fo- llicular (10–6 days before LH peak), late follicular phase (5–1 days before LH peak), the LH surge phase, and early luteal (1–5 days after LH peak), mid-luteal (days 6–10) and late luteal (days 11–15) phases. Days outside this range were ignored due to paucity of observations. For each phase, the average hormone level per patient was calculated using that patient’s daily values. Groups were compared using weighted least squares regression since within-group variance differed significantly. Hormone levels on the day of the LH surge were compared using ANOVA. Follicle growth and maximal follicle diameter before ovulation was calculated for each subject. These data were examined using ANOVA and chi-squared tests, respectively. The statistical analyses were performed using Stata Statistical Software 9 (release 9; Stata Corporation, College Station, TX, USA).

For multiple comparison correction, we performed Bonferoni’s analysis. Two-sided P < 0.05 was considered to indicate statistical significance.

Results

Basal characteristics of the subjects are shown in Table I. Table II shows mean hormone levels on cycle Day 3. No differences were found in age, body mass index (BMI), smoking and history of smoking in packyears, duration of infertility and age
of menarche between H,H and H,L groups. The age at which the mothers of the subjects experienced menopause was slightly lower in the H,H group compared with the other groups (P < 0.05).

**Cycle duration**

In the H,L group, the mean duration of the follicular phase was shorter (11.1, range 3–22, P = 0.047) compared with the H,H group (12.0, range 10–16) and the control group (15.0, range 10–22), although this difference was mainly due to one woman with a very short follicular phase. The duration of the follicular phase was determined by the peak LH value and did not include the day of the LH peak. The luteal phase did not significantly differ in length. Table III shows mean and standard deviations for all phases per group.

**Hormone levels**

** Follicle stimulating hormone**

Compared with the controls, FSH was higher in the H,H group in all phases of the cycle, and in the early follicular and early and late luteal phase (LLP) in the H,L group. In the EFP and the LLP, FSH was not significantly different in the H,H group in comparison with the H,L group. The mean peak FSH level during the LH surge was significantly higher (P < 0.001) in the H,H group, compared with the other groups.

** Luteinizing hormone**

LH in the H,H group was higher in the EFP compared with the controls. In the mid-follicular phase and in the early and mid-luteal phase, LH was higher in the H,H group compared with the H,L group (P < 0.05). In the late follicular and LLP, there were no differences between the three groups. LH peak values (LH surge) in the three groups were not significantly different.

** Estradiol**

The LH peak day showed lower estradiol levels in the H,H group compared with the H,L group and controls. In the follicular and luteal phases, we found no differences in E2 between the three groups.

** Inhibins**

In the EFP, inhibin B was significantly lower in the H,H group compared with the H,L group and controls. In the follicular and luteal phases, we found no differences in E2 between the three groups.
Inhibin B was lower in the H,H group compared with the control group in the mid-follicular phase ($P < 0.01$). At the time of the LH surge, H,H patients and H,L patients show a decreased inhibin surge ($P < 0.01$).

Inhibin A was higher in the H,L group at the periovulatory phase (late follicular phase, LH peak day and in the early luteal phase, $P < 0.05$). In the EFP of the H,H group, inhibin A was decreased ($P < 0.05$).
Progesterone

Progesterone was slightly higher in the early follicular and mid-follicular phases in the H,H group, compared with the controls ($P < 0.05$). Progesterone did not differ in any other phase of the cycle.

Anti-Müllerian hormone

Mean AMH in the control group was significantly higher compared with the H,H group and H,L group (Table II). The correlation between Day 3 AMH in the first and the second cycle was 0.84 in the H,H group, 0.82 in the H,L group and 0.87 in the control group.

Follicle growth and endometrial thickness

Growth velocity of follicles larger than 10 mm was 1.5 mm/day in the H,H group, 1.7 mm/day in the H,L group and 1.3 mm/day in the control group (n.s.). Mean maximal diameter of the follicle at ovulation was significantly lower in the H,H group (18.8 mm) compared with the H,L group (21.6 mm) ($P < 0.05$), but not lower than the control group (21.3 mm) ($P = 0.06$). Multifollicular growth was observed in all groups. In the H,H group 5/11 patients showed multifollicular growth (follicles >10 mm). Only one patient in this group had two dominant (>16 mm) follicles, one ovulating 1 day after the other. In the H,L group, 7/11 subjects showed multiple follicle growth. One patient in this group showed three dominant follicles of 19, 18 and 17 mm, respectively, of which two ovulated. In controls, 5/11 had multiple follicle growth. Occurrence of growth of multiple dominant follicles was not statistically different between the groups. Endometrial thickness was not significantly different between the groups.

Intercycle variability

In order to evaluate the mechanisms causing intercycle variability of FSH levels in the EFP, we also measured the hormone levels in the next cycle until Day 5 (second cycle). We subsequently redivided the groups according to the Day 3 FSH level of the second cycle and analysed the preceding luteal phase and EFP of the second cycle (Fig. 3). High FSH levels on Day 3 were preceded by low levels of inhibin B in the preceding mid-luteal phase and LLP ($P < 0.05$). We found no significant differences in inhibin A in the preceding luteal phase when analysed in this way.

Discussion

This study has demonstrated that relatively young women with raised Day 3 FSH concentrations, whether intermittent or persistent, have diminished ovarian reserve. However, in younger women whose FSH levels temporarily return to normal, inhibin B concentrations were found to be normal in the preceding luteal phase.

Younger women with repeatedly elevated EFP FSH levels show many of the characteristic hormonal patterns seen in older women (Lee et al., 1988; Klein et al., 1996, 2004; Burger et al., 1998; Danforth et al., 1998; Welt et al., 1999). Serum FSH is elevated throughout all phases of the cycle together with lower inhibin B levels during the follicular phase, the LH surge and again in the luteal phase. Therefore, also in these younger patients, the elevated FSH is most likely the result of diminished ovarian feedback from a smaller available cohort of follicles in relation to limited oocyte reserve. This is supported by our finding that serum concentrations of AMH, secreted by small and intermediate antral

Figure 3: Hormone values in the luteal and EFP of groups when defined according to second Day 3 value. All values are mean and SD. White bars, H,H: high, high group: elevated basal FSH in screening and in the second study cycle group, $n = 7$. Stripes bars=H,L: high, Low group: elevated basal FSH in screening and <10 IU/l in second study cycle group, $n = 15$; black bars, L,L: low, low group: control group, $n = 16$. a, different versus L,L; $P < 0.05$; b, different versus L,L; $P < 0.01$; c, different versus H,L; $P < 0.05$; d, different versus H,L; $P < 0.01$. ELP, early luteal phase; MLP, mid-luteal phase; LLP, late luteal phase; ELP2, early luteal phase study cycle 2.
follicles and considered to be a good marker for age-related ovarian reserve (Kevenaar et al., 2006; Visser et al., 2006), were much lower than in controls. We also confirm the finding that inhibin levels in the EFP are lower in these women, which would further contribute to the notion that specifically limitations of ovarian peptide hormone feedback are involved in the regulation of pituitary gonadotrophin secretion in relation to decreased ovarian reserve (Santoro et al., 1999; De Koning et al., 2000). Furthermore, the inhibin B (and E2) levels tended to be lower periovulatory and early in the luteal phase, which is also in line with earlier observations, probably reflecting a reduction in number and quality of the follicles growing during this part of the cycle (Seifer et al., 1996, 2002; Muttukrishna et al., 2000; Welt et al., 2005). Overall, it seems that inhibin B deficiency in women with elevated FSH is a reflection of loss of function of granulosa cells and/or less granulosa cell mass of follicles.

The inhibins selectively block pituitary GnRH-independent FSH secretion (Lambalk et al., 1989; Rivier and Vale, 1991). Therefore, the demonstrated lower inhibin levels are likely to be responsible for the monotropic rise of FSH. On the other hand, we have shown that pituitary FSH and also LH responsiveness to GnRH is markedly increased in these women (De Koning et al., 2000). This may explain our finding that LH concentrations were also higher in almost all phases of the cycle, which also confirms similar findings by others (Lee et al., 1988; Ahmed Ebbiary et al., 1994; Fitzgerald et al., 1994). We found slightly elevated progesterone levels in the EFP. A likely explanation for this would be that it is the result of the higher LH levels. Remarkably, both progesterone and LH levels normalized in women with a temporary normalization of EFP FSH levels.

Our study provides novel detailed information with regard to the reproductive endocrine profile in women with temporarily normalized basal FSH levels. In our study, all these patients showed low AMH levels comparable with levels found in women with persistently elevated FSH. This indicates that variably elevated basal FSH levels are also a reflection of limited ovarian reserve. Therefore, the fact that the FSH levels were still slightly higher compared with controls was not surprising. On the other hand, with regard to secretion of the other hormones, the daily measurements revealed remarkable differences. The concentrations of LH, estradiol and progesterone became comparable with controls. Literature often but not invariably reports EFP elevations of estradiol as a reflection of active ovarian feedback from protracted development of a dominant follicle (Lambalk et al., 1998; Klein et al., 2002; van Zonneveld et al., 2003). This explains the relatively normal FSH levels in patients with limited ovarian reserve and the somewhat shorter follicular phase (Evers et al., 1998). We found indeed a tendency for estradiol to be higher and for the follicular phase to be shorter in these patients compared with controls and women with permanently elevated basal FSH. Therefore, it could be that the temporally normalization of FSH is a reflection of advanced follicular development. According to our observations, the normalization of FSH was typically accompanied by normalization of the secretion of inhibin B, not only during the EFP but already during the mid-luteal phase and LLP of the preceding cycle. The main finding that inhibin B levels are normal in the luteal phase in women with temporarily normalized basal FSH (H,L) and lower in the women with repeatedly elevated EFP FSH levels (H,H), together with the fact that follicle growth rate and the rate of multifollicular growth between the three groups is similar, is in favour of the hypothesis that there is a qualitative defect in granulosa cells of the H,H women and not the H,L women. Inhibin B, the product of developing non-dominant antral follicles, can be considered as a good marker of the number of follicles that can be recruited by FSH, and as such it may be a reflection of the size of the cohort available in that particular cycle (Kwee et al., 2003; Lockwood, 2004). This implies that, according to our findings, the variability of follicular development not only with respect to timing but also in terms of quantity may be an important cause of the variability of EFP values.

A noteworthy finding in the patients with temporarily normalized basal FSH was the observation that inhibin A levels were increased during the late follicular, the periovulatory and the early luteal phase. Other publications reported higher inhibin A levels pre-ovulatory in older cycling women (Klein et al., 2004) and in the mid-follicular phase (Reame et al., 1998). This could be a result of increased quantity and/or a higher stimulation of the granulosa cells. We suggest that an increase in number of inhibin A producing cells, possibly because of more than one developing follicle, is a good explanation for the higher inhibin A levels. Indeed 7 out of the 11 H,L patients showed development of more than one growing follicle (>10 mm). So probably the temporarily larger cohort of FSH recruitable follicles, evidenced by the normal inhibin B levels in the beginning of the cycle, in combination with the slightly higher FSH levels, creates circumstances that favour selection and development of multiple dominant follicles. Relevant in this respect is that higher basal FSH levels were found in older women in whom multiple follicle development occurred (Beemsterboer et al., 2006).

The question is if this notion may be of some benefit for the clinical approach towards these patients in particular for IVF. On the basis of our finding that suggests presence of a temporary larger cohort when basal FSH normalizes, one would indeed expect a better ovarian response upon hormonal hyper-stimulation as suggested earlier by Lass et al. (2000). However, the only study published in this respect (Abdalla and Thum, 2006) compared IVF oocyte yield and pregnancy in 39 patients who were stimulated in one cycle with elevated FSH and another cycle with normal FSH, and found no differences. In this retrospective study, however, samples used for the estimation of the FSH were often not collected in the beginning of the same IVF stimulation cycle from which outcome was measured. Our study indicates that in women with low ovarian reserve, the intercycle variability of FSH is high, which means that if a basal FSH value is to be used to determine the acute size of the cohort, it should be a value in the EFP of the stimulation cycle. A prospective trial with uniform prevention of premature luteinization (short protocol GnRH agonist or a GnRH antagonist) and standard
gonadotrophin stimulation is needed to establish or rule out the possible usefulness of such a strategy.

In conclusion, this study shows that relatively younger women with either intermittently or constantly elevated Day 3 FSH levels have a diminished ovarian reserve shown by persistently low AMH levels. The endocrine profile in patients with consistently elevated basal FSH resembles that in published data from older women. However, patients who present with elevated EFP FSH but normal FSH in the subsequent cycle are characterized by normalization of inhibin B in the preceding luteal phase, suggesting a temporary increase of the available cohort.

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