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Serum anti-müllerian hormone levels and follicular cohort characteristics after pituitary suppression in the late luteal phase with oral contraceptive pills

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BACKGROUND: Anti-mullerian hormone (AMH) is a marker of ovarian function and reserve and reflects the number and size of antral follicles. The objective of this study was to evaluate the effect of FSH suppression on AMH levels, during the late luteal phase of human menstrual cycle, with the use of oral contraceptives pills (OCP). METHODS: Twenty normovulatory infertile women were included in the study. On the third day of a spontaneous menstrual cycle, the patients were submitted to a transvaginal ultrasound examination and blood sample collection. From the 20th day of this menstrual cycle, the patients took daily OCP, containing 0.030 mg of ethinyl-estradiol plus 0.15 mg of desogestrel. On the third day of the following cycle, the measurements were repeated. RESULTS: After OCP use, the levels of FSH and estradiol were significantly reduced ($P < 0.001$). The number of antral follicles measured on both occasions did not differ, although after OCP use, the follicles presented significantly lower diameters (mean 4.4 + 1.7 mm before OCP versus 3.5 + 1.2 mm after OCP $P < 0.001$). The levels of AMH were significantly reduced after pituitary suppression, with a median (inter-quartile range) of 3.02 ng/mL (1.21–6.39) before OCP and 2.22 ng/mL (0.9–3.11) after OCP, $P = 0.04$. CONCLUSIONS: The short administration of OCP in late luteal phase caused suppression of FSH secretion during the cycle transition, leading to a more homogeneous follicular cohort. The lower AMH levels observed, although simultaneous with FSH suppression, were probably not a direct effect of the reduced FSH levels, but were more likely a consequence of the lower production by the arrested follicular cohort.

Keywords: AMH; follicular cohort; FSH; OCP

Introduction

Anti-müllerian hormone (AMH) is a dimeric glycoprotein, from the transforming growth factor β family (TGFβ), and it is considered a local growth factor and a cellular differentiation factor (Durlinger *et al*., 2002a; Josso and di Clemente, 2003). In females, AMH is produced by granulosa cells of ovarian follicles, from the end of intrauterine life until ovarian failure and menopause (Durlinger *et al*., 2002a; Josso and di Clemente, 2003; Visser *et al*., 2006). From studies using rodents, it is known that AMH is expressed mainly by granulosa cells of small antral and of pre-antral follicles, and this expression is higher by those granulosa cells that surround the oocyte and the antral cavity (Baarends *et al*., 1995). Follicles that are in more advanced stages of development, as well as follicles in atresia and the primordial follicles, do not express AMH (Baarends *et al*., 1995; Durlinger *et al*., 2002b). This specific pattern of cellular expression suggests that AMH exerts its main actions during the initial phases of follicular growth and differentiation, inhibiting follicular recruitment during the monthly ovarian cycle (Durlinger *et al*., 2001, 2002b).

The physiological interaction between AMH and FSH is not yet well established. In spontaneous cycles, serum levels of AMH and FSH appear to show an inverse correlation, but since both hormone levels are related to the number of antral follicles measured (van Rooij *et al*., 2002; Fanchin *et al*., 2003a, 2003b, 2005; Pigny *et al*., 2003; Visser *et al*., 2006; Mendez-Lozano *et al*., 2006), this relation is speculated to be indirect. During the spontaneous menstrual cycle, AMH levels have been shown to remain constant due to its secretion by the pre-antral and initial antral follicle cohort, whose number and proliferation are constant despite the cycle phase (Hehenkamp *et al*., 2006; La Marca *et al*., 2006; Mendez-Lozano *et al*., 2006).

Although ovarian AMH secretion appears to be independent of FSH influence, an *in vitro* study has shown that AMH inhibits preantral follicular growth induced by FSH, mainly through diminished granulosa cell proliferation, and also through lower aromatase activity (Durlinger *et al*., 2001, 2002b). Nevertheless, another study has shown contrary results (McGee *et al*., 2001). Such controversy about FSH

and AMH interactions has motivated the present study, whose main objective was to evaluate the effects of FSH inter-cycle suppression on AMH secretion and on follicular cohort, in normoovulatory women. It was hypothesized that manipulation of FSH secretion with oral contraceptive pills (OCP) administration during the luteal–follicular phase transition would cause antral follicle growth arrest; we therefore wished to investigate the effect of this arrest on AMH secretion by the cohort of antral follicles.

Materials and Methods

Design

A prospective clinical trial was performed.

Subjects

Twenty normoovulatory infertile women were selected, from the outpatient clinic of the Obstetrics and Gynecology Department of Hospital de Clínicas de Porto Alegre, and were being seen due to tubal occlusion or male infertility. The patients fulfilled the criteria below: regular 28- to 34-day menses; maximum age of 35 years; both ovaries present; absence of any endocrinological or ovulation disorder; body mass index between 18 and 25 kg/m^2; absence of any hormonal therapy in the past 3 months; agreement to participating in the study, by signing the post-informed consent term. The research protocol was approved by the hospital Ethical Committee (IRB equivalent).

Study protocol

Patients were submitted to an ultrasound examination by one operator (EA) on Day 3 of a spontaneous cycle and also to a blood sample collection through venous puncture for later hormonal assay. Following the 20^{th} day of the same menstrual cycle, they took a daily pill, containing 30 μg of ethinyl estradiol plus 150 μg of desogestrel. On the third day of the next menstrual bleeding, or after 14 days if menstrual bleeding had not occurred, the same ultrasound scan and hormonal dosages were performed. The objective of the ultrasound scan was to measure the ovarian dimensions for further ovarian volume calculation using the ellipse formula [$(\pi/6) \times$ length $\times$ height $\times$ width] and for evaluation of the number and the mean diameter of follicles i.e. mean of two orthogonal diameters. All follicles between 2 and 10 mm of mean diameter were included in the analysis. Ultrasounds scans were performed using an 11 Hz transvaginal probe, equipped with a tissue harmonic imaging system which allowed improved image resolution and better recognition of follicular borders (Aloka SSD 1700, Dyna View, Aloka Co., Ltd.), as already described (Fanchin *et al*., 2003c, 2003d).

Hormonal analysis

Blood samples were processed in a centrifuge and serum samples were frozen for posterior analysis, at $-80^{\circ}C$. Serum AMH levels were determined through an enzyme-linked immunosorbent sensitive assay (ELISA) (DSL-10-14400, Diagnostic Systems Laboratories, Inc., Webster, USA). FSH and estradiol levels were determined through automated systems by chemiluminescence detection (IMMULITE 2000—Euro/DPC, Llanberis, England and e Elecsys 1010—Roche, Mannheim, Germany, respectively). The limits for detection were 0.1 mUI/mL for FSH, 5.0 pg/mL for estradiol and 0.017 ng/mL for AMH. The intra-assay coefficients of variance were 10% for FSH, 5.7% for estradiol and 4.6% for AMH.

Statistics

Statistics were performed using the SPSS software, version 12.0 (SPSS Inc., 2003, Chicago, IL, USA). The measure of central tendency for normally distributed variables was mean $\pm$ SD and for non-parametric variables median and inter-quartile range was used. Because of the pair-wise design of this study, data from each participant on spontaneous cycle Day 3 were compared with data from the same participant on cycle day 3 after OCP intake by using paired statistics. Normally distributed data were analysed by the Student's *t*-test for repeated measures, and the non-parametric data were analysed by the Wilcoxon's signed-rank test, for related samples. The homogeneity between follicular cohorts' measures was analysed by Levene's test. Correlation test among the variables was also performed. The sample was calculated to detect a difference of at least 1 ng/mL on AMH serum levels at >80% power at the two-sided 0.05 significance level. The differences were considered statistically significant when $P < 0.05$.

Results

The mean age of the patients included in this study was 29.1 $\pm$ 4.11 years (range 23–34). The mean BMI was 22.6 $\pm$ 2.9 kg/m^2. In 55% of the women infertility was due to masculine causes and in 45% infertility was due to tubal obstruction.

After late luteal phase OCP administration, the pituitary secretion of FSH was significantly reduced, as was the ovary secretion of estradiol. The medians (inter-quartile ranges) of FSH serum levels were 5.47 (4.81–9.24) mU/mL before and 1.99 (0.97–2.33) mU/mL after OCP administration ($P < 0.001$); and the medians of estradiol serum levels were 36.17 (26.81–47.6) before and 8.98 (6.04–16.1) pg/mL ($P < 0.001$) and after OCP use.

Serum AMH levels were also significantly reduced after OCP intake, with medians (inter-quartile range) of 3.02 ng/mL (1.21–6.39) before and 2.22 ng/mL (0.9–3.11) after ($P = 0.04$) (Table I). Moreover, the ratio of AMH to the number of follicles measured ratio (per-follicle AMH ratio) was significantly lower at the second measurement with medians and inter-quartile ranges of 0.17 (0.08–0.47) before and 0.11 (0.06–0.15) after OCP use, $P = 0.04$ (Fig. 1). The power of our data to detect such a difference between AMH levels was higher than 80%. Although AMH levels showed a non-parametric disposition, with heterogeneous values, almost all the patients had lower AMH serum levels after OCP intake (Fig. 2), and the same occurred for serum FSH and estradiol levels.

The number of antral follicles measured in both moments did not differ (15.8 $\pm$ 4.7 before and 16.6 $\pm$ 3.6 after OCP, $P = 0.44$), although after inter-cycle FSH inhibition the follicles presented significantly lower diameters (4.4 $\pm$ 1.7 mm before and 3.5 $\pm$ 1.2 mm after OCP, $P < 0.001$), with lower variance (Levene's test for equality of variances $F = 32.212$, $P < 0.001$). In the spontaneous cycle, the number of follicles per patient with mean diameter between 2 and 4 mm was lower than it was after inter-cycle FSH inhibition (see Table I); and the opposite occurred for the number of follicles with mean diameter greater than 4 mm (data not shown). Ovarian volume also diminished after use of OCPs (mean volume of 5.78 $\pm$ 2.86 cc before and 4.37 $\pm$ 1.85 cc after OCP use

Table I. Hormonal and follicular measures in a spontaneous menstrual cycle and after intercycle FSH inhibition with the use of OCP in the late luteal phase.

	Third day of a spontaneous cycle	Third day of a cycle after OCP use in luteal phase	*P*	Δ%
FSH (um/mL)	5.47 (4.81–9.24)	1.99 (0.97–2.33)	0.001	−68.29
Estradiol (pg/mL)	36.17 (26.81–47.6)	8.98 (6.04–16.1)	0.001	−69.09
AMH (ng/mL)	3.02 (1.21–6.4)	2.22 (0.9–3.11)	0.04	−15.44
Number of antral follicles	15.8 ± 4.7	16.6 ± 3.6	0.44	11.60
Number of antral follicles with 2–4 mm	7.9 ± 4.3	12.8 ± 4.7	0.001	63.62
Mean follicular diameter (mm)	4.4 ± 1.7	3.5 ± 1.2	<0.001	−20.44

Data are displayed as median (inter-quartile range) or mean ± SD. Δ% represents the mean percentual variation after late luteal phase OCP administration.

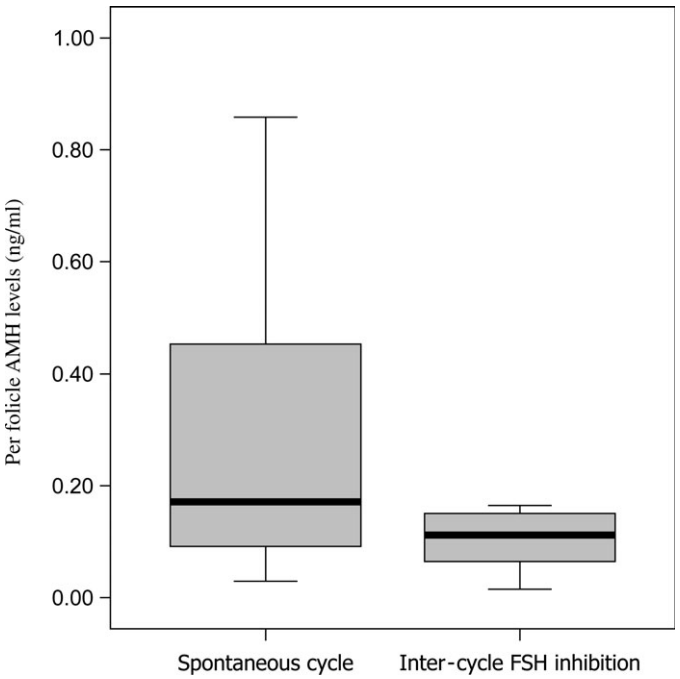

Figure 1: Per follicle levels of AMH (AMH/number of follicles) at the third day of a spontaneous menstrual cycle and after inter-cycle FSH inhibition with the use of luteal OCP. Central horizontal lines indicate the median; additional horizontal lines represent the 10th, 25th, 75th and the 90th centiles

$P = 0.002$), reflecting the smaller follicular diameters. The percentage variations (Δ%) of all these variables after OCP use, compared to the spontaneous cycle, are also shown in Table I.

Correlation tests were performed to evaluate the possible linear association of AMH serum levels with the other variables analysed. For the spontaneous menstrual cycle, the Sperman's rho coefficients were, respectively, −0.193, −0.293, −0.146 ($P > 0.05$) for AMH serum levels versus FSH serum levels, estradiol serum levels and number of follicle measured per patient. After FSH suppression, these same values were, respectively, 0.404, 0.235, 0.360 ($P > 0.05$). Although all these variables showed decreased levels after OCP administration, no correlation was found between the decrease in AMH levels and other variables. Although taking the OCPs, 50% of the patients had spontaneous uterine bleeding. When analysed separately, no significant differences were found in any of the variables between those who had spontaneous bleeding and those who had not (data not shown).

Discussion

We have performed inter-cycle FSH suppression with short administration of an OCP in the late luteal phase of menstrual cycle, as observed by the drop in FSH and estradiol levels. As a consequence, a more homogeneous follicular cohort has surged, with lower mean diameters and also with smaller variance among the follicles diameters. Surprisingly, serum AMH levels have also diminished after FSH inter-cycle inhibition. As far as we have noticed, this was the first *in vivo* study to focus on FSH influence over AMH secretion, through manipulation of endogenous FSH secretion by OCP administration.

AMH is known as an important paracrine growth factor, secreted by granulosa cells of ovarian follicles. Some studies relate its serum levels to ovarian status and follicular reserve (Fanchin *et al*., 2003a, 2003b, 2005; Laven *et al*., 2004). Our group has previously found that patients with minimal/mild endometriosis have lower AMH serum levels than do controls, and also have a more heterogeneous follicular cohort, probably due to impaired ovulatory function related to endometriosis (Cunha-Filho *et al*., 2006). Through immunohistochemistry studies with human ovarian tissue, it is known that AMH is highly expressed and secreted by preantral and small antral follicles (up to 4 mm of diameter) in which almost all granulosa cells express AMH (Weenen *et al*., 2004). Primordial follicles and antral follicles with a diameter greater than 8 mm do not express AMH, which means that AMH is a product which originates essentially from the small antral follicles (Weenen *et al*., 2004). Noticeably, those initial antral follicles (up to 2 mm of diameter), which also secrete significant amounts of AMH, are not measurable by ultrasound examination, and this explains the constancy of AMH serum levels during a spontaneous menstrual cycle, despite the dominant follicle growth (Hehenkamp *et al*., 2006; La Marca *et al*., 2006; Mendez-Lozano *et al*., 2006).

The most remarkable clinical application of the knowledge about AMH is that its serum levels are strong and positively related to the ovarian reserve and to the number of oocytes retrieved after ovarian stimulation (Fanchin *et al*., 2003a, 2005; Laven *et al*., 2004; Eldar-Geva *et al*., 2005; Fleming *et al*., 2006; Peñarrubia *et al*., 2005). Moreover, patients with low response to ovarian stimulation have lower basal levels of AMH (van Rooij *et al*., 2002; Peñarrubia *et al*., 2005; Fleming *et al*., 2006). Contradictorily, some authors have demonstrated that AMH levels decline during ovarian stimulation after exogenous FSH administration, explained by the atresia and regression of small follicles or by the increase of

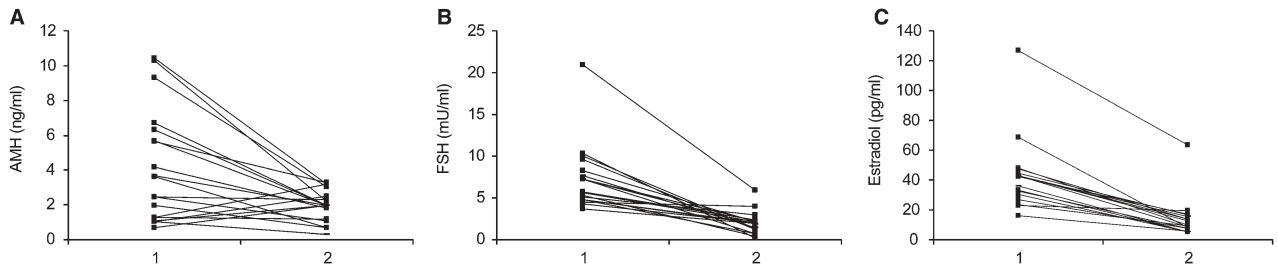

Figure 2: Individual AMH (A), FSH (B) and estradiol serum levels (C) before (1) and after (2) use of OCP in the late luteal phase to pituitary down-regulation

inhibin B and estradiol to supra-physiological levels (Fanchin *et al*., 2003b; La Marca *et al*., 2004). In ovarian stimulation cycles with exogenous FSH administration, AMH levels have strong positive correlation with the number of follicles which achieve sensitivity to FSH at each day of stimulation and which start to develop, suggesting that AMH is secreted by follicles that may respond positively to FSH stimulation (Fleming *et al*., 2006).

The physiological interaction between AMH and FSH has not been completely solved. Some studies suggest that AMH inhibits initial follicular growth (Carlsson *et al*., 2006) as well as the follicular growth induced by FSH (Durlinger *et al*., 2001, 2002a, 2002b; Visser *et al*., 2006), making the granulosa cells less sensitive to FSH stimulus. This is important during follicular recruitment, since follicles that express less AMH are those who show more granulosa cells mitosis (Durlinger *et al*., 2002a). Other studies, however, have shown that AMH could enhance follicular growth induced by FSH *in vitro*, especially growth of the primordial and the small antral follicles (McGee *et al*., 2001; Schmidt *et al*., 2005), which makes this subject a controversy in the current literature. Furthermore, in males, administration of FSH stimulates AMH secretion by the prepubertal testis (Young *et al*., 2005), probably due to direct activation of AMH gene transcription in Sertoli cells (Lukas-Croisier *et al*., 2003),

In the present study, the inverse phenomenon has been showed. When follicular growth was arrested, as demonstrated through FSH suppression and lower follicular diameters achieved after OCP administration, the follicular hormonal products were diminished. Furthermore, the measured number of follicles was not different after FSH suppression, but their diameter and AMH levels were diminished, probably because of the lower follicular activity and the suppressed granulosa cell proliferation. Whether this phenomenon is due to a direct effect of FSH on follicular AMH expression or due to indirect one, coordinated with the momentary follicular status, should be further studied. It has already been found, in a somewhat opposite situation, that women with polycystic ovarian syndrome have higher AMH serum levels than normoovulatory women, resulting from the great follicular number and activity observed (Pigny *et al*., 2003; Laven *et al*., 2004).

Although AMH serum levels have dropped after OCP use and FSH inhibition, we have failed to demonstrate any significant correlation between AMH and FSH levels, which suggests that the drop in AMH levels was not a direct response to FSH levels, but that both hormones are probably indirectly related. Indeed, the current study comprises only normoovulatory women, with normal ovarian reserve, with low variation in the number of selectable follicles, making this sample not adequate to perform an appropriate correlation test.

Another important point that calls for special attention was the presence of a progestagen in the OCP administered, which might have produced a direct effect on follicular AMH secretion. Analysing the follicular fluid of patients who underwent IVF (Fanchin *et al*., 2005b) demonstrated that the fluid collected from large follicles had lower AMH levels and higher progesterone levels compared to the fluid colleted from smaller follicles. They also have found a significant negative correlation between the levels of progesterone and AMH in the follicular fluid, suggesting that luteinization has a negative effect on the AMH production by granulosa cells (Fanchin *et al*., 2005b). OCP administration during the late luteal phase, just when the corpora lutea is dismissing, could maintain an hormonal environment similar to that found in the luteal phase, with high serum progesterone levels and low serum gonadotrophins levels.

In conclusion, the study supports the concept that the AMH secretion is related to the follicular activity. The effects of inter-cycle FSH inhibition after the short-term OCP luteal administration were evident, causing homogeneity of follicular diameters. The reduction of AMH levels, although occurred simultaneously with the FSH suppression, were probably not a direct effect of FSH stimulus, but more likely an indirect marker of follicular development arrest.

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