Endocrine profile in serum and follicular fluid differs after ovarian stimulation with HP-hMG or recombinant FSH in IVF patients

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BACKGROUND: Highly purified menotrophin (HP-hMG) has been associated with fewer oocytes retrieved and a higher proportion of top-quality embryos compared with recombinant FSH (rFSH). METHODS: A randomized, assessor-blind, multinational trial in 731 women undergoing IVF after stimulation with HP-hMG (MENOPUR) ($n = 363$) or rFSH (GONAL-F) ($n = 368$) following a long GnRH agonist protocol was conducted. Blood was collected before, during and after stimulation. Fluid was collected from follicles ≥17 mm. RESULTS: Serum androstenedione, total testosterone and free androgen index (FAI) were higher ($P < 0.001$) with HP-hMG than with rFSH after starting stimulation. At the end of stimulation, serum estradiol was higher ($P = 0.031$) with HP-hMG, whereas progesterone was higher ($P < 0.001$) with rFSH, even after adjusting for ovarian response. Serum LH was not different between treatments. Mean mid- and end-follicular hCG levels in the HP-hMG group were 2.5 and 2.9 IU/l, respectively. Follicular fluid levels of FSH, LH, hCG, androstenedione, testosterone, FAI and estradiol and ratios of estradiol : androstenedione, estradiol : total testosterone and estradiol : progesterone were higher ($P < 0.001$) with HP-hMG, whereas progesterone was higher ($P < 0.001$) with rFSH. CONCLUSION: Major differences in serum and follicular fluid endocrine profile exist after stimulation with HP-hMG or rFSH. Exogenous LH activity induces a differential endocrine environment influencing oocyte quantity and quality, which may be of relevance for clinical outcome.

Key words: endocrine/follicular fluid/IVF/highly purified menotrophin/recombinant FSH

Introduction

In the natural cycle, follicle growth is driven by a delicate interplay of FSH and LH that affects theca and granulosa cells, leading to the selection of a single dominant follicle through a series of feedback mechanisms (van Santbrink et al., 1995; Sullivan et al., 1999). FSH drives the development of the granulosa cell compartment and is essential for follicle survival and differentiation. Effects of FSH are amplified via several paracrine loops including the products of aromatization that depend on the provision of androgens by the theca cells (Hillier et al., 1994). One of the pivotal cellular actions of FSH is the induction of LH receptors on granulosa cells that will enable the granulosa cells to respond to both gonadotrophins (Zeleznik and Hillier, 1984). Through the LH receptor expression on the granulosa cells, the maturing follicle continues to develop irrespective of the physiological decline in FSH concentration that exists in the pre-ovulatory days (Zeleznik and Hillier, 1984). The concepts that threshold levels for FSH and LH are needed to achieve folliculogenesis and steroidogenesis and the effects by which inappropriately timed and/or abundant secretion of LH lead follicles into atresia (McNatty et al., 1975; Hillier, 1994) have now been clinically documented. Results from various studies have indicated that both the induction of aromatase and the presence/amount of LH and/or hCG are the principal components of the process of follicle selection (The European Recombinant Human LH Study

Group, 1998; Filicori *et al*., 1999, 2001, 2002; Sullivan *et al*., 1999; Loumaye *et al*., 2003; Platteau *et al*., 2006).

Within the field of controlled ovarian stimulation for IVF or ICSI, where high doses of gonadotrophins are administered, the role of LH activity is still heavily debated (Collins, 2003). Among the many reasons for the disagreement on the importance of LH activity are the variability of the endocrine background of the patient, the different definitions of 'LH depletion' related to the functional sensitivity and heterogeneity of the routine diagnostic tests for LH, the differential effects of various GnRH agonist protocols and the differences in biochemical composition of gonadotrophin preparations. Measuring the bioactivity of LH by using an immunoassay is inherently approximative (Niccoli *et al*., 1996). The value of a serum LH concentration depends on both the specificity and the sensitivity characteristics of the immunoassay used (Costagliola *et al*., 1994). It is also difficult to uniformly quantify the exposure to LH activity brought by the different gonadotrophin preparations, as there are different types of exogenous compounds with LH activity, i.e. LH or hCG. A recent study addressed the differences in composition in glycoproteins between the different types of menotrophins on the market (Wolfenson *et al*., 2005), which in a large part could be attributable to hCG molecules. The diverse kind of menotrophin preparations used in clinical evaluations combined with the plethora of immunoassays with different threshold figures used to quantify the degree of LH suppression leads to the large heterogeneity in published figures on the prevalence and degree of LH suppression in GnRH agonist-desensitized patients, ranging from 12 to 70% (Fleming *et al*., 2000; Westergaard *et al*., 2000; Esposito *et al*., 2001; Humaidan *et al*., 2002).

A primary distinction is that the clinical situation associated with exogenous LH/hCG supplementation during stimulation may represent a very different scenario than a pathophysiological condition in patients with elevated endogenous LH levels after pituitary down-regulation. Several earlier studies have found a negative impact of a high endogenous LH on oocyte or embryo quality and clinical outcome (Stanger and Yovich, 1985; Howles *et al*., 1986; Homburg *et al*., 1988; Watson *et al*., 1993), but the mechanisms behind this are still enigmatic. Also intriguing are the clinical reports addressing the effects of an exogenous contribution of LH/hCG on the follicular response and more particularly the quality of the oocyte cohort, its developmental competence and implantation potential (Filicori *et al*., 1999; Humaidan *et al*., 2004; Platteau *et al*., 2004; Humaidan, 2006). More insight into this important question can be acquired methodologically by using a source of exogenous LH activity supplementation (i.e. hCG) different from the source of endogenous LH activity (i.e. LH).

As part of a prospective study comparing highly purified menotrophin (HP-hMG) and recombinant FSH (rFSH) with ongoing pregnancy rate as a primary end-point (Nyboe Andersen *et al*., 2006), the evaluation of the endocrine profile in serum and follicular fluid was performed to support the concept that LH activity from the start of stimulation would induce a higher serum androgen load, impacting follicle selection, and would change the ratio of intrafollicular steroid balance (estradiol : progesterone, estradiol : androstenedione and estradiol : total testosterone), impacting embryo quality. It is suggested that the different ratio of estrogens to androgens attributed to the presence/absence of LH activity could be responsible for the difference between treatments in follicular dynamics during the recruitment and selection phase. Exposure to LH activity is expected to increase androgen load, and because androgens have a stimulatory role in intracellular regulation of granulosa cell function via effects on FSH receptor expression, on insulin-like growth factor-1 (IGF-1) and on the aromatase gene (Luo and Wiltbank, 2006), a differential status on markers of granulosa differentiation would be expected in women exposed to rFSH or HP-hMG. Exploration of intrafollicular levels and ratios of major steroids, and levels of IGF-1 and insulin and of markers of granulosa differentiation would provide an additional insight into the impact of exogenous LH activity. Furthermore, it would be interesting to study the concept that exogenous LH activity supplementation during stimulation does not lead to early luteinization, as may be detected by investigating progesterone, vascular endothelial growth factor (VEGF), inhibin A and inhibin A : inhibin B.

This prospective study included only patients eligible for IVF and not patients requiring ICSI. This was based on methodological considerations (Arce *et al*., 2005) and a previous report suggesting a differential treatment outcome between the gonadotrophin preparations tested in the present study when used in IVF cycles (Platteau *et al*., 2004). The clinical outcome of this study and the pharmacodynamic differences in follicular development, embryo quality and endometrial echogenic profile have been the object of a separate publication (Nyboe Andersen *et al*., 2006).

Materials and methods

Study population

Women with major indications for IVF such as tubal infertility or unexplained infertility including endometriosis stage I/II and partners with mild semen abnormalities not requiring ICSI were recruited to the study. Patients were 21–37 years of age with regular menstrual cycles of 21–35 days, presumed to be ovulatory. They had been infertile for at least 1 year, except for those with proven bilateral tubal infertility. They had a uterus consistent with expected normal function, presence of both ovaries and without evidence of abnormality and normal adnexa. The early follicular phase serum levels of FSH were within normal limits (1–12 IU/l). The body mass index before inclusion in the study was in the range 18–29 kg/m². Patients with polycystic ovary syndrome, endometriosis stage III/IV or partners with severe male factors requiring ICSI were not included in the study. Likewise, poor responders (those with more than three previously consecutive unsuccessful IVF cycles or previous cycles with >20 days of gonadotrophin stimulation, or cancellation due to limited follicular response, or <4 follicles of ≥15 mm) and patients with a previous IVF cycle with unsuccessful fertilization were excluded from participation. A detailed description of the study population and all inclusion and exclusion criteria are provided elsewhere along with the clinical outcome of the study (Nyboe Andersen *et al*., 2006).

Study design

This was a randomized, open-label, assessor-blind, parallel-group, multicentre, multinational study comparing HP-hMG (MENOPUR; Ferring Pharmaceuticals A/S, Copenhagen, Denmark) and rFSH (follitropin alfa, GONAL-F; Serono, Geneva, Switzerland). A total of 37 fertility clinics in 10 countries randomized patients to the study. The randomization of patients to treatment was stratified by age (<35 years

and 35–37 years) in each centre. All investigators, embryologists, laboratory personnel and sponsor staff were blinded to treatment allocation throughout the study. Patients underwent controlled ovarian hyperstimulation following down-regulation with a GnRH agonist in a long protocol for women undergoing IVF. All patients in all centres and countries received identical type and dose of concomitant fertility treatments, i.e. GnRH agonist for down-regulation, hCG for triggering final maturation and progesterone for luteal support. Pituitary down-regulation using triptorelin acetate, 0.1 mg/day s.c. (DECAPEPTYL; Ferring Pharmaceuticals A/S), was initiated 5–7 days before the estimated start of next menses and continued until the end of gonadotrophin administration. Gonadotrophin administration was initiated when down-regulation was confirmed using transvaginal ultrasound showing no ovarian cysts, a shedded endometrium with a thickness of <5 mm or serum estradiol <50 pg/ml (0.184 nmol/l). The starting dose of HP-hMG or rFSH was 225 IU for the first 5 days, followed by individual adjustments according to the patient's follicular response. The dose could be changed by 75 IU per adjustment and not more frequently than every 4 days. Choriongonadotrophin alfa, 250 µg s.c. (OVITRELLE; Serono), was administered to induce final follicular maturation within 1 day of observing three or more follicles of ≥17 mm diameter. Oocyte retrieval took place 36 h (±2 h) after hCG administration. Insemination was done via regular IVF insemination (not ICSI) at 3 h (±1 h) after oocyte retrieval. Fertilization was assessed at 20 h (±1 h), and embryo quality was assessed at 28 (±1 h), 44 (±1 h) and 68 h (±1 h) after oocyte retrieval. A top-quality embryo was defined as four to five cells on day 2, seven or more cells on day 3, equally sized blastomeres and ≤20% fragmentation on day 3, and no multinucleation. The transfer of one or two embryos of minimum quality, defined as four or more cells with no cleavage arrest (i.e. cleavage must have occurred within the last 24 h) and ≤20% fragmentation, was done on day 3 after oocyte retrieval. Vaginal progesterone gel 90 mg/day 8% (CRINONE; Serono) for luteal support was given from the day of embryo transfer till the confirmation of clinical pregnancy (5–6 weeks after embryo transfer) or negative serum βhCG test (13–15 days after embryo transfer). Ongoing pregnancy was determined 10–11 weeks after embryo transfer. The study procedures are described in detail in a separate publication (Nyboe Andersen *et al.*, 2006).

Collection and handling of serum

Blood samples were obtained on day 1 of stimulation, on day 6 of stimulation, on the last stimulation day and at oocyte retrieval. The sample on day 1 was taken before the start of gonadotrophin administration, and the samples on day 6 and last stimulation day were collected at least 8 h after the previous gonadotrophin dose. Blood samples were centrifuged for 10 min at 1800 × *g*. Serum was stored individually at –18°C or colder at the clinic for a maximum of 2 weeks before transfer to –70°C and subsequent analysis at a central laboratory. All serum samples were analysed centrally for FSH, LH and hCG by Laboratorium für Klinische Forschung (LKF; Raisdorf, Germany) and for estradiol, progesterone, androstenedione, total testosterone and sex hormone-binding globulin (SHBG) by Capio Diagnostik (Copenhagen, Denmark).

Collection and handling of follicular fluid

Follicular fluid from at least one follicle of ≥17 mm from which an oocyte was retrieved was collected at oocyte retrieval. A manual was provided to all clinics with instructions on how to obtain and prepare the follicular fluid. Fluid was preferably collected from the first follicle aspirated and from follicles where flushing had not been applied. The fluid was centrifuged for 10 min at 1000 × *g*, after which it should be clear and not contaminated with blood. The follicular fluid was stored under the same conditions as serum. The samples were analysed for FSH, LH, hCG, estradiol, progesterone, androstenedione,

total testosterone, SHBG, insulin, inhibin A, inhibin B, VEGF and IGF-1 by the hormone laboratory at Academisch Ziekenhuis Vrije Universiteit Brussel (Brussels, Belgium) and for cortisone and cortisol by the Department of Chemical Pathology, Southampton General Hospital, University of London (Southampton, UK). Fluids that were found to be contaminated either by blood cells or by flushing medium were disregarded from the analysis. Final endocrine analysis of follicular fluid was done for 335 patients in the HP-hMG group and for 341 patients in the rFSH group.

Analytical methods

An overview of analytical methods, sensitivity and total imprecision for serum and follicular fluid hormonal assessments is given in Table I. Validated laboratory immunoassay methods were selected for their sensitivity and reproducible proficiency profile to increase the precision of the measurements in serum and in follicular fluid.

Statistical analysis

For serum and follicular fluid hormone concentrations, the treatment difference between HP-hMG and rFSH with 95% confidence interval and *P*-value was estimated for each parameter based on a linear model of the log-transformed data including treatment and age strata. The treatment differences estimated in this model represent the overall treatment influences, i.e. both the direct treatment effects and the effects mediated via other hormones. To evaluate the direct treatment effects on each hormone separately, we also carried out a multi-adjusted analysis. This analysis included the endocrine profile at the specific time point, the primary reason for infertility and duration of infertility as co-variates in addition to the age strata. Overall, the findings in the multi-adjusted analysis were consistent with the analysis adjusted exclusively for age strata with only minor differences. The data presentation in the Results section is focused on the age strata-adjusted analysis, but differences observed in the multi-adjusted analysis are noted in the tables. For serum estradiol and progesterone on the last stimulation day and at oocyte retrieval, the age strata-adjusted comparison between treatment groups was also adjusted for the number of follicles and oocytes retrieved, respectively.

The relationship between estradiol and testosterone and estradiol and androstenedione on day 6 and at the end of stimulation was investigated within each group using a proportionality model. Log-transformed data were analysed in a regression analysis model, and the estimates were then transformed to the original scale. The influence of the FSH concentration was explored. Data were analysed separately for each treatment group. The influence of hCG concentration on day 6 among HP-hMG-treated patients in predicting embryo quality as assessed by the local embryologists and clinical outcome was evaluated by grouping in quartiles (i.e. 25% of the patients in each group). Test for trend in hCG was conducted by assigning an ordinal score (the median) to the grouped value and then treating this score as continuous in analysis. Data were analysed using both an age strata-adjusted and a multi-adjusted (age strata, primary reason for infertility, duration of infertility and day 1 serum levels of all endocrine parameters as co-variates) approach.

The primary end-point of this clinical study was ongoing pregnancy rate (reported in Nyboe Andersen *et al.*, 2006). No adjustment for multiplicity was performed, as there was only one primary end-point and all other end-points were considered secondary.

Results

A total of 731 patients were at the end of down-regulation randomized to either HP-hMG (*n* = 363) or rFSH (*n* = 368). Demographics, baseline characteristics and the mean levels for all endocrine parameters before the start of gonadotrophin

Table I. Analytical methods for the parameters measured in serum and follicular fluid

Parameter	Method	Sensitivity	Total imprecision (%CV)
Serum			
FSH	Electrochemiluminescence immunoassay	<0.1 IU/l	<6
LH	Electrochemiluminescence immunoassay	0.1 IU/l	<6
hCG	Electrochemiluminescence immunoassay	<0.1 IU/l	<8
Estradiol	Chemiluminescent immunometric assay	55 pmol/l	10
Progesterone	Chemiluminescent immunometric assay	0.6 nmol/l	8
Androstenedione	Radioimmunoassay	0.08 nmol/l	10
Total testosterone	Radioimmunoassay	0.17 nmol/l	5
SHBG	Chemiluminescent immunometric assay	0.02 nmol/l	10
Follicular fluid			
FSH	Electrochemiluminescence	<0.10 IU/l	<5.0
LH	Electrochemiluminescence	0.10 IU/l	<5.0
hCG	Electrochemiluminescence	<0.10 IU/l	<8
Estradiol	Electrochemiluminescence	18.35 pmol/l	<5
Progesterone	Electrochemiluminescence	0.095 nmol/l	<5
Androstenedione	Radioimmunoassay	0.13 nmol/l	<12
Total testosterone	Radioimmunoassay	0.1 nmol/l	<8
SHBG	Electrochemiluminescence	0.35 nmol/l	<5.0
Insulin	IRMA	0.2 mIU/l	7
Inhibin A	ELISA	1.0 ng/l	9
Inhibin B	ELISA	<15 ng/l	12
VEGF	ELISA	<9.0 ng/l	7
IGF-1	ELISA	0.105 nmol/l	10
Cortisol	Radioimmunoassay	20 nmol/l	<8
Cortisone	Radioimmunoassay	4 nmol/l	<10

CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; IGF-1, insulin-like growth factor-1; IRMA, immunoradiometric assay; SHBG, sex hormone-binding globulin; VEGF, vascular endothelial growth factor.

Table II. Patient demographics, baseline characteristics and serum hormone concentrations on day 1

	HP-hMG (n = 363)	rFSH (n = 368)
Baseline		
Age (years)	30.8 ± 3.2	30.9 ± 3.3
Weight (kg)	62.7 ± 8.5	61.0 ± 8.2
Body mass index (kg/m^2)	22.6 ± 2.7	22.1 ± 2.6
Primary cause of infertility		
Unexplained infertility	151 (42%)	166 (45%)
Tubal infertility	131 (36%)	125 (34%)
Mild male factor	46 (13%)	40 (11%)
Other (including endometriosis I/II)	35 (10%)	37 (9%)
Duration of infertility (years)	3.9 ± 2.3	3.9 ± 2.2
Duration of GnRH agonist before start of stimulation (days)	14.8 ± 4.1	14.8 ± 3.9
Day 1 of stimulation		
Mean ovarian volume (cm^3)	5.2 ± 3.1	5.1 ± 3.4
Antral follicles	10.9 ± 6.4	10.8 ± 6.9
FSH (IU/l)	3.9 ± 1.4	4.0 ± 1.5
LH (IU/l)	2.2 ± 1.4	2.3 ± 1.3
hCG (IU/l)	–	–
Estradiol (nmol/l)	–	–
Progesterone (nmol/l)	1.3 ± 0.6	1.3 ± 0.6
Androstenedione (nmol/l)	4.6 ± 1.8	4.4 ± 1.9
Total testosterone (nmol/l)	0.71 ± 0.3	0.66 ± 0.3
SHBG (nmol/l)	58 ± 25	57 ± 24
FAI	1.51 ± 1.1	1.36 ± 1.0

HP-hMG, highly purified menotrophin; rFSH, recombinant FSH; SHBG, sex hormone-binding globulin.
Data are mean ± standard deviations or number of patients (percentages).

administration were comparable between the two treatment groups (Table II). All but one patient received 225 IU daily for the first 5 days. On day 6, most of the patients (60% for HP-hMG and 66% for rFSH) had the dose maintained at 225 IU,

whereas 33% in the HP-hMG group and 25% in the rFSH group had the daily dose increased to 300 IU and <10% had the daily dose reduced to 150 IU. The dose of 225 IU throughout the study was maintained by 135 patients in the HP-hMG group and 171 patients in the rFSH group.

On day 6, after stimulation with 225 IU/day, the mean number of follicles >10 mm was 4.1 in the HP-hMG group and 4.9 in the rFSH group, which was significantly different ($P = 0.007$). On the last stimulation day (on average day 10, irrespective of treatment group), patients in the HP-hMG group had significantly fewer follicles, both in total and by various size distributions. The total number of follicles was on average 14.8 with HP-hMG and 15.9 for rFSH, which was significantly different ($P = 0.013$). The mean number of oocytes retrieved was significantly ($P < 0.001$) higher with rFSH (11.8) compared with HP-hMG (10.0).

Serum levels during stimulation

Mean serum levels at the time points evaluated after the start of gonadotrophins (i.e. day 6 of stimulation, last stimulation day and day of oocyte retrieval) are presented in Table III, and the relative difference between treatment groups is illustrated in Figure 1. Data are presented for the population of all patients exposed to HP-hMG or rFSH. It is relevant to note that the differences observed in endocrine profile on the last stimulation day and at oocyte retrieval between HP-hMG and rFSH were also observed among those patients who maintained the dose of 225 IU throughout the study.

FSH, LH and hCG

The serum FSH concentration was at all time points significantly higher among patients treated with HP-hMG compared

Table III. Serum hormone concentrations on day 6, at the end of stimulation and at the time of oocyte retrieval after stimulation with highly purified menotrophin (HP-hMG) or recombinant FSH (rFSH)

	HP-hMG ($n = 363$)	rFSH ($n = 368$)	P-value[a]
Day 6 of stimulation			
FSH (IU/l)	15.8 ± 3.5	15.2 ± 3.5	0.009
LH (IU/l)	1.4 ± 0.8	1.4 ± 0.8	0.465
hCG (IU/l)	2.45 ± 0.80	–	–
Estradiol (nmol/l)	1.0 ± 0.9	1.1 ± 1.0	0.004
Progesterone (nmol/l)	1.4 ± 0.6	1.5 ± 0.7	0.333
Androstenedione (nmol/l)	6.0 ± 2.5	5.5 ± 2.4	0.002
Total testosterone (nmol/l)	0.89 ± 0.45	0.77 ± 0.41	<0.001
SHBG (nmol/l)	55 ± 23	57 ± 22	0.188
FAI	1.96 ± 1.46	1.59 ± 1.09	<0.001
Last stimulation day			
FSH (IU/l)	18.3 ± 6.0	16.3 ± 4.7	<0.001
LH (IU/l)	1.8 ± 0.9	1.7 ± 0.9	0.125
hCG (IU/l)	2.94 ± 1.18	–	–
Estradiol (nmol/l)	7.2 ± 4.3	6.6 ± 4.0	0.031
Progesterone (nmol/l)	2.6 ± 1.3	3.4 ± 1.7	<0.001
Androstenedione (nmol/l)	11.9 ± 5.2	9.5 ± 3.8	<0.001
Total testosterone (nmol/l)	1.71 ± 0.88	1.31 ± 0.65	<0.001
SHBG (nmol/l)	88 ± 39	89 ± 38	0.729
FAI	2.23 ± 1.36	1.66 ± 0.96	<0.001
Oocyte retrieval			
FSH (IU/l)	9.6 ± 3.6	7.8 ± 2.8	<0.001
LH (IU/l)	0.3 ± 0.0	0.3 ± 0.2	0.397
hCG (IU/l)	108.21 ± 31.11	104.11 ± 32.79	0.055
Estradiol (nmol/l)	3.9 ± 2.1	3.4 ± 1.9	0.001
Progesterone (nmol/l)	24.5 ± 15.6	36.3 ± 25.0	<0.001
Androstenedione (nmol/l)	13.6 ± 5.5	10.8 ± 4.2	<0.001
Total testosterone (nmol/l)	2.42 ± 1.13	1.77 ± 0.82	<0.001
SHBG (nmol/l)	129 ± 54	129 ± 53	0.893
FAI	2.19 ± 1.40	1.61 ± 1.03	<0.001

SHBG, sex hormone-binding globulin.
Data are mean ± standard deviations.
The findings of the multi-adjusted analysis differ from the table above with respect to the following: day 6—progesterone was significantly lower with HP-hMG ($P < 0.001$); last stimulation day—estradiol was not significantly different ($P = 0.550$), SHBG was significantly lower with HP-hMG ($P = 0.023$); oocyte retrieval—LH was significantly lower with HP-hMG ($P < 0.001$), estradiol was not significantly different ($P = 0.515$).
[a]Analysis adjusted for age strata.

with those treated with rFSH. On average, the FSH concentration was 5% higher with HP-hMG than with rFSH on day 6, 12% higher on the last stimulation day and 23% higher at oocyte retrieval. There was no difference between treatment groups in LH concentrations at any time point. Most of the patients in the HP-hMG group had circulating levels of hCG >2 IU/l on day 6 and at the end of stimulation. hCG was not detectable in serum from patients treated with rFSH at these time points. After a single administration of 250 µg of rhCG for triggering final maturation, the mean hCG concentration at oocyte retrieval was 108 and 104 IU/l in the HP-hMG and rFSH groups, respectively, which was not significantly different.

Estradiol and progesterone

The estradiol concentration on day 6 was 20% higher in the rFSH group than in the HP-hMG group, which was significant. On the last stimulation day and at the day of oocyte retrieval, the estradiol concentration was significantly higher by 10 and 16%, respectively, with HP-hMG compared with rFSH. The significantly higher estradiol level found among patients in the HP-hMG group at the end of stimulation and at oocyte retrieval was

maintained after adjusting for either the number of follicles (15%) or the number of oocytes retrieved (29%). Estradiol >3500 pg/ml (12.85 nmol/l) on the last stimulation day was measured in 35 patients (10%) in the HP-hMG group and in 30 patients (8%) in the rFSH group. There was no significant difference in progesterone concentration between treatment groups on day 6, whereas the progesterone concentration was 23% higher with rFSH on the last stimulation day and 31% higher at oocyte retrieval. These significantly higher progesterone levels with rFSH were also maintained after adjusting for ovarian response: 28% higher at the end of stimulation when adjusting for the number of follicles and 29% higher at oocyte retrieval when adjusting for the number of oocytes retrieved. Progesterone >4 nmol/l on the last stimulation day was measured in 41 patients (11%) in the HP-hMG group and 85 patients (23%) in the rFSH group.

Androgens

Concentrations of androstenedione, total testosterone and FAI were significantly higher among patients treated with HP-hMG compared with those treated with rFSH at all time points after the start of stimulation. Androstenedione was on average 10% higher on day 6, 24% higher on the last stimulation day and 25% higher at oocyte retrieval with HP-hMG compared with rFSH. Total testosterone was 17, 32 and 36% higher in the HP-hMG group on day 6, on the last stimulation day and at oocyte retrieval, respectively. FAI was 22% higher with HP-hMG than with rFSH on day 6, 33% higher on the last stimulation day and 28% higher at oocyte retrieval.

Estrogen and androgen interplay

In the HP-hMG group, a proportional relationship with estradiol was observed on day 6 and at the end of stimulation for testosterone and on day 6 for androstenedione, and a linear relationship was observed at the end of stimulation for androstenedione. In the rFSH group, a proportional relationship with estradiol was observed for testosterone on day 6 and at the end of stimulation, whereas it was linear for androstenedione at both assessment time points. The relationships between estradiol and total testosterone and estradiol and androstenedione were not confounded by the FSH concentration.

Correlation between mid-follicular hCG and outcome

The associations between hCG levels during stimulation and outcome were assessed. Table IV summarizes embryo quality parameters and ongoing pregnancy by hCG concentrations on day 6 after a fixed daily dose of 225 IU HP-hMG. The ongoing pregnancy rate was significantly positively correlated with serum hCG concentrations on day 6 of stimulation ($P = 0.008$, trend analysis). The day 6 hCG concentration in the HP-hMG group was also significantly positively correlated with the number of top-quality embryos on day 3 after oocyte retrieval ($P = 0.002$, trend analysis) and the percentage of patients with top-quality embryos ($P = 0.003$, trend analysis).

Follicular fluid levels

Table V summarizes the geometrical means of follicular fluid hormone concentrations for each of the treatment groups, and
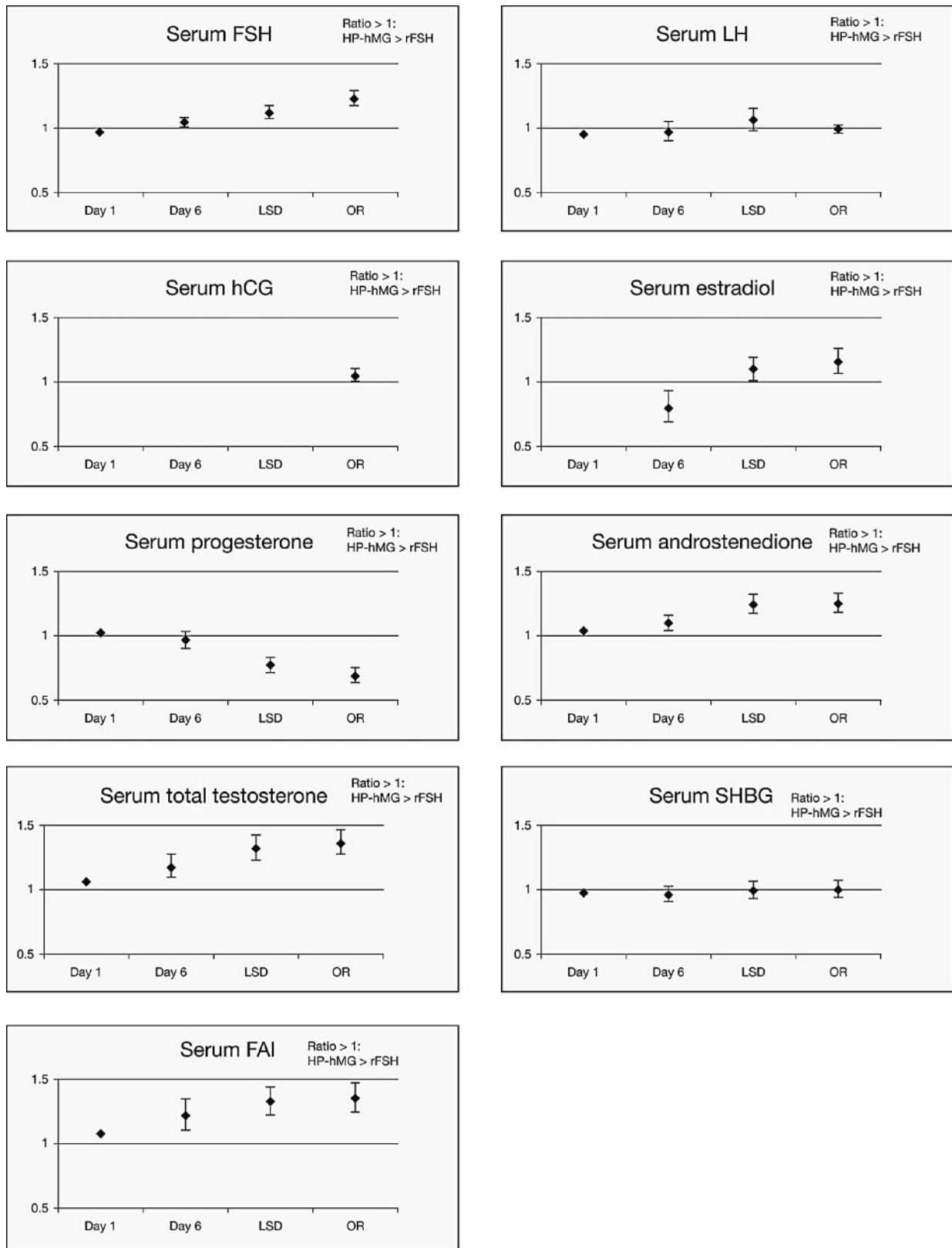
Figure 1. Serum profiles during stimulation (day 1, day 6, last simulation day and oocyte retrieval) with highly purified menotrophin (HP-hMG) and recombinant FSH (rFSH). The graphs illustrate the level of each hormone displayed by the ratio of the concentration in the HP-hMG group to the concentration in the rFSH group (ratio > 1: HP-hMG > rFSH). The point indicates the HP-hMG : rFSH ratio, and the vertical lines illustrate the 95% confidence interval. If the point is above 1, it means that the concentration is higher in the HP-hMG group than in the rFSH group. If the 95% confidence interval does not include 1, the difference between HP-hMG and rFSH is significant ($P < 0.05$). The analysis is adjusted for age strata. LSD, last stimulation day; OR, oocyte retrieval.

Table IV. Serum hCG concentrations on day 6 in patients treated with highly purified menotrophin (HP-hMG) and clinical outcome (ongoing pregnancy rate, number of top-quality embryos and patients with top-quality embryos)

	Ongoing pregnancy rate	Number of top-quality embryos	Patients with top-quality embryos
Serum hCG on day 6			
<25% (*n* = 87)	15%	0.6	33%
25–50% (*n* = 88)	25%	0.8	48%
50–75% (*n* = 90)	33%	1.2	49%
>75% (*n* = 90)	33%	1.3	57%
P-value, trend analysis[a]	0.008	0.002	0.003
P-value, trend analysis[b]	0.081	0.004	0.009

[a]Analysis adjusted for age strata.
[b]Analysis adjusted for age strata, primary reason for infertility, duration of infertility and day 1 serum endocrine parameters.

Table V. Follicular fluid concentrations at oocyte retrieval after stimulation with highly purified menotrophin (HP-hMG) or recombinant FSH (rFSH)

	HP-hMG (*n* = 335)	rFSH (*n* = 341)	*P*-value[a]
FSH (IU/l)	7.1	4.4	<0.001
LH (IU/l)	0.15	0.10	<0.001
hCG (IU/l)	37.1	30.1	<0.001
Estradiol (nmol/l)	1962	1213	<0.001
Progesterone (nmol/l)	28 075	33 621	<0.001
Androstenedione (nmol/l)	56.88	43.78	<0.001
Total testosterone (nmol/l)	17.41	12.49	<0.001
SHBG (nmol/l)	94	95	0.709
FAI	18.58	13.18	<0.001
Estradiol/progesterone	0.070	0.036	<0.001
Estradiol/androstenedione	34.49	27.71	<0.001
Estradiol/total testosterone	112.70	97.08	<0.001
Insulin (mIU/l)	2.6	2.8	0.243
Inhibin A (μg/l)	34 669	38 570	<0.001
Inhibin B (μg/l)	41 287	34 991	0.002
Inhibin A/inhibin B	0.84	1.10	<0.001
VEGF (μg/l)	2376	3011	<0.001
IGF-1 (nmol/l)	20.33	21.27	0.181
Cortisol (nmol/l)	522	514	0.638
Cortisone (nmol/l)	51	51	0.800
Cortisol/cortisone	10	10	0.765

IGF-1, insulin-like growth factor-1; SHBG, sex hormone-binding globulin; VEGF, vascular endothelial growth factor.
Data are geometric means.
The findings of the multi-adjusted analysis differ from the table above with respect to the following: hCG was not significantly different (*P* = 0.295), androstenedione was not significantly different (*P* = 0.721), VEGF was not significantly different (*P* = 0.336) and cortisol was significantly lower with HP-hMG (*P* < 0.001).
[a]Analysis adjusted for age strata.

Figure 2 illustrates the relative difference between HP-hMG and rFSH. The concentrations of FSH, LH and hCG in follicular fluid were significantly higher among patients treated with HP-hMG compared with those treated with rFSH. On average, FSH was 63% higher, LH was 56% higher and hCG was 23% higher with HP-hMG than with rFSH. The follicular fluid concentrations of estradiol and progesterone were significantly different between groups. Estradiol was 62% higher in the HP-hMG group than in the rFSH group, and progesterone was 20% higher with rFSH compared with HP-hMG. Total testosterone and FAI were significantly higher in follicular fluid in patients

stimulated with HP-hMG compared with those with rFSH. Total testosterone was 39% higher with HP-hMG compared with rFSH, whereas there was no difference in SHBG, leading to an average FAI that was 41% higher for HP-hMG compared with rFSH.

As shown in Figure 2, the ratios of estradiol : androstenedione, estradiol : total testosterone and estradiol : progesterone were all significantly higher in the follicular fluid from patients stimulated with HP-hMG compared with those stimulated with rFSH. Estradiol : androstenedione was 24% higher with HP-hMG, estradiol : total testosterone 16% higher and estradiol : progesterone 93% higher. Concerning the inhibins, the following significant observations were made: inhibin A was higher in the rFSH group by 11%, and inhibin B was higher in the HP-hMG group by 18%, with the inhibin A : inhibin B ratio being 31% higher in the rFSH group. The follicular concentration of VEGF was 27% higher among patients stimulated with rFSH, which was significant. There were no significant differences between treatment groups in follicular fluid concentrations of IGF-1, cortisol or cortisone or in cortisol : cortisone ratio.

The differences observed in follicular fluid profile between HP-hMG and rFSH were also observed among those patients who maintained the dose of 225 IU throughout the study, with the single exception of the estradiol : total testosterone ratio, which was not significantly different between HP-hMG and rFSH in this subset of patients.

Correlation between follicular fluid and serum levels

There was a good correlation between follicular fluid levels and circulating levels of FSH, LH, hCG and SHBG at the time of oocyte retrieval. The correlation coefficients (HP-hMG and rFSH, respectively) were 0.88 and 0.81 for FSH, 0.72 and 0.72 for hCG and 0.88 and 0.89 for SHBG. Ovarian steroids in follicular fluid were poorly correlated with the serum levels at oocyte retrieval, with correlation coefficients in the range of 0.18–0.31 for estradiol, 0.19–0.26 for progesterone, 0.10–0.14 for androstenedione and 0.07–0.31 for total testosterone. These correlations were not markedly improved when the serum levels were adjusted for the number of oocytes retrieved. Serum levels of LH at the time of oocyte retrieval were below the limit of quantification in 99% of the patients, and therefore, the correlation between serum and follicular fluid levels was not calculated.

Discussion

The results indicate that providing LH activity in gonadotrophin treatment from the start of stimulation, after GnRH agonist down-regulation, induces significant differences in hormone profiles in both serum and follicular fluid accompanied by differential follicle growth and selection. It is reasonable to assume that findings in follicular development and endocrine environment during ovarian stimulation until oocyte retrieval are generalizable from IVF to ICSI cycles. Despite the administration of an identical starting dose of two preparations equally calibrated for FSH bioactivity in the Steelman–Pohley assay and with similar batch-to-batch consistency (Wolfenson *et al.*, 2005), different effects on follicle recruitment and on
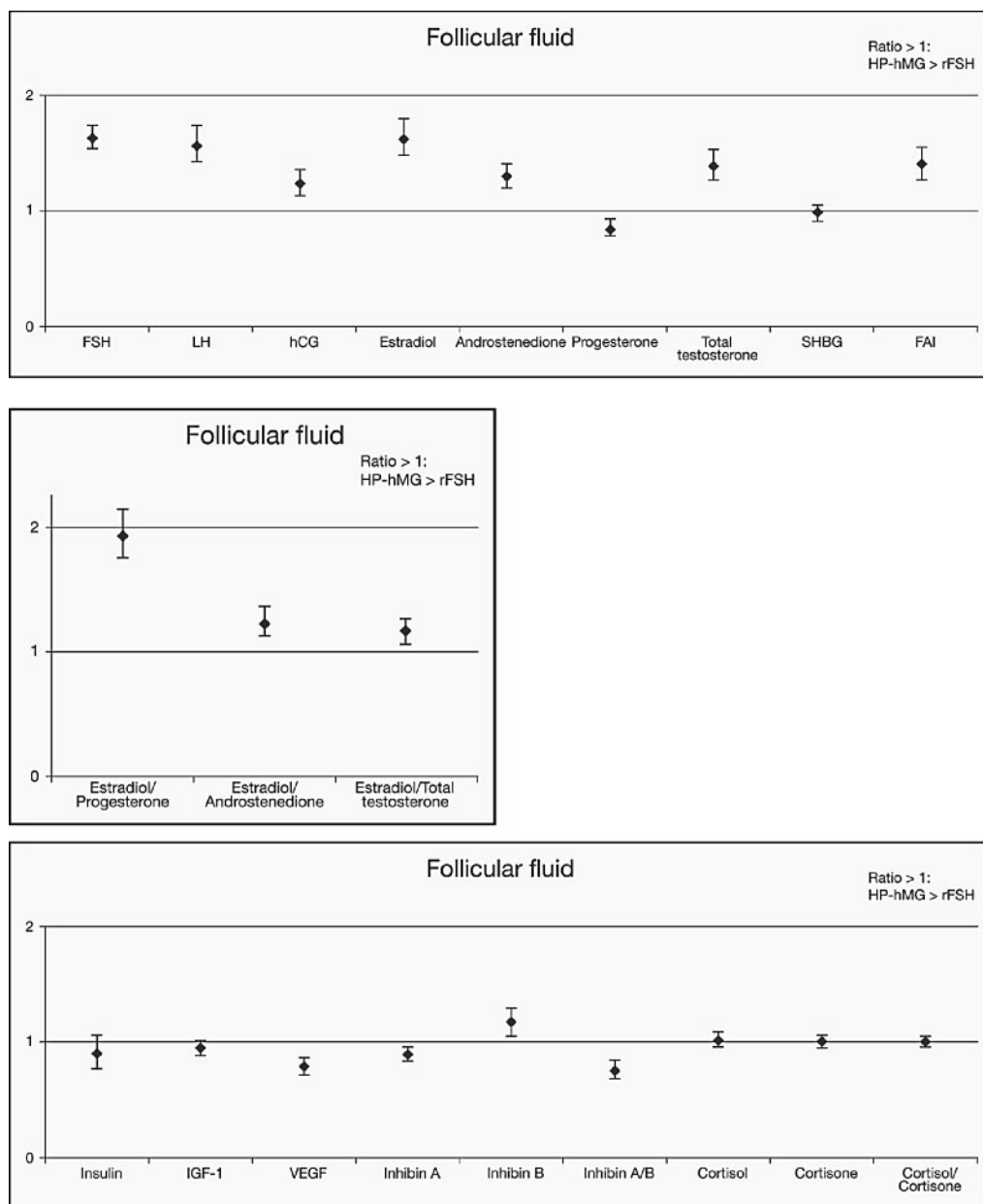
Figure 2. Follicular fluid profiles after stimulation with highly purified menotrophin (HP-hMG) and recombinant FSH (rFSH). The graphs illustrate the level of each hormone displayed by the ratio of the concentration in the HP-hMG group to the concentration in the rFSH group (ratio > 1: HP-hMG > rFSH). The point indicates the HP-hMG : rFSH ratio, and the vertical lines illustrate the 95% confidence interval. If the point is above 1, it means that the concentration is higher in the HP-hMG group than in the rFSH group. If the 95% confidence interval does not include 1, the difference between HP-hMG and rFSH is significant ($P < 0.05$). The analysis is adjusted for age strata.

circulating FSH and estradiol concentrations were observed. The FSH in the HP-hMG preparation has a longer half-life than the FSH in the rFSH preparation, as the relative difference in FSH concentrations was higher 2 days after stopping gonadotrophin treatment (time of oocyte retrieval) than at the end of stimulation. The similar LH levels between the patients exposed to HP-hMG and those to rFSH are not surprising. The timing of sampling could contribute to this finding, as LH has a very short half-life. Moreover, the HP-hMG preparation used in this study has a low LH content, with most of the LH activity derived from the hCG rather than the LH content (Wolfenson *et al.*, 2005). Exposure to hCG from the start of stimulation

might explain the differential effects observed between gonadotrophin preparations in follicle growth and selection.

It has previously been reported from the population in this study that fewer oocytes were retrieved in the HP-hMG group but that the proportion of top-quality embryos was higher than in the rFSH group (Nyboe Andersen *et al.*, 2006). The ovarian response differed between treatment groups early after the start of stimulation. On day 6, there were more follicles in the rFSH group contributing to the significantly higher estradiol concentration compared with HP-hMG. Circulating aromatizable androgen concentrations and FAI were increased with HP-hMG, and this higher serum androgen tonus is related to the

presence of circulating hCG, as the two treatments were not different with respect to LH concentration. The provision of androgens in the human ovary can be attributed to the theca cells that express functional LH receptors already during the pre-antral follicle growth stages (Erickson *et al.*, 1985). The pivotal role of androgens for follicle survival has been studied in several animal models and also in the human (Hillier and De Zwart, 1981; Horie *et al.*, 1992; Tetsuka and Hillier, 1997). During follicular growth, aromatizable androgens are either transformed by aromatase into estrogens or bound to the androgen receptor present on granulosa cells. Cyp19 aromatase promotor use can be triggered differentially by androgen subtypes, leading to an increased estrogen synthesis (Shaw *et al.*, 1989; Hamel *et al.*, 2005). Estrogens act via estrogen receptors β in granulosa cells, opposing the negative effects of androgens in the follicle (Cheng *et al.*, 2002). Androgens can, via the androgen receptor, increase the sensitivity of the follicle to FSH via up-regulation of FSH receptor (Luo and Wiltbank, 2006). However, an amplification of the FSH-stimulated cAMP-mediated postreceptor signalling at an early stage of follicular development will induce the arrest of proliferation in granulosa cells (Hillier *et al.*, 1991). A variety of paracrine and autocrine loops can contribute to the estrogen–androgen tonus that determines the fate of the follicle (Erickson *et al.*, 1989; Hillier *et al.*, 1991). It is hypothesized that the shift in favour of androgens by hCG in HP-hMG from the start of stimulation led to a more selective follicle growth.

It has been speculated that elevated LH levels during ovarian stimulation have a detrimental impact on pregnancy rates (Stanger and Yovich, 1985; Howles *et al.*, 1986; Homburg *et al.*, 1988). It is, however, important to distinguish between exogenous LH/hCG supplementation and elevated endogenous LH levels.

In HP-hMG-treated patients, the contribution of exogenous LH activity supplementation can be evaluated independently from the confounding factor of endogenous LH levels. Among the HP-hMG-treated patients, those with the highest day 6 hCG levels had the highest ongoing pregnancy rates. Also, the number of top-quality embryos and the proportion of patients with top-quality embryos in the HP-hMG group were positively correlated with circulating levels of hCG. Oocytes in the HP-hMG group were found to have a similar fertilization rate as oocytes in the rFSH group, but the proportion of top-quality embryos was higher with HP-hMG (Nyboe Andersen *et al.*, 2006). Improved embryo quality with exogenous LH has recently been reported from another study, where a higher incidence of grade 1 and 2 embryos was observed when supplementing the FSH stimulation with LH in women undergoing a long agonist protocol (Lisi *et al.*, 2005). In the context of most recent studies involving LH receptor action on human cumulus cells (Assou *et al.*, 2006), the direct effects of LH/hCG on this important cellular compartment linked to the maturing oocyte and subsequent embryo quality (McKenzie *et al.*, 2004) could become part of the working hypothesis.

It was interesting to observe that progesterone was more elevated with rFSH-alone treatment. Several mechanisms could explain the increased progesterone that was also found in twice as many patients in the rFSH group compared with the HP-hMG group (23 versus 11%). Although increases in progesterone have been historically associated with LH in the context of premature LH surges, recent articles attribute the increase in progesterone to FSH exposure (Hofmann *et al.*, 1993; Ubaldi *et al.*, 1996; Filicori *et al.*, 2002). Patients in the HP-hMG group had longer exposure time, higher serum FSH and presence of hCG, but this did not lead to increased progesterone compared with the rFSH group. The fact that there were more follicles with rFSH cannot entirely explain the increased progesterone tonus observed with this preparation, as progesterone remained significantly different between treatment groups even when adjusting for the number of developed follicles. Changes in the paracrine regulation could explain differences in progesterone production in the follicle. FSH-stimulated granulosa cells produce paracrine factors that either stimulate the production of progesterone and androgens such as IGF-1 and inhibins (Hillier *et al.*, 1991; Smyth *et al.*, 1993; Campbell and Baird, 2001) or decrease the action of $P450_{c17\alpha}$ enzymes such as transforming growth factor (TGF)-β (Magoffin *et al.*, 1995). TGF-β, whose intrafollicular production is normally inhibited by LH, acts as a strong direct suppressor of the enzyme converting progesterone to androgens (Hernandez *et al.*, 1990; Fournet *et al.*, 1996) and as a stimulator of the steroidogenic genes [$P450_{scc}$ and 3β-hydroxysteroid dehydrogenase (HSD)] necessary for progesterone production (Fournet *et al.*, 1996). TGF-β has been found to be increased in follicular fluid from GnRH agonist-down-regulated patients stimulated with rFSH compared with patients stimulated with a menotrophin preparation leading to an increased progesterone accumulation (Fried *et al.*, 1998). Although it is generally assumed that there is sufficient remaining active LH present after GnRH agonist down-regulation to support steroid production during the initial phase of follicle selection (reviewed by Fauser, 1997), this study found that supplementary hCG activity increases estrogenization of the follicle without increasing the luteinization process. The higher progesterone tonus has been shown to have a biological correlate at the endometrial echogenicity level with hyperechogenicity, leading to a reduced ongoing pregnancy rate (Nyboe Andersen *et al.*, 2006). The propensity of rFSH to induce early luteinization and the impact on endometrial transformation would obviously be equally harmful in IVF as in ICSI cycles.

Regarding intrafollicular assessments, FSH was significantly higher in HP-hMG-stimulated patients, in line with the observations in serum FSH. LH concentrations were also significantly higher with HP-hMG, although the levels were just above the functional sensitivity of the immunoassay. Intrafollicular hCG was also significantly higher for HP-hMG, but the contribution of intrafollicular hCG during stimulation cannot be known from these measurements, as samples were taken after hCG injection for triggering ovulation massively influencing the intrafollicular hCG levels after the end of stimulation. The higher production of intrafollicular androgens and estrogens in the HP-hMG group is attributed to hCG. There was no correlation between serum estradiol, androstenedione or testosterone and follicular fluid levels for these hormones, nor when serum steroid values were adjusted by the number of oocytes retrieved. The simultaneous increase in estradiol and androgens

did not induce a different adaptation in SHBG production between the two treatment groups. The calculated intrafollicular estrogen : androgen ratios demonstrate that HP-hMG induced a more estrogenic than androgenic microenvironment. These data suggest that paracrine factors induced by hCG, and mediated by androgens, activate aromatase, leading towards a more estrogenic intrafollicular climate by the end of stimulation. The estradiol : total testosterone values have been found to be significantly higher in follicular fluid from pregnancy-associated follicles than in fluid containing oocytes that did not produce viable embryos (Yding Andersen and Ziebe, 1992; Yding Andersen, 1993). The potential to effectively convert androgens to estradiol may be considered a marker of health of the follicle, and an inability to convert androgens to estradiol may represent an early atretic change.

Intrafollicular progesterone concentrations measured after hCG administration reflect the massive response of LH receptor-expressing granulosa cells. The hCG ovulatory dose induces a partial inhibition of 17-hydroxylase in theca cells; as a result, more progesterone and less androgens are produced. Immediately following the hCG injection, the pre-ovulatory granulosa cells will have a decreased production of estradiol because of a lack of androgen provision and a secondary decrease in aromatase, resulting in a change from estradiol to progesterone synthesis (Erickson et al., 1985; Couse et al., 2005). In this study, progesterone concentrations in follicular fluid were higher in rFSH-stimulated patients. Similar observations have been made previously (Westergaard et al., 2004). The estradiol : progesterone ratio in follicular fluid was significantly higher with HP-hMG compared with rFSH. It is hypothesized that providing continuous levels of hCG throughout stimulation (as is the case of HP-hMG) causes desensitization to major signalling (Hausdorff et al., 1990). An exaggerated shift in the steroid balance from androstenedione and estradiol to progesterone after the administration of hCG as ovulatory trigger has been associated with a negative effect on early embryo development (Dumesic et al., 2002, 2003). It can be speculated that hCG exposure throughout stimulation would dampen the effect of the hCG as ovulatory stimulus and would reduce the magnitude of the androgen and estrogen shift to progesterone, described to be detrimental to early embryogenesis.

The rapid growth of multiple follicles needs the development of a vascular network. VEGF is a major mediator of neovascularization and permeabilization. Its production by granulosa cells is influenced by gonadotrophins and steroids and is highly increased during luteinization (Christenson and Stouffer, 1997). The intrafollicular VEGF concentration in rFSH compared with HP-hMG might be compatible with an earlier luteinization. Inhibin A and B production in antral follicles is highly dependent on gonadotrophin stimulation and on the stage of granulosa differentiation. Inhibin B secretion by small follicles reflects the increase in the number of granulosa cells and inhibin A secretion their increased differentiation (Campbell and Baird, 2001). Assuming that inhibin A : inhibin B is a good marker for the follicle developmental progression, the data suggest that large follicles in the rFSH group might be more advanced towards end-differentiation. Considering the increase in progesterone, inhibin A : inhibin B ratio and VEGF in the rFSH group, it could be

suggested that this treatment leads granulosa cells more rapidly to their end-differentiation stage or pre-luteinization.

In conclusion, the hormonal data from this comparative study show that the presence of LH activity in gonadotrophin preparations has a differential effect on the antral follicle selection process. LH activity supplementation induces a more selective follicle recruitment process, presumably because of the significant shift in the balance between estrogens and androgens. The distinct endocrine profiles between HP-hMG and rFSH appear to have genuine biological effects at the end organ level, as reflected in the embryo quality and endometrial changes reported for the population included in this trial (Nyboe Andersen et al., 2006). The intrafollicular endocrine response upon the ovulatory hCG injection supports the hypothesis that folliculogenesis in the absence of sufficient amounts of LH activity leads the granulosa cells more quickly to luteinization.

Conflict of interest

Johan Smitz and Paul Devroey have conducted clinical research sponsored by Ferring Pharmaceuticals, Serono and Organon. Anders Nyboe Andersen has conducted clinical research sponsored by Ferring Pharmaceuticals, Serono, Organon, Novo Nordisk and Medicult. Joan-Carles Arce is employee of Ferring Pharmaceuticals.

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