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ORIGINAL ARTICLE *Reproductive endocrinology*

A randomized controlled dose–response pilot study of addition of hCG to recombinant FSH during controlled ovarian stimulation for *in vitro* fertilization

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STUDY QUESTION: Is it possible to define an optimal dose of hCG in combination with rFSH from the first day of stimulation in the GnRH agonist protocol applied to IVF?

SUMMARY ANSWER: Supplementation with hCG from the first day of stimulation may increase the number of top-quality embryos per patient. Daily doses of hCG up to 150 IU are compatible with good live birth rates. A ceiling level of estradiol (E_2) was reached with hCG doses above 100 IU/day. A positive dose–response was seen for pre-ovulatory progesterone, but concentrations remained below values for which an impairment of endometrial receptivity has been previously reported. We suggest a large clinical trial to be proceeded with a group given 100 IU hCG daily versus a control group.

WHAT IS KNOWN AND WHAT THIS PAPER ADDS: Prospective multicentre studies have indicated increased live birth rates and increased number of top-quality embryos when low doses of hCG were associated with FSH. We analysed the clinical, embryological and endocrine aspects of adding increasing doses of hCG to rFSH from the first day of stimulation for IVF.

DESIGN: A prospective randomized, controlled, open-label dose–response pilot study was conducted between February 2009 and June 2010 at Copenhagen University Hospital, Rigshospitalet, Denmark. Adequate allocation concealment was assured from sequentially numbered, opaque, sealed envelopes prepared from a computer-generated list. Scoring of the embryos was done in an assessor-blinded way.

PARTICIPANTS AND SETTING: Endocrinologically normal IVF patients aged 25–37 years, BMI 18–30 kg/m^2, regular cycles and FSH <12 IU/l, were treated with a fixed dose of rFSH 150 IU/day and randomized to daily hCG dose of 0, 50, 100 or 150 IU from Day 1 of stimulation. Primary end-point was the total number of top-quality embryos on Day 3.

DATA ANALYSIS METHOD: Data were analysed by analysis of variance, Kruskal–Wallis test, chi-squared test or Poisson distribution count.

MAIN FINDINGS: A total of 62 patients were randomized into four hCG dose groups: Dose 0 (D0; $n = 16$), Dose 50 (D50; $n = 15$), Dose 100 (D100; $n = 16$) and Dose 150 (D150; $n = 15$). Two patients in D150 were withdrawn after randomization because of major (10- to 30-fold) hCG dosing errors, leaving 13 patients in this group. Thus, the results are based on the per protocol population. The mean numbers of top-quality embryos per patient were D0: 0.8 ± 1.2, D50: 0.5 ± 0.7, D100: 1.2 ± 1.7 and D150: 1.5 ± 1.7 ($P = 0.04$). All pregnancies were singleton gestations, and the live birth rates per started cycle were D0: 25%, D50: 27%, D100: 25% and D150: 31% ($P = 0.98$). Steady state level of serum (s)-hCG was reached on Day 6 of stimulation. S-hCG levels (IU/l) on the day of hCG administration were D0: <0.1, D50: 3.1 (2.6–3.6), D100: 5.5 (4.1–7.4) and D150: 11.0 (8.9–13.6) ($P < 0.01$). The patients receiving hCG supplementation were

stratified by 33 and 66% percentiles into three groups according to the concentration of s-hCG on Day 6 of stimulation: 0.5–3.5 IU/l ($n = 16$), 3.5–8.0 IU/l ($n = 14$) and 8.0–21.1 IU/l ($n = 14$). The mean numbers of top-quality embryos in the three groups were 0.5 ± 0.9, 1.1 ± 1.8 and 1.5 ± 1.5, respectively ($P = 0.03$). The progesterone increments from stimulation Day 1 to the day of hCG triggering were D0 = 49%, D50 = 79%, D100 = 110% and D150 = 160% ($P = 0.02$). S-androstenedione level was highest in D150 ($P < 0.01$). S-E_2 was 2-fold higher in the D100 and D 150 compared with D0 ($P = 0.09$).

BIAS, LIMITATION, GENERALISABILITY: Our study has a limited sample size. Supplementation with daily hCG dose up to 150 IU throughout stimulation has never been used before. Hence, this had to be tested in a small study before conducting a larger trial.

STUDY FUNDING/COMPETING INTERESTS: Ferring Pharmaceuticals, Research and Development, provided funds for the endocrine measurements.

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Key words: hCG / dose–response / recombinant FSH / IVF / GnRH agonist

Introduction

Several studies have shown that hCG can replace rFSH during the final days of controlled ovarian stimulation (COS) (Filicori *et al.*, 2005; Blockeel *et al.*, 2009; Kosmas *et al.*, 2009), but no dose–response study has investigated the effects of addition of hCG to rFSH from the first day of stimulation.

Prospective multicentre studies and meta-analyses have shown that pregnancy and live birth rates may be improved after IVF by using highly purified-human menopausal gonadotrophins (HP-hMG) rather than r-FSH (Andersen *et al.*, 2006; Platteau *et al.*, 2008; van Wely *et al.*, 2011). HP-hMG contains hCG and compared with rFSH, COS with HP-hMG has been shown to induce a different endocrine profile, different follicular dynamics, a larger proportion of top-quality embryos and more favourable endometrial receptivity (Andersen *et al.*, 2006; Smitz *et al.*, 2007; Ziebe *et al.*, 2007).

The LH activity of urinary-derived commercially available menotrophins varies, but in the HP menotrophins (hMG; Menopur), the main LH activity is related to hCG and for 75 IU of FSH, the drug contains around 10 IU of hCG (Wolfenson *et al.*, 2005). When using HP-hMG for COS, hCG will be present throughout the stimulation. The differences in endocrine profiles observed using HP-hMG versus rFSH have in part been attributed to the hCG content. The circulating levels of hCG on Day 6 in the long agonist protocol have been shown to be positively correlated with live birth rates and number of top-quality embryos (Smitz *et al.*, 2007).

The purpose of the present study was to define a possible optimal dose of hCG that could be added to rFSH throughout stimulation. This was investigated through a regular dose–response pilot study analysing the clinical, embryological and endocrine aspects. An additional aim was to define possible ceiling levels, i.e. levels above which there were no additional beneficial effects or potentially harmful effects of supplementation with hCG. The primary end-point was the number of top-quality embryos per patient on Day 3 after fertilization.

Materials and Methods

Study design

This was a single-centre, prospective, randomized, controlled, parallel-group dose–response study conducted between February 2009 and June 2010 at the Fertility Clinic, Copenhagen University Hospital, Rigshospitalet, Denmark. The Danish National Committee on Biomedical Research Ethics (HB-2008-146) and The Danish Medicines Agency (2612–3928, EudraCT number 2008-008355-42) approved the study. All the patients participated after giving verbal and written informed consent. The study was conducted according to the International Conference on Harmonization (ICH) guidelines and Good Clinical Practice (GCP). An unpaid independent external monitor from the GCP unit, Copenhagen Region, monitored the study (2008–257). The trial was registered in the ClinicalTrial.gov as NCT00844311.

Participants

The CONSORT (Consolidated Standards of Reporting Trials) diagram shows the flow of participants through each stage of the study (Fig. 1). Seventy-five patients were assessed for eligibility and 62 were randomized to obtain 60 patients for the per protocol (PP) analysis. The patients were 'standard' patients scheduled for IVF.

Inclusion and exclusion criteria

The inclusion criteria were as follows: (i) women with indication for COS and IVF; (ii) age 25–37 years; (iii) BMI >18 and <30 kg/m^2; (iv) a regular menstrual cycle of 24–35 days, presumed to be ovulatory; (v) two ovaries; (vi) tubal or unexplained infertility, including endometriosis Stage I/II and mild male factor; (vii) a uterus consistent with expected normal function (e.g. no clinically interfering uterine fibroids) documented by transvaginal ultrasound at the screening; (viii) male partner with sperm quality compatible with fertilization via an IVF procedure or previous clinical pregnancy; (ix) early follicular phase serum FSH levels of 1–12 IU/l; (x) early follicular phase total antral follicle (2–10 mm) count ≥6 and (xi) willing and able to sign informed consent.

The exclusion criteria included (i) history of or current PCOS, endometriosis Stage III/IV or severe male factor requiring ICSI; (ii) history of severe ovarian hyperstimulation syndrome (OHSS); (iii) presence of unilateral or bilateral hydrosalpinx at ultrasound; (iv) more than three previous COS cycles; (v) previous poor response on an IVF cycle, defined as >20 days of gonadotrophin stimulation, cancellation due to limited follicular response or less than four follicles of ≥15 mm diameter; (vi) previous IVF cycle with unsuccessful fertilization, defined as fertilization of ≤20% of the retrieved oocytes; (vii) history of recurrent miscarriage; (viii) FSH >12 IU/l or LH >12 UI/l (early-follicular phase); (ix) contraindications for the use of gonadotrophins or GnRH analogues; (x) recent history of current epilepsy, HIV infection, diabetes or cardiovascular, gastrointestinal, hepatic, renal or pulmonary disease; (xi) pregnancy, lactation or contraindication to pregnancy; (xii) current or past (last 12 months) abuse of alcohol or drugs; (xiii) history of chemotherapy (except for gestational conditions) or radiotherapy; (xiv) undiagnosed vaginal bleeding; (xv)

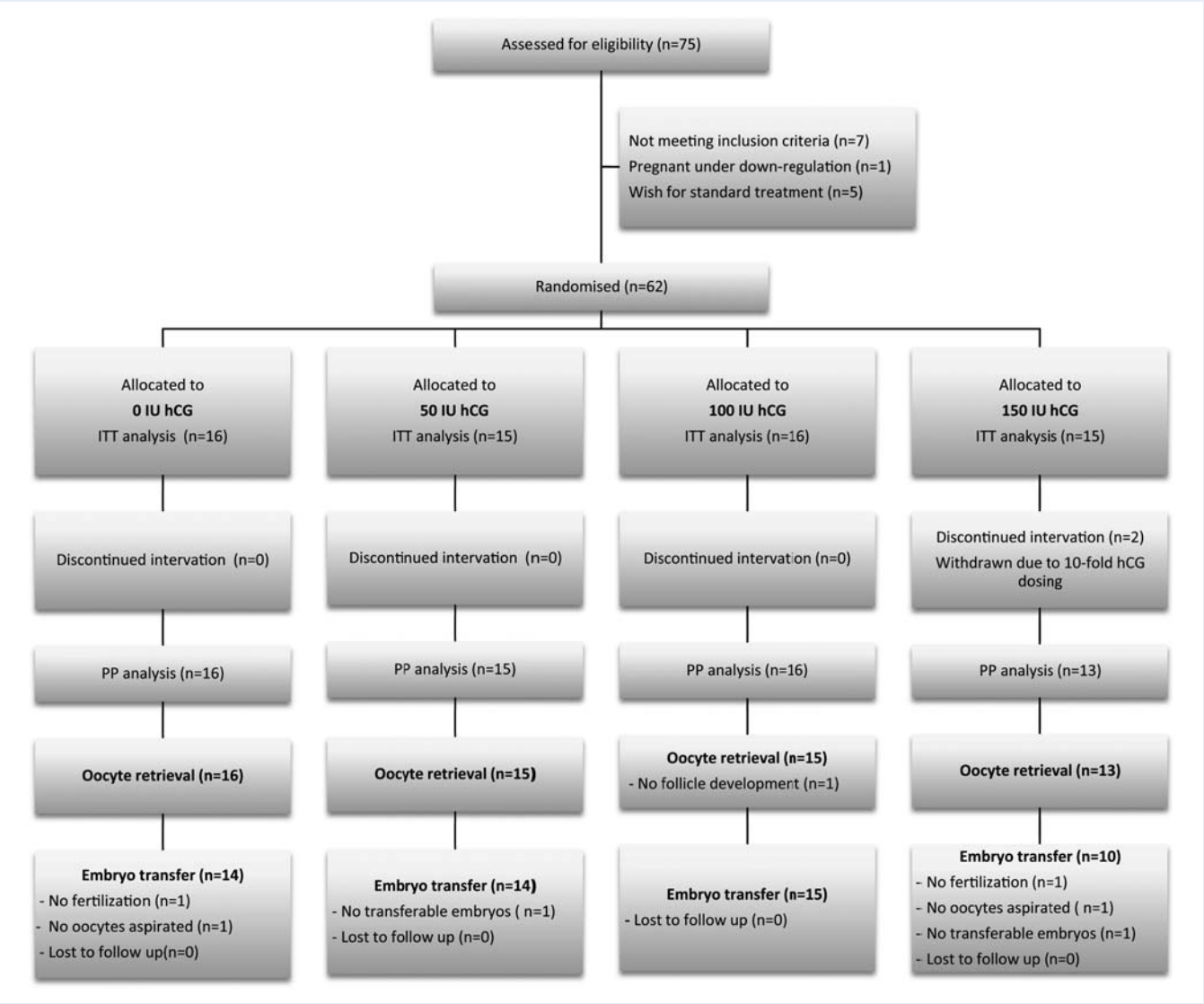

Figure 1 CONSORT diagram showing the flow of participants through each stage of the trial. ITT, intention to treat; PP, per-protocol.

tumours of the ovary, breast, adrenal gland, pituitary or hypothalamus and malformation of sexual organs incompatible with pregnancy; (xvi) abnormal karyotype of the patient (if karyotyping was performed) and (xvii) hypersensitivity to any trial product.

Inclusion and exclusion criteria were thus in essence identical with the criteria used in the MERIT trial (Andersen *et al.*, 2006).

Inclusion of patients

Patients were assessed for eligibility on cycle Days 2–5. Those who agreed to participate and fulfilled the inclusion criteria signed the informed consent and had endocrine testing [s-FSH, s-LH, s-Anti-Mullerian hormone (AMH)] and a transvaginal sonography with recording of antral follicle count and ovarian volume. After down-regulation, the patients were randomized.

Treatment protocol and randomization procedures

Patients started down-regulation on cycle Day 21 (range cycle Day 19–25). All used nafarelin 200 μg × 3 (nasal administration) (Synarela®, Pfizer ApS, Denmark) for at least 2 weeks. Once down-regulation had been confirmed through presence of menstrual bleeding and shedding of the endometrium on sonography, the patient was randomly assigned to one of the four treatment arms (Fig. 1). All the randomized patients were treated with rFSH 150 IU/day (Puregon®, N.V. Organon, Oss, The Netherlands) in a fixed dose regimen on Day 1 of stimulation. Supplementation with different doses of hCG (Predalon® 500 IU, Organon, Berlin, Germany) started on Day 1 of stimulation after the randomization. The four arms were (i) control arm (D0): 150 IU/day of rFSH alone, (ii) hCG low dose (D50): 150 IU/day of rFSH + 50 IU/day of hCG, (iii) hCG medium dose (D100): 150 IU/day of rFSH + 100 IU/day of hCG and (iv) hCG high dose (D150): 150 IU/day of rFSH + 150 IU/day of hCG (Fig. 1).

On stimulation Day 1, the study nurse instructed the patient how to inject the medicine and how to open a new Predalon® ampoule every day. The first sonography was done 5 days after stimulation. Thereafter, follicular development was monitored by ultrasound at least every second day. The dose of rFSH was kept constant; however, it was permissible, according to the protocol, to reduce the dose of rFSH after Day 6 in

case of imminent risk of OHSS. The target for the ovarian stimulation was set to be 7–15 oocytes at retrieval and the total duration of stimulation was a maximum of 20 days. HCG, 10 000 IU s.c. (Pregnyl, N.V. Organon, Oss, The Netherlands), was administered to induce final follicular maturation within one day when three or more follicles of ≥17–18-mm diameter were observed.

Oocyte retrieval took place 36 h (±2 h) after hCG administration. Subsequently, IVF was performed. Transfer of preferably one embryo was done on Day 3 after oocyte retrieval. Vaginal tablets of progesterone 200 mg × 3/day (Utrogestan®, Besins Healthcare, Brussels, Belgium) were administrated for luteal support and given from the day of embryo transfer until confirmation of pregnancy or negative serum βhCG test 13–15 days after embryo transfer. In case of positive s-hCG, the patient was scanned at gestational week 6–7 (clinical pregnancy) and at week 10–12 for diagnosis of ongoing pregnancy. All pregnancies were followed up to delivery. In addition, frozen embryos derived from the study were followed 1 year after study completion.

Fertilization rate and embryo quality

Fertilization was done with standard IVF procedure at 3 h (±1 h) after oocyte retrieval. Experienced embryologists blinded to the treatment protocol assessed embryo quality at 20 h (±1 h), 28 h (±1 h), 44 h (±1 h) and 68 h (±1 h) after oocyte retrieval. According to the routine procedures of the clinic, embryos were scored on agreement between two embryologists who simultaneously judged each embryo using a microscope with a ×400 magnification and a connected monitor. Two-pronuclear oocytes as well as oocytes that subsequently cleaved were considered fertilized. The individual fertilization rate was calculated as the number of fertilized oocytes divided by the number of all the intact oocytes retrieved. The embryo quality evaluation consisted of assessment of cell number and three parameters of embryo morphology: degree of fragmentation, blastomere uniformity and multinucleation.

All the oocytes were followed individually and quality assessed on the day of retrieval, Day 1, 2 and 3 after oocyte retrieval, respectively.

Sonography

Transvaginal sonography was performed using a BK Medical Pro Focus scanner on cycle Days 2–5, on stimulation Days 1 and 6 and thereafter, every second day until hCG administration. On cycle Days 2–5 and on the first day of stimulation, the number of antral follicles 2–4, 5–7 and 8–10 mm were counted and ovarian volume was calculated from the measurements of the maximum longitudinal (D1), anterior–posterior (D2) and transverse (D3) diameters, using the ellipse formula D1 × D2 × D3 × 0.523. Every follicle and its size were recorded at every examination. Follicular size was determined as mean diameter. Assessment of the endometrium (endometrial thickness, triple-layer pattern and echogenicity) was done on Day 1, Day 6 and the last stimulation day.

Serum endocrinology

Blood samples were drawn for assays of baseline FSH, LH and AMH on cycle Days 2–5. Blood sampling for endocrine parameters was done before the daily drug injection on stimulation Days 1, 3, 6 and thereafter, every second day until the day of hCG administration. HCG, androstenedione, estradiol (E_2), progesterone, FSH and LH were measured. Blood samples were centrifuged for 12 min at 3000*g*, and serum was stored individually at −24°C and later analysed with all the samples quantified in the same run. The analyses of hCG, androstenedione and progesterone were done at the Laboratorium für Klinische Forschung (LKF; Raisdorf, Germany). E_2, FSH, LH and AMH were analysed at the Department of Clinical Biochemistry, Rigshospitalet, Denmark.

The sensitivity and inter- and intra-assay coefficients of variation were for hCG <0.1 IU/l, ≤7.4 and ≤2.8%; progesterone 0.03 ng/ml, 4.8 and 2.9%; androstenedione 0.03 ng/ml, 9.8 and 5.6%; FSH 0.1 IU/l, 4.5 and 2.8%; LH 0.1 IU/l, 2.2 and 1.2%; E_2 0.02 nmol/l, 4.7 and 3.3% and for AMH 0.7 pmol/l, 14.2 and 12.3% (AMH/MIS kit, Immunotech).

Study end-points

The primary end-point was the total number of top-quality embryos on Day 3. A top-quality embryo was defined as four to five blastomeres on Day 2, seven or more blastomeres on Day 3, equally sized blastomeres and ≤20% fragmentation on Day 3 and no multinucleation. These end-points were the same as those used in the MERIT trial (Ziebe *et al.*, 2007).

Secondary end-points included follicular development, number of oocytes retrieved, number of oocytes, fertilization, fertilization rate, number of embryos transferred, implantation rate, duration of stimulation, total dose of rFSH, serum levels of endocrine parameters, endometrial status, pregnancy rates and miscarriage rates.

Sample size calculation

The study was planned according to an adaptive design. This design is defined as a prospective study allowing for future planned design modifications during the course of the study (Sagkriotis and Scholpp, 2008). The adaptive design allows for transition from one phase of clinical development to the next within a single trial and consists of two stages separated by an interim phase. In Stage 1, patients are randomized into four different groups. Either the study stops at Stage 1 or proceeds to Stage 2, where only two groups showing the most promising differences continue. Sample size at Stage 2 is determined on the basis of the results of the interim analysis and through simulations of the results of Stage 1. We have now completed the first stage of the study, which basically is identical to a pilot study, and the findings are described in this article. As suggested by Lachin *et al.* (1988), such studies of adaptive designs can start with 15 patients randomized to each of the groups in Stage 1. The approach for Stage 2 is described in the 'Discussion' section.

Randomization

A randomization list was generated by computerized block randomization. Block size was 12 resulting in a balanced allocation in treatment groups for each 12 patients. The blocking was unknown to all individuals involved in allocating treatments and data collection. Participants were randomized to one of the four treatment arms 'control', '50 IU', '100 IU' or '150 IU' at a ratio of 1:1. From the randomization list, a secretary prepared sequentially numbered, opaque sealed envelopes. Independent monitors from the GCP Unit at Copenhagen University verified that the envelopes were sealed. The envelopes were opened in consecutive order by one of the investigators after verbal and written informed consent, after the ultrasound examination on the first day of stimulation and immediately before starting rFSH stimulation. The monitors verified the order in which the patients were included and that all spare envelopes were unopened at the end of the study. After randomization, the study was open without blinding for the investigator and the patients; however, the lab technicians scoring the embryos were blinded to the treatment protocol.

Statistical analysis

The statistical analyses presented are based on the PP population. The intention-to-treat (ITT) principle would not be appropriate in this dose–response study because two patients in the group D150 had their cycle cancelled because of major (10- to 30-fold) hCG dosing errors (described in the 'Results' section). No adjustments for multiplicity were made regarding secondary end-points.

Data are presented as mean or median according to data distribution. A two-sided *P*-value of <0.05 was considered statistically significant. For between-group analyses, comparisons of continuous variables were carried out by analysis of variance (ANOVA) or by the Kruskal–Wallis test where appropriate. Comparisons of proportions between the groups were made by the chi-squared test or Fisher's exact test where appropriate. Serum analyses were logarithmically transformed before the between-group analyses were done. The logarithmic means were transformed into the original scale, and thus, the presented means are the geometric means. Additionally, the number of top-quality embryos was analysed as a Poisson-distributed count as the range was relatively small (0–6) with the highest frequencies close to zero and the mean values were compared for the four groups.

The statistical analysis of the data was performed in collaboration with statisticians affiliated to the Juliane Marie Center Rigshospitalet from the Biostatistics Department Copenhagen University using the Statistical Package for the Social Sciences 18.0 software for Windows (SPSS Inc., Chicago, IL, USA).

Results

Demographic characteristics

As presented in Fig. 1, 75 patients were assessed for eligibility. A total of 62 patients were randomized into the four hCG dose groups: D0 ($n = 16$), D50 ($n = 15$), D100 ($n = 16$) and D150 ($n = 15$).

The results are based on the PP analysis. The reason for withdrawal of two initiated patient cycles from the analysis was that 10- to 30-fold dosing errors occurred in the main investigational drug (hCG). These two patients were randomized to 150 IU/day of hCG and had their cycle cancelled 9 and 3 days after stimulation, respectively. Instead of 150 IU/day, one patient injected 1500 IU daily over 3 days; the other patient injected 1500 IU on Day 2 of stimulation.

Demographics, clinical characteristics and endocrine profile at screening on cycle Days 2–5 are shown in Table I. Although properly randomized, there were more patients with tubal factors in D0 (50%) compared with D50 (13%) ($P = 0.04$). Furthermore, the frequency of primary infertility differed between D50 (73%) and D150 (31%) ($P = 0.03$). Otherwise, there were no statistical differences between treatment groups of any of the baseline characteristics.

Primary end-point

The number of top-quality embryos per patient was analysed as a Poisson-distributed count (Table II). The mean numbers of top-quality embryos per patient were D0: 0.8 ± 1.2, D50: 0.5 ± 0.7, D100: 1.2 ± 1.7 and D150: 1.5 ± 1.7 with a statistically significant higher mean count in D150 compared with D50 ($P = 0.04$) (Table II).

The patients receiving hCG supplementation were stratified by 33 and 66% percentiles into three groups according to the concentration of s-hCG on Day 6 of stimulation: <3.5 IU/l ($n = 16$), 3.5–8.0 IU/l ($n = 14$) and >8.0 IU/l ($n = 14$). The mean numbers of top-quality

Table I Demographic characteristics at screening.

Characteristics	Dose 0 ($n = 16$)	Dose 50 ($n = 15$)	Dose 100 ($n = 16$)	Dose 150 ($n = 13$)
Age (years)	31.5 ± 2.4	31.7 ± 3.2	32.7 ± 2.7	33.6 ± 2.1
BMI (kg/m^2)	22.3 ± 2.6	21.4 ± 1.3	22.6 ± 3.6	22.3 ± 2.6
Never-smoker[a]	8 (50)	8 (53)	8 (50)	5 (39)
Duration of infertility[b] (years)	2.5 (1.6–3.5)	2.1 (1.7–2.5)	2.5 (1.7–3.2)	2.4 (1.6–3.2)
Primary infertility[b]	8 (50)	11 (73)	10 (63)	4 (31)
Parous women[c]	2 (13)	0 (0)	3 (19)	3 (23)
Infertility diagnosis				
Mild male factor	3 (19)	4 (27)	2 (13)	2 (15)
Tubal factor	8 (50)	2 (13)	4 (25)	3 (23)
Mild endometriosis	0	0	1 (6)	0
Unexplained	4 (25)	6 (40)	5 (31)	5 (39)
Combined	1 (6)	3 (20)	4 (25)	3 (23)
First treatment cycle	15 (94)	12 (80)	13 (81)	10 (77)
Antral folicle count[d]	19.3 ± 7.7	19.9 ± 7.1	21.3 ± 7.6	18.9 ± 10.3
Ovarian volume[e]	11.2 ± 4.4	10.0 ± 4.4	11.6 ± 4.0	11.1 ± 5.6
Endocrinology, cycle day 2–5				
FSH (IU/l)	6.7 (6.0–7.3)	6.1 (5.2–7.2)	6.5 (5.4–7.9)	6.6 (5.9–7.5)
LH (IU/l)	6.2 (5.4–7.1)	5.6 (4.7–6.8)	5.6 (4.2–7.4)	5.2 (4.2–6.5)
AMH (pmol/l)	16.5 (12.0–22.8)	16.9 (11.3–25.2)	15.4 (10.2–23.2)	13.6 (6.1–30.3)

Values are mean ± SD/95% CI or number (column percentage); (AMH, Anti-Mullerian hormone).
[a]One current smoker in D0.
[b]Women without a previous pregnancy (biochemical, abortion, delivery).
[c]Two parous women; one in D0 and one in D100 had a live birth.
[d]Total count, left + right ovary.
[e]Total volume (ml), left + right ovary.

Table II Primary end-point.

	Dose 0 (n = 16)	Dose 50 (n = 15)	Dose 100 (n = 16)	Dose 150 (n = 13)	P-value
Total no. of embryos (n)	102	71	99	88	
Total no. of transferable embryos (n)	49	35	58	47	
Total no. of top-quality embryos (n)	12	7	19	19	
Top-quality embryos per patient[a]	0.8 ± 1.2	0.5 ± 0.7	1.2 ± 1.7	1.5 ± 1.7	0.04
Transferable embryos per patient[b]	3.1 ± 3.9	2.3 ± 2.3	3.6 ± 2.6	3.6 ± 3.5	0.65
Embryos per patient[b]	6.4 ± 4.7	4.7 ± 3.6	6.2 ± 4.3	6.8 ± 5.7	0.44
Patients with top-quality embryos [n (%)][c]	6 (38)	5 (33)	8 (50)	8 (62)	0.43

Values are mean ± SD or number (column percentage).
[a]Poission distribution.
[b]ANOVA.
[c]χ^2 test.

Table III Stimulation characteristics.

	Dose 0 (n = 16)	Dose 50 (n = 15)	Dose 100 (n = 16)	Dose 150 (n = 13)	P-value
Day of hCG triggering					
Follicles ≤10 mm	8.6 ± 4.8	7.7 ± 4.2	7.2 ± 4.2	7.9 ± 4.5	0.86
Follicles >10 mm	14.6 ± 7.7	13.6 ± 7.3	15.0 ± 7.2	13.6 ± 7.0	0.94
Follicles 11–14 mm	7.9 ± 5.8	6.7 ± 5.1	6.5 ± 4.0	5.3 ± 4.4	0.58
Follicles ≥15 mm	5.8 ± 2.0	6.4 ± 2.6	7.9 ± 3.6	7.5 ± 2.9	0.16
Endometrium (mm)					
Stimulation day 1	3.0 ± 0.9	2.9 ± 1.0	2.6 ± 0.6	2.7 ± 0.8	0.48
Stimulation day 6	6.2 ± 1.7	6.8 ± 2.2	6.7 ± 1.6	6.8 ± 1.8	0.71
Day of hCG triggering	9.6 ± 1.8	9.7 ± 2.3	10.1 ± 1.9	11.2 ± 2.5	0.20

Values are mean ± SD. ANOVA is used.

embryos in the three groups were 0.5 ± 0.9, 1.1 ± 1.8 and 1.5 ± 1.5, respectively (P = 0.03).

Secondary end-points

Stimulation characteristics

Stimulation characteristics are shown in Table III. On the day of hCG triggering, the total number of follicles according to different size categories was similar in the four groups. The mean endometrial thickness (mm) on the day of hCG triggering was 9.6 mm in D0 and 11.2 mm in D150 (P = 0.20). No differences were observed between the groups regarding the echogenicity of the endometrium.

Clinical outcome

The clinical outcome according to treatment groups is listed in Table IV. Oocyte retrieval was performed for all patients except in one patient in the D100 group, as no mature follicles developed. Transfer was not reached for seven patients. This was due to no oocytes at retrieval (n = 2), fertilization failure (n = 2) and lack of transferable embryos due to extensive fragmentation (n = 2) (Fig. 1). Because of poor embryo quality, five patients had two embryos transferred instead of one embryo. No significant differences were found between the four groups regarding treatment duration, oocytes retrieved, implantation rates and pregnancy rates (Table IV). Despite the lack of any difference between the four groups, D150 had the highest number of oocytes retrieved, the lowest fertilization and cleavage rates together with the fewest number of patients (77%) reaching embryo transfer. Among the four groups, D50 had the shortest duration of stimulation, resulting in the lowest consumption of the rFSH. The FSH dose was kept constant, except in cases (n = 3) with an imminent risk OHSS. All live births after fresh transfer were the result of single-embryo transfers. D100 had the highest cumulated (fresh + frozen embryo transfers) positive hCG rate 13–15 days after transfer (69%). The cumulated live birth rate ranged between 31 and 44%.

As hCG can be metabolized differently in different patients, it was decided that the results should be analysed in relation to serum concentration on Day 6. Patients receiving hCG supplementation were stratified into three groups by 33 and 66% percentiles according to the concentration of s-hCG on Day 6 of stimulation: <3.5 IU/l (n = 16), 3.5–8.0 IU/l (n = 14) and >8.0 IU/l (n = 14). In these groups, there were no differences in live birth after the stimulated

Table IV Cycle outcome.

	Dose 0 (n = 16)	Dose 50 (n = 15)	Dose 100 (n = 16)	Dose 150 (n = 13)	P-value
Treatment duration (days)	10.3 ± 1.4	9.3 ± 1.4	9.9 ± 1.3	10.4 ± 1.1	0.14
Total dose of rFSH (IU)	1538 ± 209	1385 ± 232	1475 ± 195	1562 ± 163	0.10
Patients reaching oocyte retrieval [n (%)]	16 (100)	15 (100)	15 (94)	13 (100)	0.42
Oocytes retrieved/per retrieval	9.3 ± 6.3	8.5 ± 4.4	9.2 ± 4.2	11.3 ± 5.7	0.53
Fertilization rate (%)[a]	0.77 ± 0.27	0.72 ± 0.27	0.83 ± 0.23	0.67 ± 0.34	0.51
Cleavage rate[b]	0.72 ± 0.27	0.66 ± 0.27	0.77 ± 0.23	0.60 ± 0.34	0.41
Patients with embryo transfer [n (%)]	14 (88)	14 (93)	15 (94)	10 (77)	0.48
Embryos transferred	1.1 ± 0.27	1.1 ± 0.27	1.2 ± 0.41	1.0 ± 0.0	0.37*
SET[c] [n (% per embryo transfer)]	13 (93)	13 (93)	12 (80)	10 (100)	0.37
Total implantation rate[d] (%)	4/15 (27)	4/15 (27)	5/18 (28)	4/10 (40)	0.78
Patients with embryos cryopreserved	8 (50)	8 (53)	12 (75)	8 (62)	0.48
Cryopreserved embryos per patient	2.8 ± 3.7	2.2 ± 2.6	2.8 ± 2.4	3.2 ± 3.4	0.87
Positive hCG/cycle started	7 (44)	5 (33)	7 (44)	5 (39)	0.92
Clinical pregnancy/cycle started	4 (25)	4 (27)	6 (38)	4 (31)	0.87
Live births[e]/cycle started	4 (25)	4 (27)	4 (25)	4 (31)	0.98
Number of FER[f] cycles	6	7	8	2	
Positive hCG after FER/FER cycles	1 (17)	2 (29)	4 (50)	1 (50)	0.57
Live births after FER (n)[g]	1	1	3	1	
Cumulated no. of live births[h]/started 'fresh cycle'	5 (31)	5 (33)	7 (44)	5 (39)	0.89

Values are mean ± SD or number (column percentage). ANOVA or χ^2 test is used.
[a]Oocytes fertilized per intact oocyte retrieved.
[b]More than 1 cell Day 2 per intact oocyte aspirated.
[c]Single embryo transfer.
[d]Gestational sacs per embryos transferred.
[e]All fresh transfers were singletons.
[f]Frozen embryo replacement.
[g]In addition to these numbers, two more patients had a FER pregnancy after delivery.
[h]Two multiple pregnancies after FER.
*Non-parametric test.

cycle with 5 (31%), 3 (21%) and 4 (29%) live births, respectively (P = 0.83). Cumulated live birth rates including the frozen embryo replacement (FER) treatments were 6 (38%), 5 (36%) and 6 (43%) in the three groups, respectively (P = 0.92).

Serum endocrinology

Serum hormone levels are graphically shown in Fig. 2 and presented in Supplementary data, Table SI.

Human chorionic gonadotropin

Steady state level of s-hCG was reached on Day 6 of stimulation. On the day of hCG triggering, serum hCG levels (IU/l) were D0: <0.1, D50: 3.1 (2.6–3.6), D100: 5.5 (4.1–7.4) and D150: 11.0 (8.9–13.6) (P < 0.001).

Androstenedione

The levels of s-androstenedione increased significantly with higher doses of hCG (P < 0.01). The pre-ovulatory level was >2-fold higher after 150 IU/day (4.6 ng/ml; 3.5–6.1) of hCG compared with no hCG (2.1 ng/ml;1.6–2.6). The level in D100 was approximately twice as much as D0.

Estradiol

On the day of hCG, the two groups given the highest hCG doses had the highest s-E_2 levels. The peak pre-ovulatory levels were twice as high in D100 (12.8 nmol/l; 9.5–17.2) compared with D0 (6.3 nmol/l; 3.7–10.8) (P = 0.09).

Progesterone

Serum progesterone levels on the day of hCG triggering were significantly different between the treatment arms. The lowest serum progesterone was found in D0 group (0.72 ng/ml) and the highest (1.17 ng/ml) in the D150 group. The progesterone increments were calculated to elucidate the actual elevation of progesterone with respect to different baseline values. The increment was defined as the level on the day of hCG triggering in relation to stimulation Day one. The progesterone increments were D0 = 49, D50 = 79, D100 = 110 and D150 = 160% (P = 0.02).

With progesterone levels ≤1.5 versus >1.5 ng/ml on the day of hCG triggering, the live birth rate was 28% (15/53) and 20% (1/5) (P = 0.58), respectively. For patients without and with live birth, the mean s-progesterone level was 1.03 ± 0.59 versus 1.05 ± 0.38 ng/ml (P = 0.90).

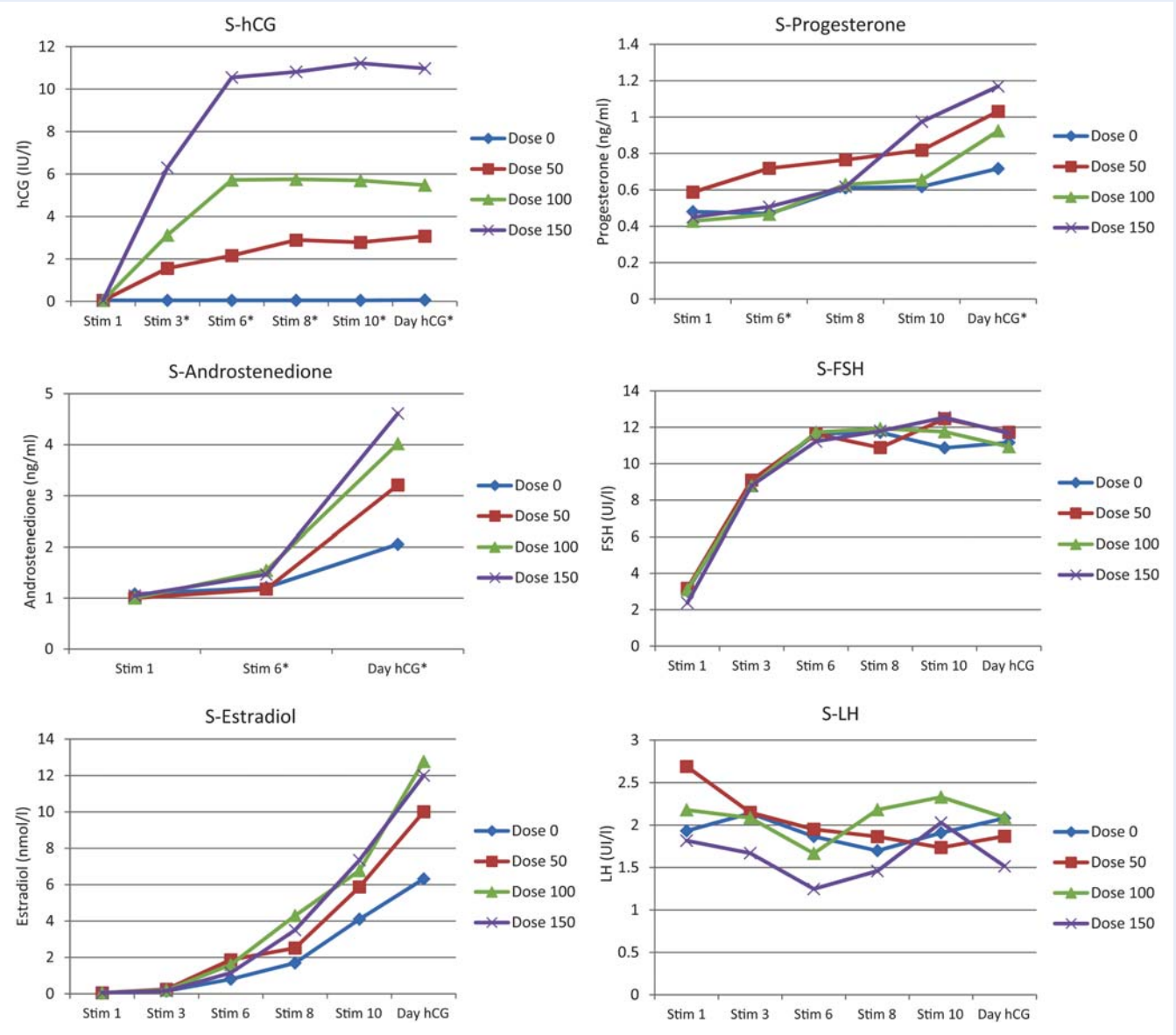

Figure 2 Serum endocrinology. Daily serum concentrations (means) of hCG, androstenedione, estradiol, progesterone, FSH and LH during the stimulation. *Statistical significance ($P < 0.05$) between the groups using ANOVA. Stim, stimulation day; Day hCG, the day of hCG triggering; 'S-, serum.

Gonadotrophins

The levels of FSH and LH did not vary between the groups.

Adverse reactions/events

OHSS was not seen in the two highest groups of hCG; however, one patient in group D0 was diagnosed with mild OHSS (outpatient) and one patient in D50 was admitted to hospital for 2 days because of moderate OHSS. Six days after embryo transfer, one patient in D50 was hospitalized for 2 days because of suspected ovarian torsion, but no operation was done. Two patients had transfer postponed to Day 5 because of abdominal pain, but the embryos were assessed on Day 3 as in other patients. The total number of pregnancy losses was five: two ectopic pregnancies (D50, D150) and three early miscarriages (D100).

Discussion

This is the first prospective randomized dose–response pilot study investigating the role of supplementation of hCG to rFSH from Day 1 of COS for IVF. Using the long agonist protocol for COS, we found a significant influence of hCG on the predefined primary end-point, the number of Day 3 top-quality embryos per patient, with the highest number in the group given 150 IU of hCG per day. Among the hCG-exposed patients, we also found an association between the mean number of top-quality embryos per patient and serum hCG

levels on Day 6 of stimulation. The live birth rates were good, even with a high daily hCG administration of 150 IU of hCG—a dose that far exceeds the levels of currently commercialized gonadotrophin preparations with a 1:1 ratio of FSH and LH activity.

By administering hCG from Day 1 of stimulation, repeated measurements during the follicular phase showed a consistent pattern of serum hCG levels, which increased from Day 1 to Day 6. From Day 6 of stimulation and onwards a plateau was found, and circulating hCG levels were around 3 IU/l after 50 IU/day, 6 IU/l after 100 IU/day and 11 IU/l after 150 IU/day of hCG.

In earlier studies where 200 IU hCG/day was given for a few final days of follicular maturation, serum levels of hCG continued to increase with a pre-ovulatory s-hCG around 8–9 IU/l (Filicori *et al.*, 2005; Blockeel *et al.*, 2009). In a long agonist protocol where HP-hMG doses of 225 IU per day provide a daily hCG dose of 30 IU of hCG (Wolfenson *et al.*, 2005), the resulting hCG level in serum on the last day of stimulation was 2.9 ± 1.2 IU/l (Andersen *et al.*, 2006). Using the same hCG assay, this serum level approaches our D50 level with an average of 3.1 ± 1.3 IU/l on the day of hCG triggering.

An additional aim of this study was to find a dose of hCG that would reach the ceiling level above which some of the parameters measured would be twisted into a negative direction. Estrogen secretion under a constant FSH tonus reflects LH bioactivity; at hCG doses above 100 IU/day, no further E_2 increase was measured. According to the 2-cell two-gonadotrophin concept, LH/hCG promotes androgen production and FSH stimulates the aromatization of androgen to estrogen in the granulosa cells. Hence, a clear dose-dependent response in serum androstenedione was observed with doses up to 150 IU/day of hCG. Estradiol (E_2), however, did not increase further with doses above 100 IU/day, indicating a saturated aromatase system. Peak pre-ovulatory E_2 levels were twice as high after 100–150 IU/day of hCG compared with no hCG administration. As the number of follicles was similar, it suggests that hCG induced more estrogenized follicles—a factor that could be linked to the observed improved embryo quality (Carson *et al.*, 1982; Andersen, 1993; Xia and Younglai, 2000). Hence, the better embryo quality obtained from oocytes from patients stimulated with hCG may be explained by the hCG supplementation.

The data show that the highest hCG dose group was associated with significantly higher pre-ovulatory progesterone concentrations. However, average progesterone levels remained below the critical level of 1.3 ng/ml (4.1 nmol/l), previously shown to decrease ongoing pregnancy rate (Ubaldi *et al.*, 1997; Andersen *et al.*, 2006; Bosch *et al.*, 2010). In a long agonist protocol, stimulation with HP-hMG resulted in lower pre-ovulatory progesterone level compared with stimulation with rFSH (Andersen *et al.*, 2006). This finding could be due to the hCG content of HP-hMG but the amounts of hCG were below the doses tested in the current study. It has been hypothesized that progesterone can be converted to androgens in theca cells and that hCG/LH can drive this conversion via 17-hydroxylase and as such reduce the progesterone concentration in the general circulation (Andersen *et al.*, 2006; Smitz *et al.* 2007; Bosch *et al.*, 2010; Fleming and Jenkins, 2010). It has also been demonstrated in the bovine model that aromatisable androgens can up-regulate the aromatase enzyme complex. Hence, the increased androgen pool by hCG treatment could improve the metabolization of the upstream steroid compounds in theca cells by driving aromatase (Luo and Wiltbank, 2006). As we observed a dose-dependent increase in progesterone level in this study, we propose that at hCG doses of 50 and higher the hCG-driven stimulation of LH receptors in granulosa is more pronounced than any possible hCG-driven increased capacity of the theca to metabolize progesterone through conversion into androgens.

Establishment of pregnancy requires a healthy embryo in combination with a receptive endometrium. As found in the present trial, hCG may promote top-quality embryos, but doses of hCG over 150 may also cause inappropriate advancement of the endometrium due to pre-ovulatory increased progesterone. However, we could not find evidence for impaired receptivity in our study. Even though addition of hCG seems to give increase progesterone, it may also increase the estrogen response, and the balance between estradiol and progesterone could be of importance for the endometrium receptivity. HCG may also have direct effects on the endometrium. In a recent study, Mansour *et al.* (2011) showed that administration of hCG 500 IU as an intrauterine injection before transfer significantly improved implantation and pregnancy rates.

To date, hCG supplementation of rFSH from the start of stimulation has been investigated in antagonist cycles only. One prospective, randomised, pilot study including 114 patients compared low-dose hCG (200 IU) with rLH (200 IU) added to rFSH only for the first 5 days of the ovarian stimulation in the short antagonist protocol (Drakakis *et al.*, 2009). The number of follicles and oocytes, as well as the implantation and pregnancy rates, were significantly higher in the hCG group. However, the overall implantation rate in this study was low, especially in the LH group. Another retrospective observational study investigated co-administration with hCG to rFSH throughout the stimulation (Van Horne *et al.*, 2007). Between 50 and 100 IU/l of hCG was administered concomitantly with rFSH in 94 GnRH antagonist cycles, and compared with cycles with only rFSH ($n = 96$). Low-dose hCG administration was associated with lower rFSH requirements, fewer oocytes and fewer embryos, but the group had higher implantation and pregnancy rates than the rFSH alone group. In our study, similar pregnancy rates were obtained in the four groups; however, with the limited sample size, our study was not designed to detect any differences in pregnancy or live birth rates. From combining those retrospective observational studies and the present trial, we conclude that hCG doses in the range 50–150 IU/day are consistent with good clinical responses and pregnancy rates, thereby challenging the possible benefits of 'LH free' rFSH preparations and the concept of 'LH-ceiling' (Hillier and Tetsuka, 1997; Shoham, 2002; Westergaard *et al.*, 2004).

In the latest Cochrane review (Mochtar *et al.*, 2007), the authors concluded that there were no improved pregnancy rates with rLH supplementation. Nevertheless, all pooled pregnancy estimates pointed towards a beneficial effect of co-treatment with rLH for patients with earlier pregnancy loss and for poor responders. The doses used varied from 37.5 to 150 IU/l rLH from stimulation Day 7 to the day of hCG administration. It is possible that despite the same bioactivity between hCG and LH in the rat seminal vesicle assay, the effects are different in humans because of differences in metabolisation. In contrast to earlier studies with LH supplementation, hCG in our study was administered from the start of the stimulation

and with hCG doses equivalent to or much higher than the LH doses previously tested.

The present study has several potential limitations. First, the sample size is limited. As the highest doses of hCG had not previously been used from the first day of stimulation, we aimed to assess the effects of this treatment in a small group before a larger trial was conducted. Second, the open-label design with clinicians and patients being aware of the treatment assignment is also a limitation of the study. However, scoring of the embryos was done in an assessor-blinded way, reducing the bias of the open-label design.

Our intention in this pilot study was not to give final conclusions and recommendations regarding future treatment practice, but rather to explore possible benefits of a changed treatment regime, which would have to be confirmed in a subsequent study. Hence, it is important for the planning of Stage 2 in the adaptive design to determine sample size and decide which groups to proceed with. As the primary end-point, the mean number of top-quality embryos, was highest in D150, one suggestion would be to continue with group D150 and the control group D0. Based on the differences in the mean number of top-quality embryos in D0 and D150 from 0.75 to 1.46, a Poisson power analysis showed that in order to detect such a difference with a power of 90% using a test level of 5%, each group should include 44 patients. However, considering an increased pre-ovulatory s-progesterone level and a possible saturated aromatase system, another alternative option could be to proceed with D100. Calculations on D0 versus D100 with mean numbers of top-quality embryos of 0.75 and 1.19, respectively, would necessitate inclusion of 103 patients in each group. The last alternative is our choice.

To conclude, adding hCG to rFSH in COS for IVF from the start of stimulation resulted in a significant increase in the number of top-quality embryos per patient and this increase was related to serum levels of hCG. The study showed a dose-dependent response throughout the hCG dose range regarding androstenedione levels, whereas a ceiling level of estradiol was reached with hCG doses above 100 IU/day. Supplementation of hCG induced a dose-related increase in the progesterone increments; nevertheless, the pregnancy rates in the four groups were similar.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

A.N.A., A.L., J.S. and L.L.T. were responsible for the study design. L.L.T. and A.N.E. were responsible for sample and data collection. L.L.T. and J.H.P. performed the data analyses. L.L.T. drafted the paper. All the authors provided critical discussion and manuscript review.

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Conflict of interest

None declared.

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