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Influence of ovarian stimulation with HP-hMG or recombinant FSH on embryo quality parameters in patients undergoing IVF

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BACKGROUND: There are limited data on the impact of different gonadotrophin preparations on embryo quality. METHODS: This evaluation was part of a randomized, assessor-blind, multinational trial, conducted in 731 women undergoing IVF after stimulation with highly purified human menopausal gonadotropin (HP-hMG; MENOPUR) (n = 363) or recombinant FSH (rFSH; GONAL-F) (n = 368). Ongoing pregnancy was the primary end-point [HP-hMG 27% and rFSH 22%; odds ratio (OR) (95% confidence interval, CI) 1.25 (0.89-1.75)]. All 7535 oocytes retrieved were evaluated daily until day 3 (embryo transfer) in a blinded manner both by local site embryologists and a central panel of three embryologists. RESULTS: The proportion of top-quality embryos per oocyte retrieved was higher with HP-hMG (11.3%) compared with rFSH (9.0%) (P = 0.044) in the local assessment, but comparable in the central assessment (9.5 and 8.0%, respectively). Significant differences in favour of HP-hMG were observed for number of blastomeres and degree of fragmentation, while uniformity of blastomere sizes, localization of fragments, frequency of multinucleation and homogeneous cytoplasm were comparable between HP-hMG and rFSH. The live birth, ongoing pregnancy and ongoing implantation rates for top-quality embryos were higher with HP-hMG than rFSH [48 versus 32% (P = 0.038), 48 versus 32% (P = 0.038), 41 versus 27% (P = 0.032)]. Both the proportion of embryos with at least 50% surviving blastomeres after cryopreservation and embryos resuming mitosis were more frequent with HP-hMG compared with rFSH. CONCLUSIONS: Composition of gonadotrophin preparations used during ovarian stimulation has an impact on some embryo quality parameters. The capacity to implant of the top-quality embryos derived from stimulation with HP-hMG appears to be improved, although the mechanism needs to be elucidated.

Keywords: embryo quality; IVF; rFSH; HP-hMG

Introduction

In recent years, the focus of ovarian stimulation has shifted from trying to obtain the maximum possible number of oocytes to trying to obtain an adequate cohort of good-quality embryos, i.e. from quantity to quality (Macklon and Fauser, 2003). It is well known that the quantitative aspects can be modulated by the doses of gonadotrophins, the type of gonadotrophin used and by the endocrine environment associated with stimulation (Sullivan *et al.*, 1999; Filicori *et al.*, 2001,2002; Loumaye *et al.*, 2003). In young women, high doses of gonadotrophins during stimulation are usually associated with an increased ovarian response. The ovarian stimulation regimen appears to have an effect on aneuploidy rate in embryos (Munné *et al.*, 1997; Katz-Jaffe *et al.*, 2005). Milder stimulation protocols are proposed to minimize the risk of aneuploidy (Baart *et al.*, 2007). In addition, administration of exogenous LH activity can modulate the magnitude of the follicular response (Sullivan *et al.*, 1999; Filicori *et al.*, 2001,2002;

Loumaye *et al.*, 2003) and perhaps impact the quality of the oocyte cohort (Platteau *et al.*, 2004; Lisi *et al.*, 2005).

Randomized controlled trials comparing gonadotrophin preparations have primarily focused on clinical aspects and have been designed to evaluate number of oocytes retrieved or to a lesser extent pregnancy rates. Despite the fact that embryo quality is considered an important predictor of implantation and pregnancy (Cummins *et al.*, 1986; Puissant *et al.*, 1987; Staessen *et al.*, 1992; Shulman *et al.*, 1993; Ziebe *et al.*, 1997; Thurin *et al.*, 2005), embryology aspects have practically been neglected in such trials. There is a need to understand if there are relevant differences in embryo quality parameters as result of the gonadotrophin preparation used for controlled ovarian hyperstimulation.

Many studies have shown that morphological structures in the embryo can be used as biomarkers of embryonic quality (Puissant *et al.*, 1987; Shulman *et al.*, 1993; Giorgetti *et al.*, 1995; Van Royen *et al.*, 1999) and that embryo selection based on morphology assessment is important to increase the percentage of chromosomally normal embryos (Ziebe *et al.*, 2003) and to improve implantation and pregnancy rates (Hill *et al.*, 1989; Erenus *et al.*, 1991; Giorgetti *et al.*, 1995; Ziebe *et al.*, 1997; Van Royen *et al.*, 1999,2001,2003). It is critical to consider the impact of individual quality parameters for construction of appropriate embryo grading systems. Most of the existing scoring systems are based on combinations of kinetics and several morphological parameters like number of cells, embryonic fragmentation and blastomere uniformity (Puissant *et al.*, 1987; Erenus *et al.*, 1991; Steer *et al.*, 1992; Giorgetti *et al.*, 1995). It has previously been demonstrated that the occurrence of early cleavage may be a good prognostic factor (Lundin *et al.*, 2001; Van Montfoort *et al.*, 2004) and that early-cleaved embryos of non-top quality had an improved implantation rate compared with late-cleaving embryos of the same quality (Lundin *et al.*, 2005). Furthermore, studies have shown that the competent embryos cleave at a certain rate (Ziebe *et al.*, 1997; Van Royen at al., 1999), different for ICSI or regular IVF (Nagy *et al.*, 1998), and that too slow or too fast cleavage both seem to indicate a compromised potential to develop to blastocysts (Alikani *et al.*, 2000) and to implant (Van Royen *et al.*, 2001). It has also previously been shown that the appearance of many fragments indicates a reduced developmental potential, while the presence of minor amounts of fragments is associated with high implantation and pregnancy rates, and no different from the rates associated with embryos without fragments (Staessen *et al.*, 1992; Ziebe *et al.*, 1997). The presence of unequally-sized blastomeres in the embryo may reflect either asymmetric distribution of cell material during cleavage or asynchrony in cleavage rate between blastomeres, resulting in an embryo composed of blastomeres from different cell generations. An increased rate of chromosomal abnormality has been shown in embryos containing unequally-sized blastomeres (Hardarson *et al.*, 2001; Ziebe *et al.*, 2003). Embryos with unequally-sized blastomeres have increased rates of multinuclearity and there is a coupling between blastomere size and multinuclearity (Hardarson *et al.*, 2001; Hnida *et al.*, 2004,2005). Multinucleated embryos have increased rates of chromosomal abnormalities (Kligman *et al.*, 1996; Balakier and Cadesky, 1997; Hardarson *et al.*, 2001) and transfer of embryos with multinucleated blastomeres is associated with decreased implantation, pregnancy and birth rates (Jackson *et al.*, 1998; Pelinck *et al.*, 1998; Van Royen *et al.*, 2003).

Several methodological aspects should be considered in embryo quality evaluation. To increase objectivity and to minimize bias, a systematic approach to embryo quality assessment should be implemented by using different assessors, multiple time points and pre-established definitions for each embryo quality parameter and grading. As embryo quality heavily depends on the kinetics, exact time points and strictly controlled timing interval for each embryo evaluation should also be predefined. The present investigation assessed the impact of two different gonadotrophin preparations with and without LH activity on individual embryo quality parameters as well as on the overall embryo quality grading using these considerations. The functional properties of these embryos were subsequently evaluated by assessing their ability to implant and to result in pregnancy and live birth. Furthermore, the extent of survival and the potential to implant in cryopreserved embryos were also evaluated. This evaluation was part of a large, randomized, assessor-blind study comparing highly purified human menopausal gonadotropin (HP-hMG) and recombinant FSH (rFSH) in women undergoing an IVF cycle with ongoing pregnancy rate as the primary end-point. The clinical outcome of this study and the endocrine profile in serum and follicular fluid have been the object of separate publications (Nyboe Andersen *et al.*, 2006; Smitz *et al.*, 2007).

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. The participating women 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 BMI before inclusion in the study was in the range 18–29 kg/m^2. Patients with polycystic ovary syndrome, endometriosis stage III/IV or partners with severe male factor 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 $\geq$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 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 five 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 four days. Choriongonadotrophin alfa, 250 μg s.c. (OVITRELLE; Serono), was administered to induce final follicular maturation within one day of observing three or more follicles of ≥17 mm diameter. Oocyte retrieval took place 36 ± 2 h after hCG administration. Oocytes were cultured individually (1 oocyte per well or per droplet) throughout culture, from the time of retrieval until the assessment on day 3, allowing for continued individual assessment of each oocyte/embryo. Information on culture media (type, batch and manufacturer) used from retrieval to transfer was documented. Cumulus mass appearance was assessed at oocyte retrieval. Insemination was done via regular IVF insemination (not ICSI) at 3 ± 1 h after oocyte retrieval. Fertilization was assessed at 20 ± 1 h, and embryo quality was assessed at 28 ± 1 h (day 1), 44 ± 1 h (day 2) and 68 ± 1 h (day 3) after insemination. Transfer of one or two embryos fulfilling at least the minimum-quality criteria (see definition in the following section) 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 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 overall study procedures and those specifically related to endocrine assessments of serum and follicular fluid have been described in detail elsewhere (Nyboe Andersen *et al.*, 2006; Smitz *et al.*, 2007).

Oocyte/embryo assessments

An atlas with representative pictures of all morphological parameters was prepared as a visual aid and distributed to all local embryologists before start of the clinical trial. Furthermore, a common training session with scoring of embryos and production of digital images was held with the responsible embryologist from each of the clinics participating in the trial.

Assessment of cumulus mass appearance was done by the local embryologist. The evaluation consisted of three components: cumulus mass layer, cumulus expansion and contact between cumulus cells and oocyte. Cumulus mass layer was assessed as ≤3, 4–9 or ≥10 layers. Cumulus expansion was assessed as tight/dense cells, moderate expansion, fully expanded or overexpanded. Contact between cumulus cells and oocyte was assessed as fully enclosed, incomplete covering ≥50%, incomplete covering <50% or naked.

Embryo quality was assessed daily by the local embryologists at the inverted microscope. The embryo quality parameters evaluated were the number of blastomeres (0, 1, 2, 3. . .. and compaction) and the following morphology aspects: degree of fragmentation [0, ≤10, 11–20, 21–50, >50% fragmentation, or totally fragmented (no blastomeres recognized)], localization of fragments (locally or dispersed), blastomere uniformity [equally sized or unequally sized (largest blastomere >25% larger in average diameter compared with the smallest blastomere)], visual sign of multinucleation (yes or no) and cytoplasmic appearance (homogeneous or dark, granulated, vacuolated). If there was only one blastomere, none of the embryo morphology parameters were assessed.

The overall classification of embryos was based on the individual embryo scoring parameters at different time points according to pre-established definitions. 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. A minimum-quality embryo (lowest quality allowed for transfer) was defined as four or more blastomeres on day 3 with no cleavage arrest (i.e. cleavage must have occurred within the last 24 h) and ≤20% fragmentation.

A representative picture of each embryo was taken at day 1, day 2 and day 3. A custom-made version of the commercially available software system FertiGRAB (IHMedical, Copenhagen, Denmark), which kept track of the images for each oocyte and time point, was used for processing and storage of the images. Based on these digital images, the number of blastomeres and embryo morphology at all three time points were assessed by a panel of three central embryologists. The central embryologists were blinded to each other's evaluation, the local embryologist's evaluation and treatment allocation. The central score for embryo quality was the majority decision or the median (in case all three central embryologists assessed numerical parameters differently). All embryos were evaluated by all three central embryologists. We have previously provided a detailed description of the methodology established for the central evaluation of embryos in this study (Arce *et al.*, 2006).

In addition to the local and central score, a consolidated score was constructed *a priori* as an overall embryo classification (top-quality, minimum-quality) based on the individual embryo parameter assessments, considering both the local and central assessments. The central embryologists's assessment on degree of fragmentation and blastomere uniformity, the local embryologists's assessment on cleavage stage and the central and/or local embryologist's assessment (worst case) on multinucleation were used for the consolidated score.

As follow-up of the study, the protocol specified to follow cryopreserved embryos for three years and to analyse and report the data after pre-defined time points of one year and three years after study completion. The 1-year data have been collected and are presented here. Cryopreservation and thawing were performed according to local site procedures, but it was requested to freeze embryos in individual cryostraws. For individually cryopreserved embryos, the number of blastomeres was assessed at thawing and comparison to the number of blastomeres at freezing was used as a measure for blastomere survival. Assessment at thawing and transfer were done by the local embryologist. Clinical aspects of the frozen embryo replacement cycles were according to local practice, with the only restriction of not mixing the cryopreserved embryos with embryos from a fresh cycle. Pregnancy and delivery information were collected for those patients who had transfer of cryopreserved embryos.

Statistical analysis

The primary end-point of the study was ongoing pregnancy per started cycle and the sample size calculations were based on the primary end-point (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.

Logistic regression models were applied for binary data, while continuous data were analysed using ANOVA models. All analyses were adjusted for age (<35 and ≥ 35 years) implying that all reported *P*-values and treatment comparisons are adjusted for age. Oocytes with three or more pronuclei were regarded as having fertilization failure and were excluded from all analyses of embryo quality. The individual embryo quality parameters (number of blastomeres and morphology) and the overall embryo classification were displayed for both the local and central embryologists. Embryo end-points on a patient level (e.g. oocytes retrieved, top-quality embryos) were presented descriptively among patients with oocyte retrieval. The analysis of ratios (e.g. top-quality embryos of oocytes retrieved) was based on patients with oocyte retrieval, while the difference between treatments in terms of other end-points was based on all patients in the intent to treat population.

Live birth, ongoing pregnancy and ongoing implantation were presented according to overall embryo classification for the consolidated score. The differences between treatments in live birth rate and ongoing pregnancy rate were estimated using a logistic regression model, while ongoing implantation was investigated using a generalized estimation equation method to account for the possible correlation between embryos from the same patients.

Ongoing implantation rates by overall embryo classification were calculated for patients with 0 or 100% ongoing implantation rate after single- or double-embryo transfer.

Mean blastomere survival rate was calculated based on the survival rate for each thawed embryo. Cryosurvival data were analysed using a logistic regression model adjusting for age.

Results

Of the 731 (HP-hMG 363, rFSH 368) patients who initiated controlled ovarian hyperstimulation in the study, 691 (HP-hMG 344, rFSH 347) patients underwent oocyte retrieval of whom 688 had oocytes retrieved. From these patients, a total of 7535 oocytes were obtained and inseminated via regular IVF procedures: 3454 in the HP-hMG group and 4081 in the rFSH group ($P < 0.001$). On average, 10.0 oocytes were retrieved in a patient stimulated with HP-hMG and 11.8 oocytes in a patient exposed to rFSH ($P < 0.001$). The flow of oocyte/embryo assessments is illustrated in Fig. 1.

Number of cumulus mass layers, degree of cumulus expansion and contact between cumulus cells and oocyte were comparable between the HP-hMG and rFSH groups. The proportion of cumulus-oocyte complexes with ≥ 10 layers of

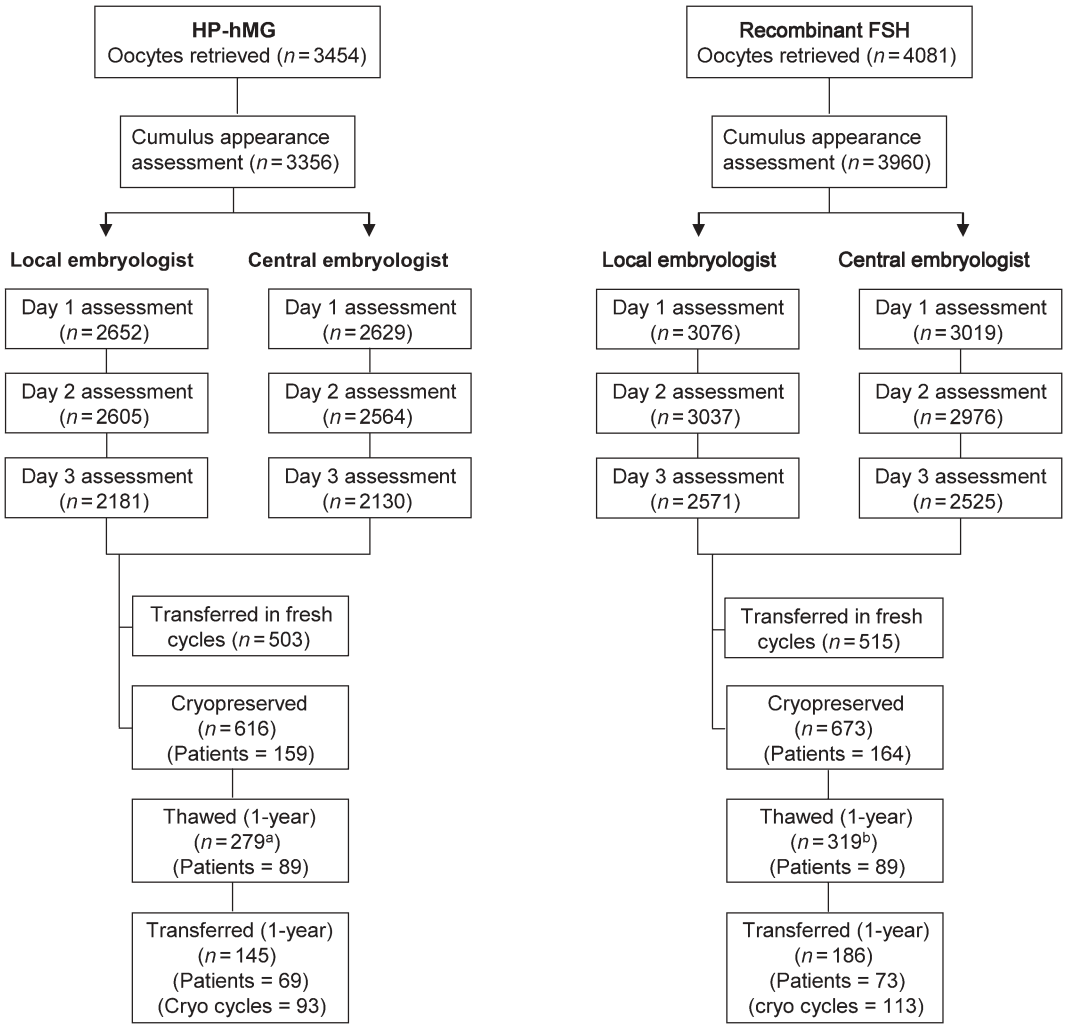

Figure 1: Study flow chart and disposition of oocytes/embryos by study evaluation
[a] Number of blastomeres at freezing and thawing was assessed for $n = 237$ embryos. [b] Number of blastomeres at freezing and thawing was assessed for $n = 300$ embryos

cumulus cells (HP-hMG 59%, rFSH 56%) or 4–9 layers (HP-hMG 34%, rFSH 39%) were the most frequent observations in both groups, compared with complexes with ≤3 layers (HP-hMG 6%, rFSH 5%). Fully expanded cumulus (HP-hMG 54%, rFSH 55%) and moderate expansion (HP-hMG 37%, rFSH 35%) were the most common recordings, followed by tight dense cells (HP-hMG 8%, rFSH 8%) and overexpansion (HP-hMG 2%, rFSH 2%). Most of the cumulus-oocyte complexes were fully enclosed (HP-hMG 92%, rFSH 91%). The fertilization rate was 51.6% with HP-hMG and 52.5% with rFSH ($P = 0.650$). The number (mean ± SD) of oocytes with 2-pronuclei at 20 h post-insemination was 5.1 ± 4.0 and 6.0 ± 4.3 for HP-hMG and rFSH, respectively ($P = 0.003$). The distribution of culture media according to manufacturer was similar for the two treatment groups: Medicult (HP-hMG 53%, rFSH 56%), Cook (HP-hMG 20%, rFSH 20%) and Vitrolife (HP-hMG 16%, rFSH 15%) (other manufacturers were each used for <1–8% of the embryos).

Embryo quality parameters

Table 1 presents the individual embryo parameters by treatment group, as assessed by both the local and central embryologists. The number of blastomeres on day 2 was significantly higher with HP-hMG compared with rFSH. On day 3, the number of blastomeres in the HP-hMG group was borderline significantly higher than in the rFSH group according to the local observer and significantly higher according to the central embryologists. Among the embryo morphology parameters, a significantly higher proportion of oocytes in the HP-hMG group had ≤20% fragmentation compared with rFSH, both on day 2 and day 3. There were no significant differences between HP-hMG and rFSH with respect to proportion of equally-sized blastomeres, localization of fragments, multinucleation or homogenous cytoplasm.

The total number of embryos on day 3 was 6.3 ± 4.7 in the HP-hMG group, which was significantly lower than the 7.4 ± 5.0 in the rFSH group. However, there was no significant difference in the proportion of embryos available or embryos of minimum quality on day 3 per oocytes retrieved. The number of top-quality embryos was similar in both groups, with the proportion of top-quality embryos per oocytes retrieved significantly higher in the HP-hMG group (11.3%) compared with the rFSH group (9.0%) for the local assessors (Table 2). The observations by the central embryologists were in the same direction (HP-hMG 9.5 versus rFSH 8.0%), but significance was not reached. Based on the consolidated assessment, the proportion of patients who had top-quality embryos was 48% for HP-hMG and 44% for rFSH.

The highest proportion of top-quality embryos was found among oocytes that at retrieval had more than three cumulus mass layers and moderate or fully expanded cells. For oocytes with more than three cumulus mass layers, 11.2% (353/3147) in the HP-hMG group and 9.4% (354/3754) in the rFSH group developed into top-quality embryos [age-adjusted OR 1.21 (1.04; 1.42), $P = 0.016$] and for oocytes with moderate or fully expanded cells the frequency

Table 1: Individual embryo quality parameters (local and central assessment)

	Local embryologists				Central embryologists			
	HP-hMG	rFSH	Difference/OR [95% CI]	*P*-value	HP-hMG	rFSH	Difference/OR [95% CI]	*P*-value
Day 2 (44 ± 1 h post-insemination)								
Within subject rate of ≥4 cells (%)	33.0 ± 26.6	29.7 ± 24.1	3.31 [−0.47; 7.09]	0.086	30.2 ± 25.7	26.6 ± 23.5	3.65 [−0.01; 7.32]	0.051
Number of blastomeres	2.78 ± 1.56	2.70 ± 1.51	0.08 [0.00; 0.16]	0.048	2.68 ± 1.52	2.56 ± 1.49	0.12 [0.04; 0.19]	0.005
Equally-sized blastomeres	60% (1072/1791)	61% (1260/2065)	0.95 [0.84; 1.09]	0.465	65% (1121/1723)	67% (1288/1936)	1.06 [0.93; 1.22]	0.382
≤20% fragmentation	83% (1488/1798)	77% (1604/2070)	1.40 [1.19; 1.64]	<0.001	84% (1468/1754)	81% (1611/1997)	1.24 [1.05; 1.46]	0.014
Localization of fragments: locally	52% (760/1469)	51% (887/1744)	1.04 [0.90; 1.19]	0.610	34% (422/1247)	35% (513/1453)	0.94 [0.80; 1.10]	0.427
No multinucleation	92% (1640/1792)	92% (1892/2065)	0.98 [0.78; 1.23]	0.859	96% (1649/1723)	96% (1849/1936)	0.96 [0.70; 1.31]	0.777
Homogenous cytoplasm	86% (1545/1791)	85% (1759/2065)	1.10 [0.92; 1.32]	0.314	98% (1688/1723)	98% (1897/1936)	0.99 [0.63; 1.58]	0.979
Day 3 (68 ± 1 h post-insemination)								
Within subject rate of ≥7 cells (%)	21.9 ± 22.9	20.2 ± 21.2	1.73 [−1.55; 5.02]	0.300	17.4 ± 20.2	15.9 ± 19.2	1.56 [−1.38; 4.50]	0.299
Number of blastomeres	5.08 ± 2.58	4.93 ± 2.64	0.14 [−0.01; 0.29]	0.062	4.68 ± 2.46	4.44 ± 2.60	0.23 [0.09; 0.38]	0.002
Equally-sized blastomeres	58% (1042/1806)	55% (1134/2075)	1.14 [1.00; 1.29]	0.050	57% (974/1715)	56% (1082/1945)	0.95 [0.84; 1.09]	0.486
≤20% fragmentation	79% (1427/1816)	73% (1518/2091)	1.39 [1.20; 1.61]	<0.001	80% (1402/1762)	76% (1554/2039)	1.23 [1.05; 1.43]	0.010
Localization of fragments: locally	36% (549/1537)	35% (635/1820)	1.04 [0.90; 1.20]	0.606	15% (194/1336)	17% (258/1557)	0.86 [0.70; 1.05]	0.137
No multinucleation	94% (1698/1797)	94% (1943/2058)	1.01 [0.76; 1.33]	0.968	99% (1690/1715)	99% (1916/1945)	0.98 [0.57; 1.68]	0.943
Homogenous cytoplasm	75% (1354/1806)	76% (1583/2076)	0.94 [0.81; 1.09]	0.398	93% (1595/1713)	94% (1824/1945)	0.89 [0.69; 1.16]	0.405

Table 2: Early cleavage and embryo quality classifications (local and central assessment)

	Local Embryologists				Central Embryologists			
	HP-hMG	rFSH	Difference [95% CI]	*P*-value	HP-hMG	rFSH	Difference [95% CI]	*P*-value
Oocytes retrieved	10.0 ± 5.4	11.8 ± 5.7	−1.6 [−2.5; −0.8]	<0.001	–	–		–
Early-cleaved embryos	1.6 ± 2.2	1.7 ± 2.3	−0.12 [−0.44; 0.20]	0.469	1.6 ± 2.1	1.7 ± 2.3	−0.09 [−0.40; 0.23]	0.597
Early-cleaved embryos/ oocytes retrieved	17.3%	14.9%	2.47 [−0.58; 5.51]	0.112	16.7%	14.3%	2.46 [−0.53; 5.44]	0.107
Top-quality embryos	1.1 ± 1.6	1.1 ± 1.6	−0.01 [−0.24; 0.23]	0.937	0.9 ± 1.3	0.9 ± 1.5	−0.05 [−0.25; 0.15]	0.623
Top-quality embryos/oocytes retrieved	11.3%	9.0%	2.25 [0.06; 4.44]	0.044	9.5%	8.0%	1.49 [−0.55; 3.53]	0.151
Minimum-quality embryos	3.6 ± 3.4	3.8 ± 3.4	−0.20 [−0.69; 0.30]	0.432	3.5 ± 3.4	3.8 ± 3.5	−0.28 [−0.78; 0.23]	0.279
Minimum-quality embryos/ oocytes retrieved	36.4%	33.9%	2.50 [−1.23; 6.23]	0.188	34.2%	32.7%	1.49 [−2.26; 5.25]	0.435

Table 3: Clinical outcome by quality of the transferred embryos (consolidated assessment)

	HP-hMG	rFSH	OR [95% CI]	*P*-value
Live birth rate				
Per started cycle	26% (96/363)	22% (82/368)		
Transfer of only top-quality embryos	48% (36/75)	32% (24/74)	2.04 [1.04; 4.05]	0.038
Transfer of non-top-quality embryos	27% (60/223)	25% (58/229)	1.03 [0.68; 1.58]	0.883
Ongoing pregnancy rate				
Per started cycle	27% (97/363)	22% (82/368)		
Transfer of only top-quality embryos	48% (36/75)	32% (24/74)	2.04 [1.04; 4.05]	0.038
Transfer of non-top-quality embryos	27% (61/223)	25% (58/229)	1.06 [0.69; 1.61]	0.801
Ongoing implantation rate				
Overall	24% (119/503)	20% (102/515)		
Transfer of only top-quality embryos	41% (44/107)	27% (30/112)	2.02 [1.14; 6.60]	0.032
Transfer of non-top-quality embryos	19% (75/396)	18% (72/403)	1.03 [0.72; 1.48]	0.889

of top-quality embryos was 11.2% (340/3043) for HP-hMG and 9.6% (342/3554) for rFSH [age-adjusted OR 1.18 (1.01; 1.38), $P = 0.041$].

Clinical outcome according to embryo quality

Embryo transfer was performed in 298 patients in the HP-hMG group and 303 patients in the rFSH group. The total number of embryos transferred was 503 in HP-hMG treated patients and 515 in rFSH-treated patients. Transfer of only top-quality embryos was done in 25 and 24% of the cycles with transfers in the HP-hMG and rFSH groups, respectively. Among the transfers with only top-quality embryos, single-embryo transfers accounted for 57% in the HP-hMG group and 49% in the rFSH group. Table 3 displays the clinical outcome by the quality of the transferred embryos. The live birth, ongoing pregnancy and ongoing implantation rates when transferring only top-quality embryos were significantly higher in the HP-hMG group compared with the rFSH group with a live birth rate of 48 versus 32%, an ongoing pregnancy rate of 48 versus 32% and an ongoing implantation rate of 41 versus 27%. For non-top-quality embryos, the live birth rate was 27% for HP-hMG and 25% for rFSH, the ongoing pregnancy rate was 27 versus 25%, and the ongoing implantation rate was 19 versus 18%, respectively.

Cryopreservation

On day 3, the number of embryos cryopreserved per patient with oocyte retrieval was comparable between groups: 1.8 ± 2.8 in the HP-hMG group and 1.9 ± 2.9 in the rFSH group ($P = 0.463$). As illustrated in Fig. 1, the 1-year follow-up of the cryopreserved embryos included data for 178 patients (HP-hMG 89, rFSH 89) who had embryos thawed in the specified time period, of whom 142 patients (HP-hMG 69, rFSH 73) had underwent 206 embryo transfer cycles (HP-hMG 93, rFSH 113) using 331 embryos (HP-hMG 145, rFSH 186) (Fig. 1).

Table 4: Summary of cryopreserved embryos (local assessment)

	HP-hMG	rFSH	Difference/OR	*P*-value
Mean blastomere survival	79%	74%	5.89 [−0.19; 11.96]	0.058
Embryos with 100% surviving blastomeres	64% (151/237)	58% (173/300)	1.30 [0.91; 1.84]	0.145
Embryos with ≥50% surviving blastomeres	83% (196/237)	75% (226/300)	1.59 [1.04; 2.45]	0.033
Embryos with resumption of mitosis following thawing[a]	93% (56/60)	79% (62/78)	3.95 [1.22; 12.8]	0.022

[a]analysed only for embryos where transfer took place one or more days after thawing.

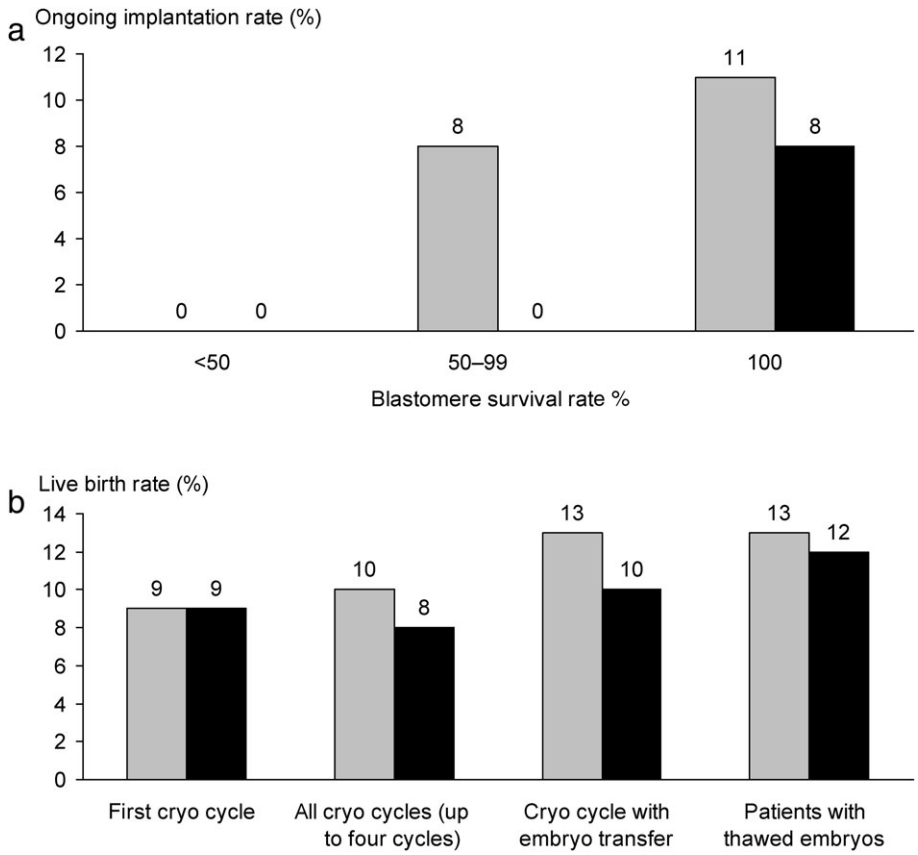

Figure 2: Summary of clinical outcome of the cryopreserved cycles in the HP-hMG (light bar) and rFSH (dark bar) groups (**a**) ongoing implantation rate by blastomere survival rate of the transferred embryos and (**b**) live birth rate for the first cryo cycle, live birth rate for all cryo cycles, live birth rate by cryo cycle with embryo transfer and live birth rate by patients with thawed embryos

After the 1-year of follow-up, a total of 178 patients (HP-hMG 87, rFSH 91) still have frozen embryos left. The embryo cryosurvival data after the first year are summarized in Table 4. The mean blastomere survival rate was 79% in the HP-hMG group and 74% in the rFSH group. The percentage of embryos with 100% survival rate (all blastomeres intact at thawing) was 64% in the HP-hMG group and 58% in the rFSH group. At thawing, the frequency of embryos with $\geq$50% surviving blastomeres was 83% in the HP-hMG group, which was significantly higher than the 75% in the rFSH group. Among the embryos that were cultured for at least one day after thawing (HP-hMG 60, rFSH 78), the percentage of embryos with resumption of mitosis before transfer was significantly higher with 93% for HP-hMG compared with 79% for rFSH (Table 4). The ongoing implantation rate by blastomere survival rate is illustrated in Fig. 2. The ongoing implantation rate for embryos with 100% survival rate was 11% for HP-hMG and 8% for rFSH. No implantation occurred with embryos with $<$50% blastomere survival (HP-hMG 1, rFSH 12).

A total of 206 frozen-thawed embryo transfers in 142 patients were performed during the 1-year follow-up. The number of embryos transferred in a cryo cycle was 1.6 ± 0.5 in the HP-hMG group and 1.6 ± 0.6 in the rFSH group. However, as there were more cryo cycles with transfer of thawed embryos in the rFSH group, the number of embryos transferred per patient was numerically higher with rFSH than with HP-hMG; 2.5 ± 1.6 embryos versus 2.1 ± 1.1 embryos, respectively. The clinical outcome of the cryo cycles in terms of ongoing implantation rate and live birth rate is summarized in Fig. 2. In the first cryo cycle, the live birth rate was 9% in both the HP-hMG and rFSH groups. The live birth rate in the cryo cycles with embryo transfer was 13% for HP-hMG and 10% for rFSH.

Discussion

The present clinical study represents the first attempt to provide a comprehensive and systematic evaluation of embryo quality in patients undergoing ovarian stimulation with two different gonadotrophin preparations, following a similar stimulation protocol and similar pre-randomization and post-randomization procedures. The findings suggest that stimulation with gonadotrophins containing LH activity positively impacts embryo developmental competence, as detected by subtle changes in cleavage rates and morphological features associated with good prognostic outcome. There is a need for dissociating oocyte quality from quantity and for identifying treatment strategies that optimize embryo quality and subsequently evaluate the impact on treatment outcome. In the present study, despite the fact that more oocytes were retrieved after stimulation with rFSH, the number of top-quality embryos was the same in the two groups. Thus, a higher percentage of

top-quality embryos were obtained per oocyte retrieved from stimulation with HP-hMG. The subgroup of top-quality embryos obtained from HP-hMG-treated women were associated with increased ongoing implantation and pregnancy rates and live birth rate compared with those derived from rFSH cycles. The data from this study suggest a differential endocrine environment associated with ovarian stimulation using HP-hMG versus rFSH (Smitz *et al.*, 2007) inducing an oocyte cohort of less quantity, but with some indication of improved embryo morphology and implantation of those embryos of the best quality.

The embryo quality parameters used in the present study are well known and reflect the developmental potential of the embryo. Among the indicators used in this study to define embryo quality, number of blastomeres on day 2 and day 3 post-insemination and degree of fragmentation showed significant differences in favour of stimulation with HP-hMG. The most relevant difference is the ~5% higher proportion of embryos with fragmentation ≤20% in the HP-hMG group compared with the rFSH group, as fragmentation above 20% has been shown to reduce developmental potential (Staessen *et al.*, 1992; Ziebe *et al.*, 1997). A previous investigation classified embryos as having no fragments, <20% fragmentation or >20% fragmentation and found no difference between the implantation rate of the embryos with no or <20% fragmentation, while embryos with >20% fragmentation had a reduced implantation rate (Staessen *et al.*, 1992).

The integration of these individual embryo quality parameters into the constructed definitions of different embryo gradings (i.e. top-quality embryo, etc) were established prior to initiation of the study. The algorithms for these embryo classifications were consistent for both local and central embryo evaluations. We have previously shown that embryo quality classifications can be determined with a good to excellent degree of interobserver agreement independently of whether microscope or digital images are used (Arce *et al.*, 2006). As number of blastomeres on day 2 and 3 as well as fragmentation ≤20% were included in the definition of top-quality embryo, the higher proportion of top-quality embryos in the HP-hMG group can be explained by the numerically higher proportion of ≥4-cells on day 2 and ≥7-cells on day 3, and the significantly higher percentage of embryos with fragmentation ≤20%. It is interesting to note that differences in quality aspects were noted already at an early stage, as the proportion of top-quality embryos developed from cumulus-oocyte complexes with more than three cumulus mass layers and from complexes with moderate or fully expanded cells was higher in the HP-hMG group compared with the rFSH group. The findings from the 1-year follow-up on the cryopreserved embryos are supportive of the conclusions from the fresh cycles. More embryos in the HP-hMG group survived thawing with >50% of the blastomeres intact compared with the rFSH group, and also more embryos in the HP-hMG group resumed mitosis between thawing and transfer. Resumption of mitosis has previously been associated with increased implantation and pregnancy potential (Ziebe *et al.*, 1998). The 1-year follow-up data presented here cover around half of the cryopreserved embryos. A 3-year follow-up expected to include the majority of the remaining cryopreserved embryos is planned, facilitating cumulative results.

The mechanisms for how LH activity could mediate improvements in some oocyte/embryo quality parameters in IVF cycles are not fully understood. Cumulus cells are considered an ideal surrogate for assessment of oocyte development potential (McKenzie *et al.*, 2004). It is speculated that a set of cumulus genes may determine oocyte maturation, fertilization potential and embryo quality (McKenzie *et al.*, 2004). There are data from sibling human oocytes suggesting that the quality of embryos improve when oocytes are allowed to interact with cumulus cells, indicative of an improvement of cytoplasmic maturation (Hassan, 2001). Cumulus cells have been suggested to have a protective and beneficial effect on embryo development (Magier *et al.*, 1990). Recent gene expression data have provided some molecular evidence for a mediation of the cumulus cells in embryo development (Assou *et al.*, 2006). It has been proposed that LH activity might influence the cumulus cells surrounding the oocyte (Platteau *et al.*, 2004), affecting the oocyte-cumulus interactions, the cytoplasmic maturation of the oocyte and its competency development. Cumulus cell gene expression may provide a direct assessment of fertility potential and a measure of embryo quality.

The data from the present study showed an increased proportion of top-quality embryos in the HP-hMG group, and also that the top-quality embryos derived from stimulation with HP-hMG were associated with an improved capacity to implant, establish a pregnancy and result in a live birth compared with top-quality embryos in the rFSH group. Increased implantation and/or pregnancy rates after addition of LH activity to FSH compared with FSH alone have previously been reported (Gordon *et al.*, 2001; Lisi *et al.*, 2005). The difference found in ongoing implantation and pregnancy rates and live birth rate in the present study for the subgroup of top-quality embryos could perhaps also be attributed to differences in other embryo morphological aspects than those evaluated in this trial, to embryo metabolic/functional parameters and/or to a difference in the endometrial receptivity between groups. Actually, in this study there were more patients with an unfavourable endometrium in the rFSH group compared with the HP-hMG group (Nyboe Andersen *et al.*, 2006). The higher proportion of patients with hyperechogenic endometrium was attributed to the higher progesterone level in response to stimulation with rFSH (Nyboe Andersen *et al.*, 2006). Independently of the mechanism, the present study shows that in order to improve the results of controlled ovarian hyperstimulation there is a need to better understand the impact of different types of gonadotrophins on morphological/functional aspects of embryo quality, as well as the embryo's potential to implant in a well-characterized endometrium.

In conclusion, differences in some morphology quality parameters were found between embryos derived from women stimulated with HP-hMG and rFSH. Evaluation of the clinical outcome by quality of the embryos transferred suggested an improved implantation, ongoing pregnancy and live birth rate among the top-quality embryos derived from stimulation with HP-hMG compared with rFSH. Elucidation of whether this is related to embryo functionality not captured by morphology

assessment, or to endometrial aspects requires further investigation. Confirmation of the mechanism is expected to contribute to optimization of treatment regimens and protocols.

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