Article

Mid-follicular LH supplementation in women aged 35–39 years undergoing ICSI cycles: a randomized controlled study

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Abstract

This single-centre, randomized, parallel group, comparative study aimed to identify potential benefits of mid-follicular recombinant human LH (r-HLH) supplementation in women aged 35–39 years undergoing ovarian stimulation for intracytoplasmic sperm injection (ICSI). The main endpoint was the number of metaphase II oocytes retrieved. After pituitary suppression with a gonadotrophin-releasing hormone agonist, ovarian stimulation was initiated with recombinant human FSH (r-HFSH; 300–450 IU/day). On stimulation day 6, patients were randomized to receive r-HFSH alone or r-HFSH + r-HLH (r-HLH 150 IU/day) for the remainder of the stimulation period. Final follicular maturation was triggered with 250 µg of recombinant human chorionic gonadotrophin. After assessing oocyte nuclear maturity, oocyte were fertilized by ICSI and afterwards embryo quality was analyzed. Of the 131 women enrolled, 68 were allocated to r-HFSH alone and 63 to r-HFSH + r-HLH. No significant differences were observed in markers of either oocyte or embryo quality or quantity. However, higher rates of implantation and live birth per started cycle were observed with r-HLH supplementation than with r-HFSH alone. Although additional large studies are required to further investigate these findings, r-HLH supplementation for women aged 35–39 years undergoing ICSI is recommended as it may have a beneficial action on implantation.

Keywords: *ICSI, implantation, metaphase II, pregnancy, r-HFSH, r-HLH*

Introduction

The role of LH during ovarian stimulation in IVF is controversial (Griesinger *et al.*, 2005). There is no doubt that endogenous LH is necessary for follicular development in the natural cycle. It has been demonstrated that both FSH and LH are needed for optimal follicular development in women with hypogonadotrophic hypogonadism (European Recombinant Human LH Study Group, 1998). In the remaining cases of ovarian stimulation, follicular growth may not necessarily require exogenous LH administration. It has been speculated that this could be due to the endogenous production of small amounts of LH, even when a gonadotrophin-releasing hormone (GnRH) analogue regimen is used. Despite this, it has been suggested that in certain patient subgroups, such as in women of advanced reproductive age, exogenous LH administration could lead to more optimal follicular development and therefore an increased pregnancy rate (Alviggi *et al.*, 2006).

Previous reports on this topic have revealed conflicting outcomes. Some studies have been suggestive of a beneficial role for LH, while others report no differences in outcomes when LH is added to the treatment regimen. Many of the discrepancies in the results of these studies may be due to the heterogeneity of the studies in terms of design, sample

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size, inclusion criteria, GnRH analogue protocol, LH administration (recombinant human LH (r-HLH), human menopausal gonadotrophin (HMG), dose, beginning, duration) and general IVF procedures. In a recent meta-analysis among unselected patients treated with FSH and GnRH analogues for IVF, the addition of r-HLH had no beneficial effect (Kolibianakis *et al.*, 2007). In a recent Cochrane review, however, it was shown that LH supplementation was associated with a significantly higher pregnancy rate in poor responders (Mochtar *et al.*, 2007). Additionally, in some studies performed in women aged over 35 years undergoing intracytoplasmic sperm injection (ICSI), r-HLH administration has been associated with improved outcomes (Humaidan *et al.*, 2004; Marrs *et al.*, 2004). Contrary to these findings, in other studies with similar designs, such a beneficial effect was not evident (Fábregues *et al.*, 2006). Complicating the picture further, other studies report a similar lack of benefit in unselected populations when a GnRH antagonist was employed (Cédrin-Durnerin *et al.*, 2004; Griesinger *et al.*, 2005), but in women aged over 35 years, a benefit of LH supplementation was demonstrated (Bosch *et al.*, 2008).

While live birth rate represents the most important outcome when evaluating therapeutic strategies in IVF, using live birth rate as an endpoint would require a very large sample size (for instance, for a 5% difference in live birth rate and a 90% power, 3253 patients would be needed). Patient numbers such as these would simply not be feasible for the majority of individual centres. Additionally, there are many factors within an IVF clinic that can have an impact upon live birth rates, including the patient population studied. As an alternative, the selection of certain oocyte characteristics such as the number of metaphase II (MII) oocytes could be considered as an endpoint. In this context, the analysis of oocyte characteristics can be properly performed only in ICSI, not in conventional IVF, because in IVF the cumulus is intact, thus not allowing for a full assessment of the oocytes.

The aim of this study was to determine, by means of a prospective, randomized, controlled study, whether r-HLH supplementation in women aged 35–39 years undergoing ICSI is associated with an improvement in the number and maturity of oocytes retrieved when compared with patients receiving r-HFSH alone. A number of additional endpoints were also considered, including pregnancy rates.

Materials and methods

The study was a randomized, open-label, controlled, prospective clinical trial in infertile women aged 35–39 years, who were eligible for the ICSI programme at the institution (EudraCT number 2004-001503-36). The study was approved by the local ethics committee, authorized by the competent authority (PROT25186) and conducted in accordance with good clinical practice guidelines. All patients provided written consent.

The population under investigation was comprised of 131 consecutive couples undergoing ICSI at the treatment unit between January 2005 and November 2006. The women were normo-ovulatory (cycle length 25–35 days) and were scheduled to undergo ovarian stimulation in a long agonist protocol.

Inclusion criteria were: (i) body mass index between 18 and 30 kg/m^2; (ii) baseline FSH $\leq$10 IU/l; (iii) baseline LH and oestradiol within the normal range for the institution; (iv) the presence of both ovaries and uterine cavity capable of sustaining a pregnancy; (v) clomiphene or gonadotrophin wash out $\geq$30 days prior to starting GnRH agonist; (vi) confirmed absence of pregnancy; and (vii) signed informed consent. Patients were excluded if they met any of the following criteria: (i) human immunodeficiency virus or hepatitis B virus/hepatitis C virus positive; (ii) clinically significant condition preventing them from undergoing gonadotrophin treatment; (iii) more than two previous assisted cycles; (iv) cancellation of two previous cycles; (v) cryopreserved embryos available from previous assisted reproduction treatment; (vi) unexplained gynaecological bleeding; (vii) polycystic ovary or an ovarian cyst of unknown aetiology; (viii) pregnancy contraindication; (ix) active substance abuse; (x) simultaneous participation in another trial or re-entry in the current trial; and (xi) refusal or inability to comply with the procedures set forth in the protocol.

Hormonal analysis was performed using an ADVIA Centaur XP Automated Chemiluminescence System (Bayer, Siemens, Germany). The sensitivity and variability coefficient were 10% and 13.6% for oestradiol, 0.07% and 2.7% for LH, and 0.3% and 3.9% for FSH, respectively. No LH determinations were performed during the stimulation cycle.

Treatment for all patients was initiated with a GnRH agonist (triptorelin acetate; Decapetyl, Ipsen, Madrid) on day 20–22 of the preceding cycle, at a dose of 0.1 mg/day. Once pituitary desensitization was documented (oestradiol values <30 pg/ml and absence of follicles >10 mm), the GnRH agonist dose was decreased by half and ovarian stimulation was initiated. According to the standard protocol at the study centre, ovarian stimulation began with 300–450 IU of r-HFSH (follitrophin alpha; GONAL-f, Merck Serono, Spain) at a fixed dose until day 6 of stimulation (S6). On S6, women were randomized, by means of a computer-generated number list contained in sealed envelopes, into one of two groups. The control group continued with r-HFSH alone, whereas the study group also received supplemental r-HLH (lutrophin alpha; Luveris, Merck Serono), 150 IU/day, subcutaneously until the end of ovarian stimulation. This dose of r-HLH was selected according to the protocol of Marrs *et al.* (2004). In both groups, after S6, the r-HFSH dose was adjusted, if needed.

For all patients, final follicular maturation and ovulation were triggered when there were at least three follicles with a mean diameter $\geq$18.5 mm with 250 *g* of recombinant human chorionic gonadotrophin (r-HCG; choriogonadotrophin alpha; Ovitrelle®, Merck Serono S.A.). Oocyte retrieval was performed 36 h after the administration of r-HCG.

All ICSI procedures and assessments were performed by RM and AE from the investigational team. The cumulus corona

cell complexes were scored under an inverted microscope at ×100 magnification. When an oocyte–cumulus-complex was found, the stage of maturity was assessed by noting the volume, density and condition of the surrounding coronal and cumulus cells, according to published criteria (Veeck, 1988) and classified into one of four categories: (i) mature; (ii) slightly immature; (iii) completely immature; or (iv) slightly hyper-mature. The oocytes were then incubated for 2 h. Immediately prior to micromanipulation for the ICSI procedure, the cumulus corona cells were removed and each oocyte was examined under the microscope to assess the maturation stage and integrity. MII oocytes were defined by the absence of the germinal vesicle and the presence of an extruded polar body. Fertilized oocyte scoring involved a careful analysis of the pronuclei (PN) and the nucleoli within the nuclei from single observation 16–18 h following fertilization. Embryo morphology was determined by the number, size and shape of blastomeres, the proportion of fragments and the presence of multinucleated blastomeres, as per the recommendations of Veeck (1988).

Embryo transfer was performed on day 2–3 after HCG administration under ultrasound guidance (as previously reported) (Matorras *et al.*, 2002, 2004). The majority of transfers were performed on day 2, although some transfers were performed on day 3 if this offered a better selection of embryos. Internal embryo transfer policy allowed the transfer of up to three embryos, when available. The luteal phase was supplemented with micronized progesterone (Utrogestan; Laboratorios Seid, Spain), vaginally 200 mg/12 h. Fourteen days after embryo transfer, a β-HCG determination was obtained and considered positive if the value was greater than 10 mIU/ml. Clinical pregnancy was defined as the visualization of a gestational sac 6 weeks after embryo transfer. Pregnancy outcomes, including pregnancy loss or live birth were also documented.

The main efficacy endpoint was the number of MII oocytes retrieved. Secondary endpoints included the number and size of developed follicles, serum oestradiol concentration at the end of stimulation, the number of days of stimulation, the cumulative r-HFSH dose, the cancellation rate, the number of oocytes retrieved, the number of 2PN fertilized zygotes, fertilization rate, embryo number and grading, the number of embryos transferred, implantation rate, clinical pregnancies, delivery rate and clinical complications.

The study was designed as a superiority trial. To perform the power calculation, it was assumed, based on previous experience, that 75% of retrieved oocytes would be MII oocytes, thus a mean of seven MII oocytes would be recovered in the control (r-HFSH-alone) group. The study was designed to have a power of 80% to detect a significant difference of 20% in the number of MII oocytes retrieved and provided for a significance level of 0.05. The resulting calculation required a total of 124 enrolled patients, 62 of whom who would be randomized to the control group with the same number to be assigned to the experimental group.

The statistical plan defined the analysis as an intention-to-treat (ITT) analysis, which means that subjects were analysed according to their treatment allocation and not the treatment actually received. A second, per protocol (PP), analysis was also conducted and this included patients strictly adhering to the protocol. The Kolmogorov–Smirnov test was used for non-categoric variables; for normally distributed parameters, analysis of variance (ANOVA)s was then applied, whereas for non-normally distributed data, the Mann–Whitney *U*-test was applied. For categoric parametric variables and for the proportion analysis, the chi-squared test was used. The statistical significance limit was defined as alpha = 0.05.

Results

Patient disposition and populations

Of the 138 women screened, 131 were considered eligible for enrollment. Of these, 68 were randomized to receive r-HFSH alone and 63 patients were randomized to receive r-HFSH plus mid-follicular r-HLH (r-HFSH + r-HLH). The patient disposition for the trial is presented in **Figure 1**. Seven patients were excluded during the selection process: five chose not to participate in the study, one did not meet the inclusion criteria and one failed to start ovarian stimulation.

Three patients were excluded from the PP analysis due to protocol violations prior to randomization: one patient entered the study with a baseline FSH of 13 mIU/ml (not reaching pregnancy); a second patient was not included because their starting dose of FSH was lower than specified by the protocol (patient error: 37.5 IU during 6 days; cancelled by low response); and a third patient was not included because the starting dose of r-HFSH during the first 4 days of stimulation was only 150 IU (triplet pregnancy). All three of these patients were in the group that received r-HFSH alone.

In two other cases, the randomization allocation was not followed. One patient who was assigned to the r-HFSH + r-HLH group received only r-HFSH. The results for this patient, which resulted in a pregnancy, were included in the r-HFSH + r-HLH group for the ITT analysis and in the r-HFSH-alone group for the PP analysis. In another case, the opposite happened. The treatment assigned was r-HFSH alone, but the patient received r-HFSH + r-HLH (no pregnancy). This patient was included in the r-HFSH-alone ITT analysis and in the r-HFSH + r-HLH PP analysis.

Homogeneity of groups

The majority of the baseline demographic characteristics of enrolled women and their male partners were similar in both groups (**Table 1**). However, in the r-HFSH-alone group, the mean ± SD basal LH concentrations assessed on cycle day 3 (prior to stimulation) were higher (10.5 ± 10.3 mIU/ml) than the LH concentrations in the r-HFSH + r-HLH group (8.0 ± 8.4 mIU/ml); this difference was statistically significant ($P = 0.04$). There was also a trend towards higher mean ± SD baseline oestradiol values in the r-HFSH-alone group than in the r-HFSH +

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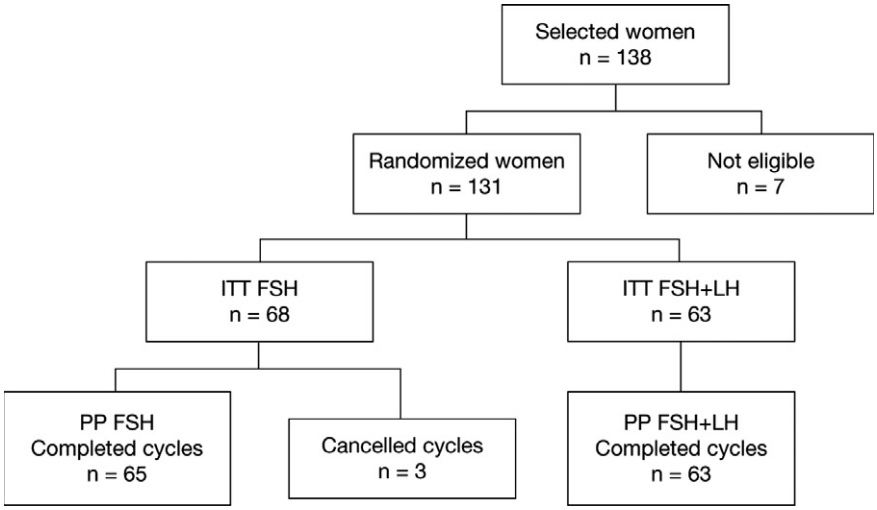

Figure 1. Flow chart of patient participation in the trial. ITT = intention-to-treat; PP = per protocol.

Table 1. Baseline characteristics of the two study groups.

Characteristic	*r-HFSH* (n = *68*)	*r-HFSH + r-HLH* (n = *63*)	P-*value*
Age (years)	36.7 ± 1.5	36.6 ± 1.6	NS
BMI (kg/m^2)	22.6 ± 2.7	22.7 ± 2.8	NS
Infertility duration (years)	4.4 ± 2.3	4.7 ± 2.4	NS
Basal plasma FSH (mIU/ml)	5.7 ± 2.4	5.1 ± 1.9	NS
Basal plasma LH (mIU/ml)	10.5 ± 10.3	8.0 ± 8.4	0.04
Basal plasma oestradiol (pg/ml)	126.0 ± 149.1	60.9 ± 81.0	NS
No. previous children (%)	50 (73.5)	51 (80.9)	NS
Aetiology (%)			
Tubal factor	3 (4.4)	2 (3.2)	NS
Male factor	31 (45.6)	31 (49.2)	NS
Endometriosis	3 (4.4)	1 (1.6)	NS
Mixed cause	22 (32.4)	11 (17.5)	NS
Unknown cause	9 (13.2)	18 (28.6)	NS
No. of previous IVF/ICSI cycles (%)			
0	34 (50.0)	30 (47.6)	NS
1	21 (30.9)	23 (36.5)	NS
2	13 (19.1)	10 (15.9)	NS
Sperm parameters			
Volume (ml)	3.5 ± 4.5	3.7 ± 2.4	NS
Concentration (10^6/ml)	47.7 ± 31.7	50.9 ± 31.1	NS
Mobile forms (%)	17.2 (13.6)	20.1 (13.4)	NS

Values are mean ± SD or *n* (%). BMI = body mass index; ICSI = intracytoplasmic sperm injection; NS = not statistically significant; r-HFSH = recombinant human FSH; r-HLH = recombinant human LH.

r-HLH group (126.0 ± 149.1 versus 60.9 ± 83.3, respectively); however, this difference was not significant ($P = 0.06$).

There were no follicular, endometrial or hormonal differences between the groups at the start of stimulation or at the time of randomization on day S6 (**Table 2**).

Cycle parameters at the end of ovarian stimulation

There were also no differences between the two groups regarding the mean days of agonist treatment, the mean dose or duration of r-HFSH stimulation or in the mean number of follicles on the day of HCG. The mean number

Table 2. Comparison of cycle parameters in both groups at the start of stimulation, at randomization and at the end of stimulation.

Characterisic	*r-HFSH (n = 68)*	*r-HFSH + r-HLH (n = 63)*
At the start of stimulation		
Follicles >10 mm	0.05 (0.2)	0.1 (0.4)
Endometrial thickness (mm)	1.8 (3.4)	0.6 (1.5)
Plasma oestradiol (pg/ml)	21.9 (8.8)	21.2 (6.5)
At randomization (S6)		
Follicle size (mm)		
>10 and ≤14	4.0 (3.6)	4.0 (3.2)
>14 and ≤18	0.7 (2.0)	0.6 (1.6)
>18	0.0 (0.1)	0.1 (0.4)
Plasma oestradiol (pg/ml)	265 (341)	259 (397)
Endometrial thickness (mm)	5.9 (3.2)	6.0 (3.2)
At the end of stimulation		
GnRH agonist (days)	26.6 (5.7)	25.6 (5.1)
Duration of ovarian stimulation (days)	11.7 (2.1)	11.4 (2.4)
Cumulative r-HFSH dose (IU)	3962 (1288)	4039 (1246)
On day of HCG follicle size (mm)		
≥10 and <14	2.6 (2.8)	2.3 (2.1)
≥14 and <16 mm	2.1 (1.9)	2.2 (2.0)
≥16 and <18 mm	2.7 (2.1)	2.3 (2.0)
≥18 mm	3.7 (3.0)	4.0 (3.0)

Values are mean (SD). No significant differences were observed between the two groups.
GnRH = gonadotrophin-releasing hormone; HCG = human chorionic gonadotrophin; r-HFSH = recombinant human FSH; r-HLH = recombinant human LH; S6 = day 6 of stimulation.

of follicles per patient and follicular size are presented in **Table 2**. The results of the PP analysis for these parameters were similar (data not shown).

Oocyte characteristics

A similar mean ± SD number of oocytes were retrieved in the r-HFSH and r-HFSH + r-HLH treatment groups (8.9 ± 4.9 versus 8.3 ± 4.7, respectively (**Table 3**). The mean ± SD number of MII oocytes (primary endpoint) was also similar in the r-HFSH and r-HFSH + r-HLH groups (7.0 ± 4.8 versus 6.7 ± 4.1, respectively). In addition, no significant differences in these outcomes were observed between the two groups in the PP analysis (data not shown).

Fertilization rate and embryo characteristics

The number of inseminated oocytes was similar in both groups, as was the fertilization rate (0.48 ± 0.25 versus 0.49 ± 0.26) and also the number of embryos in the different assigned grades (**Table 4**). In addition, the number of transferred embryos was almost identical in both groups (2.1 ± 1.2 versus 2.3 ± 1.1). The proportion of patients with day-2 and day-3 transfers was similar in both groups (73.8% and 26.2% for the r-HFSH-alone group versus 73.0% and 27.0% for the r-HFSH + r-HLH group, respectively). The PP analysis also revealed no significant differences between the two groups (data not shown).

Cycle cancellations

There were three cycle cancellations, all of which occurred in the group receiving r-HFSH alone (3/68) with none occurring in the group receiving r-HFSH + r-HLH (4.4% versus 0%, not significant). Two of these cancellations were because of poor response and one cancellation was due to the potential risk for ovarian hyperstimulation syndrome.

Pregnancy outcomes

Table 5 presents the data on pregnancy rates and outcomes for the two treatment groups. In the ITT analysis, the clinical pregnancy rate per started cycle was 14.7% (10/68) in the r-HFSH-alone group and 27.0% (17/63) in the r-HFSH + r-HLH group. The clinical pregnancy rates per transfer were 18.5% (10/54) in the r-HFSH-alone group and 30.4% (17/56) in the r-HFSH + r-HLH group. The implantation rate was 11.3% (16/141) in the r-HFSH-alone group, which was significantly lower than the implantation rate in the r-HFSH + r-HLH group (18.1% [26/144], $P = 0.049$).

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Table 3. Recovered oocytes and follicles after ovarian stimulation.

	r-HFSH (n = 68)	*r-HFSH + r-HLH (n = 63)*
Retrieved oocytes	8.9 (4.9)	8.3 (4.7)
Follicles >10 mm on day of HCG	11.5 (5.6)	11.0 (5.5)
Oocyte retrieval rate	0.71 (0.35)	0.71 (0.33)
Oocyte maturity		
Metaphase II oocytes	7.0 (4.8)	6.7 (4.1)
Metaphase I oocytes	0.7 (1.1)	0.7 (1.3)
Germinal vesicle oocytes	0.4 (0.8)	0.7 (1.1)
Atretic oocytes	0.3 (0.8)	0.1 (0.3)

Values are mean (SD). No significant differences were observed between the two group.
GnRH = gonadotrophin-releasing hormone; HCG = human chorionic gonadotrophin; r-HFSH = recombinant human FSH; r-HLH recombinant human LH.

Table 4. IVF cycle data.

	r-HFSH (n = 68)	*r-HFSH + r-HLH (n = 63)*
Inseminated oocytes	7.0 (4.8)	6.6 (3.9)
Fertilized oocytes (2PN)	3.3 (2.6)	3.1 (2.3)
Fertilization rate	0.49 (0.26)	0.48 (0.25)
Grade 1 embryos	1.3 (1.4)	1.1 (1.1)
Grade 2 embryos	1.2 (1.1)	1.1 (1.5)
Grade 3 embryos	0.5 (0.9)	0.5 (0.6)
Grade 4 embryos	0.2 (0.6)	0.3 (0.5)
Grade 5 embryos	0.2 (0.6)	0.3 (1.0)
Transferred embryos	2.1 (1.2)	2.3 (1.1)

Values are mean ± (SD). No significant differences were observed between the two groups.
2PN = 2 pronuclei; r-HFSH = recombinant human FSH; r-HLH recombinant human LH.

Table 5. Pregnancy rates and outcomes.

	r-HFSH (n = 68)	*r-HFSH + r-HLH (n = 63)*	P-*value*[a]
ITT population analysis			
Positive β-HCG test	16.2 (11/68)	28.6 (18/63)	NS
Clinical pregnancy rate	14.7 (10/68)	27.0 (17/63)	NS
Clinical pregnancy rate per transfer	18.5 (10/54)	30.4 (17/56)	NS
Implantation rate	11.3 (16/141)	18.1 (26/144)	0.049
Live birth rate per started cycle	7.4 (5/68)	19.0 (12/63)	0.047
Live birth rate per transfer	9.3 (5/54)	21.4 (12/56)	NS
PP population analysis			
Positive β-HCG test	18.5 (12/65)	25.4 (16/63)	NS
Clinical pregnancy rate	16.9 (11/65)	25.4 (16/63)	NS
Clinical pregnancy rate per transfer	21.2 (11/52)	28.6 (16/56)	NS
Implantation rate	10.0 (14/140)	17.4 (24/138)	NS
Live birth rate per started cycle	7.7 (5/65)	17.5 (11/63)	NS
Live birth rate per transfer	9.6 (5/52)	19.6 (11/56)	NS

Values are percentage (number/total).
HCG = human chorionic gonadotrophin; ITT = intention-to-treat; NS = not statistically significant; PP = per protocol; r-HFSH; recombinant human FSH; r-HLH = recombinant human LH.
[a]Chi-squared test.

Similar trends were observed following analysis of PP data as for the ITT population. The clinical pregnancy rate was 16.9% (11/65) in the r-HFSH-alone group versus 25.4% (16/63) in the r-HFSH + r-HLH group. The clinical pregnancy rate per embryo transfer was 21.2% (11/52) and 28.6% (16/56) in the r-HFSH-alone and r-HFSH + r-HLH groups, respectively. The PP implantation rate was 10.0% (14/140) in the r-HFSH-alone group versus 17.4 (24/138) in the r-HFSH + r-HLH group.

There were four multiple pregnancies in the r-HFSH-alone group (two twin and two triplet pregnancies) compared with seven in the r-HFSH + r-HLH group (five twin and two triplet pregnancies). Thus, multiple pregnancies represented 40% and 41% of pregnancies in the r-HFSH-alone and r-HFSH + r-HLH groups, respectively.

In the ITT analysis, the live birth rate per started cycle was significantly lower in the r-HFSH-alone group (7.4%) than in the r-HFSH + r-HLH group (19.0%) ($P = 0.047$), while the live birth rates per transfer were 9.3% versus 21.4%, respectively. In the PP analysis, there was also a trend to higher live birth rates in r-HFSH + r-HLH group, but statistical significance was not reached.

In terms of clinical pregnancy outcomes, in the r-HFSH-alone group, there were three singleton deliveries, one twin delivery, one triplet delivery (as previously described, this pregnancy was excluded from the PP analysis), three miscarriages/immature deliveries and two ectopic pregnancies. One of the triplets spontaneously reduced into a twin pregnancy and delivery. One twin pregnancy resulted in a miscarriage. In the r-HFSH + r-HLH group, there were eight singleton deliveries, four twin deliveries and five miscarriages/immature deliveries. The two triplet pregnancies spontaneously reduced into twin pregnancies and deliveries. Three twin pregnancies resulted in miscarriage/immature delivery.

Safety

There were no serious adverse events reported by patients in the r-HFSH group. In the r-HFSH + r-HLH group, there was one case of severe ovarian hyperstimulation syndrome and one case of pelvic inflammatory disease. Both patients required hospital admission but cases resolved without sequelae after medical treatment.

Discussion

The aim of this prospective, randomized, controlled study was to determine whether r-HLH supplementation in women aged 35–39 years undergoing ICSI is associated with an improvement in the number and maturity of oocytes retrieved when compared with patients receiving r-HFSH alone. No differences were observed regarding the conventional markers of oocyte quality or quantity associated with r-HLH supplementation in women aged 35–39 years undergoing ICSI, nor were there any differences in embryo quality or quantity. Interestingly, in spite of the similarities in embryo parameters between the two groups, there was a lower implantation and live birth rate in patients treated with r-HFSH alone relative to patients receiving r-HFSH + r-HLH.

The role of LH in ovarian stimulation in assisted reproduction treatment remains controversial. LH administration has been shown to have beneficial effects in some indications such as hypogonadotrophic hypogonadism (Couzinet *et al.*, 1988; European Recombinant Human LH Study Group, 1998). However, the benefits of LH are less clear in other indications. Many of the differences in results between studies are due to their heterogeneity: study design (retrospective or prospective), sample size, inclusion criteria (unselected population, poor response to stimulation, IVF or ICSI), GnRH analogue protocol (agonist or antagonist), LH administration (r-HLH, HMG, dose, start and duration of treatment) or IVF protocols (number of transferred embryos). Other differences include the endpoints analysed, such as oocytes, embryos, pregnancy rates or newborns.

It has been shown, in retrospective studies, that low concentrations of circulating follicular phase LH in women undergoing GnRH-agonist long-protocol cycles might be associated with impaired oestradiol synthesis and/or a low oocyte yield as well as low fertilization rates, low pregnancy rates and high miscarriage rates (Westergaard *et al.*, 2000; Esposito *et al.*, 2001; Humaidan *et al.*, 2002). However, in prospective randomized trials (Humaidan *et al.*, 2004; Marrs *et al.*, 2004; Fábregues *et al.*, 2006; Barrenetxea *et al.*, 2008), r-HLH supplementation for ovarian stimulation in long agonist protocols was not associated with better outcome in IVF. In addition, a number of interventional trials using an antagonist protocol and supplemental r-HLH failed to demonstrate a positive effect of this measure on the numbers of oocytes retrieved and resultant embryos or the rates of implantation and clinical pregnancy (Cédrin-Durnerin *et al.*, 2004; Griesinger *et al.*, 2005). However, it has been shown that the combination of r-HLH and GnRH antagonists in women at risk of poor ovarian response is associated with a higher number of MII oocytes than the standard flare-up protocol using GnRH agonists (De Placido *et al.*, 2006).

Whereas it has been shown in some studies performed in unselected populations that r-HLH supplementation is not associated with improved outcomes (Lisi *et al.*, 2002; Tarlatzis *et al.*, 2006), controversy still exists concerning the effect of supplemental LH in ovarian ageing and ICSI cycles. It has been suggested previously that, although LH administration may be of no benefit in IVF, LH administration could be associated with improved results in women aged over 35 years undergoing ICSI (Marrs *et al.*, 2004). Contradicting this are the results from another study, targeted at women aged over 35 years undergoing IVF–ICSI, where a benefit was not apparent (Fábregues *et al.*, 2006). No benefit was documented in another study in women aged over 40 years undergoing IVF–ICSI (Barrenetxea *et al.*, 2008). Finally, in a recent report in women undergoing IVF–ICSI under antagonist protocol, better results were obtained in those aged 35–39 years (Bosch *et al.*, 2008).

However, a number of differences have been reported in relation to LH administration on different parameters

reflecting follicular maturation. In antagonist cycles, higher concentrations of oestradiol have been reported in patients treated with r-HFSH and r-HLH supplementation than in patients treated with r-HFSH alone (Griesinger *et al.*, 2005). In agonist cycles, LH supplementation has been associated with a reduction in total FSH consumption (Humaidan *et al.*, 2004), shorter ovarian stimulation (Fábregues *et al.*, 2006) and an increase in small antral follicles and normally fertilized embryos (Durnerin *et al.*, 2008). It has also been suggested that the effect of exogenous LH depends on the concentrations of endogenous LH (Tesarik and Mendoza, 2002).

This study was designed based around two specific characteristics: (i) women aged 35–39 years, where a beneficial effect of LH has been suggested previously; and (ii) inclusion of only ICSI cycles, where oocyte characteristics could be accurately documented and analysed. The study failed to show a difference in the markers of follicular growth, oocyte maturity or embryo quality. Both the primary endpoint (the number of MII oocytes) and other outcomes analysed (including the cumulative dose of r-HFSH and oestradiol concentrations) were similar in both groups. Thus, from a quantitative point of view, no beneficial effect of r-HLH administration was observed in follicular maturation or oocyte quality. Furthermore, embryo quality (according to the categories analysed) seemed to be identical in both groups. It must be highlighted that this study also focused on live birth rates, the most important outcome in IVF studies, although not considered in a number of trials.

Interestingly, this study provided pregnancy outcome data that suggested a favourable effect of r-HLH supplementation. Although the study was underpowered to detect a significant difference in pregnancy outcomes, this increase in pregnancy rate is considered to be clinically relevant. Furthermore, the rates of implantation and live birth per started cycle in the ITT population were significantly higher in the r-HFSH + r-HLH than in the r-HFSH-alone group. Although significance was not reached in the pregnancy rate per embryo transfer or in the PP analyses, similar trends toward better outcomes in the r-HFSH + r-HLH than in the r-HFSH-alone group were observed.

In the interpretation of the data it should not be overlooked that, although the study was randomized, there were some subtle differences in the hormonal profile between both groups. The r-HFSH-alone group had a higher mean basal LH value (which could have a favourable influence) together with a trend to higher mean oestradiol value (which could have an adverse effect). Taking into account the randomized nature of the study, the magnitude of the differences and their opposing actions, it seems unlikely that the hormonal profile had a relevant influence on the study outcome. On the other hand, it must be acknowledged that this study did not include a placebo arm, unlike the multicentre study in unselected women of Tarlatzis *et al.* (2006), which showed no benefits with the use of r-HLH. In another study with an unselected population, which had a similar design, no differences were found either in the general population or in the subgroup of women aged over 35 years (Nyboeandersen *et al.*, 2008). However, unlike this study, the report was not specifically powered towards women over 35 years of age and it included both IVF and ICSI cases, which may explain the discrepancy between results.

When the results from patients included in this study were compared with those of other patients from the study centre in the same age group, who received a similar high gonadotrophin dose as well as LH activity during stimulation but who were not included in the study, a much lower cancellation rate was noted due to low response in the present study. This is probably related to the fact that the main endpoint of this study was the number and quality of oocytes. Compared with the patients not included in the study, pregnancy rates per started cycle were lower in the group receiving r-HFSH alone and were in the normal range for the group receiving r-HFSH + r-HLH.

The apparent beneficial effect of r-HLH on implantation and pregnancy outcomes in this population could be explained by two different mechanisms. Firstly, the embryo quality could be superior in the group supplemented with r-HLH. LH receptors have been found in bovine oocytes, embryos and blastocysts (Mishra *et al.*, 2003). Recent data from a retrospective study indicate that long-protocol LH-supplemented ovarian stimulation improves embryonic ploidy compared with pure FSH stimulation (Weghofer *et al.*, 2008). It is quite possible that the conventional, subjective, visual morphology scoring used in this study may not be adequate to identify embryos with best implantation potential. Secondly, there may be an effect of LH supplementation on the endometrium, which could promote implantation. Endometrial effects of LH have been demonstrated in animals (Shemesh, 2001; Mishra *et al.*, 2003; Blitek and Ziecik, 2005) and also in humans (Reshef *et al.*, 1990).

In summary, this study failed to show an improvement in oocyte quality or quantity, as assessed by conventional markers, with r-HLH supplementation over r-HFSH alone in women aged 35–39 years undergoing ICSI. However, significantly higher rates of implantation and live birth per started cycle were observed and there was a trend toward higher clinical pregnancy rates in those women receiving r-HLH supplementation. Although further studies are clearly required, LH supplementation for women aged 35–39 years undergoing ICSI is recommended as it may have a beneficial action on implantation.

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References

Alviggi C, Clarizia R, Mollo A *et al.* 2006 Who needs LH in ovarian stimulation? *Reproductive BioMedicine Online* **12**, 599–607.

Barrenetxea G, Agirregoikoa JA, Jiménez MR *et al.* 2008 Ovarian response and pregnancy outcome in poor-responder women: a

randomized controlled trial on the effect of luteinizing hormone supplementation on in-vitro fertilization cycles. *Fertility and Sterility* **89**, 546–553.

Blitek A, Ziecik AJ 2005 Effect of LH on prostaglandin F2alpha and prostaglandin E2 secretion by cultured porcine endometrial cells. *Reproduction* **130**, 105–112.

Bosch E, Labarta E, Simón C *et al.* 2008 The impact of luteinizing hormone supplementation in gonadotropin releasing hormone antagonist cycles. An age adjusted randomized trial. *Fertility and Sterility* **90**(suppl. 1), S41.

Cédrin-Durnerin I, Grange-Dujardin D, Laffy A *et al.* 2004 Recombinant human LH supplementation during GnRH antagonist administration in IVF/ICSI cycles: a prospective randomized study. *Human Reproduction* **19**, 1979–1984.

Couzinet B, Lestrat N, Brailly S 1988 Stimulation of ovarian follicular maturation with pure follicle-stimulating hormone in women with gonadotropin deficiency. *Journal of Clinical Endocrinology and Metabolism* **66**, 552–556.

De Placido G, Mollo A, Clarizia R *et al.* 2006 Gonadotropin-releasing hormone (GnRH) antagonist plus recombinant luteinizing hormone vs a standard GnRH agonist short protocol in patients at risk for poor ovarian response. *Fertility and Sterility* **85**, 247–250.

Durnerin CI, Erb K, Fleming R *et al.* 2008 Effects of recombinant LH treatment on folliculogenesis and responsiveness to FSH stimulation. *Human Reproduction* **23**, 421–426.

Esposito MA, Barnhart KT, Coutifaris C, Patrizio P 2001 Role of periovulatory luteinizing hormone concentrations during assisted reproductive technology cycles stimulated exclusively with recombinant follicle-stimulating hormone. *Fertility and Sterility* **75**, 519–524.

European Recombinant Human LH Study Group 1998 Recombinant human luteinizing hormone (LH) to support recombinant human follicle-stimulating hormone (FSH)-induced follicular development in LH- and FSH-deficient anovulatory women: a dose finding study. *Journal of Clinical Endocrinology and Metabolism* **83**, 1507–1514.

Fábregues F, Creus M, Peñarrubia J *et al.* 2006 Effects of recombinant human luteinizing hormone supplementation on ovarian stimulation and the implantation rate in down-regulated women of advanced reproductive age. *Fertility and Sterility* **85**, 925–931.

Griesinger G, Schultze-Mosgau A, Dafopoulos K *et al.* 2005 Recombinant luteinizing hormone supplementation to recombinant follicle-stimulating hormone induced ovarian hyperstimulation in the GnRH-antagonist multiple-dose protocol. *Human Reproduction* **20**, 1200–1206.

Humaidan P, Bungum M, Bungum L, Yding Andersen C 2004 Effects of recombinant LH supplementation in women undergoing assisted reproduction with GnRH agonist down-regulation and stimulation with recombinant FSH: an opening study. *Reproductive BioMedicine Online* **8**, 635–643.

Humaidan P, Bungum M, Bungum L, Andersen C 2002 Ovarian response and pregnancy outcome related to mid-follicular LH levels in women undergoing assisted reproduction with GnRH agonist down-regulation and recombinant FSH stimulation. *Human Reproduction* **17**, 2016–2021.

Kolibianakis EM, Kalogeropoulou L, Griesinger G *et al.* 2007 Among patients treated with FSH and GnRH analogues for in-vitro fertilization, is the addition of recombinant LH associated with the probability of live birth? A systematic review and meta-analysis. *Human Reproduction Update* **13**, 445–452.

Lisi F, Rinaldi L, Fishel S *et al.* 2002 Use of recombinant LH in a group of unselected IVF patients. *Reproductive BioMedicine Online* **5**, 104–108.

Marrs R, Meldrum D, Muasher S *et al.* 2004 Randomized trial to compare the effect of recombinant human FSH (follitropin alfa) with or without recombinant human LH in women undergoing assisted reproduction treatment. *Reproductive BioMedicine Online* **8**, 175–182.

Matorras R, Mendoza R, Expósito A, Rodríguez-Escudero FJ 2004 Influence of the time interval between embryo catheter loading and discharging on the success of IVF. *Human Reproduction* **19**, 2027–2030.

Matorras R, Urquijo E, Mendoza R *et al.* 2002 Ultrasound-guided embryo transfer improves pregnancy rates and increases the frequency of easy transfers. *Human Reproduction* **17**, 1762–1766.

Mishra S, Lei ZM, Rao ChV 2003 A novel role of luteinizing hormone in the embryo development in cocultures. *Biological Reproduction* **68**, 1455–1462.

Mochtar MH, Vander Veen, Ziech M, vanWely M 2007 Recombinant luteinizing hormone (rLH) for controlled ovarian hyperstimulation in assisted reproductive cycles. *Cochrane Database of Systematic Reviews* 2007, CD005070.

Nyboeandersen A, Humaidan P, Fried G *et al.* 2008 Recombinant LH supplementation to recombinant FSH during the final days of controlled ovarian stimulation for in-vitro fertilization. A multicentre, prospective, randomized, controlled trial. *Human Reproduction* **23**, 427–434.

Reshef E, Lei ZM, Rao CV *et al.* 1990 The presence of gonadotropin receptors in nonpregnant human uterus, human placenta, fetal membranes, and decidua. *Journal of Clinical Endocrinology and Metabolism* **70**, 421–430.

Shemesh M 2001 Actions of gonadotrophins on the uterus. *Reproduction* **121**, 835–842.

Tarlatzis B, Tavmergen E, Szamatowicz M *et al.* 2006 The use of recombinant human LH (lutropin alfa) in the late stimulation phase of assisted reproduction cycles: a double-blind, randomized, prospective study. *Human Reproduction* **21**, 90–94.

Tesarik J, Mendoza C 2002 Effects of exogenous LH administration during ovarian stimulation of pituitary down-regulated young oocyte donors on oocyte yield and developmental competence. *Human Reproduction* **17**, 3129–3137.

Veeck LL 1988 Oocyte assessment and biological performance. *Annals of the New York Academy of Science* **541**, 259–274.

Weghofer A, Munné S, Brannath W *et al.* 2008 The impact of LH-containing gonadotropins on diploidy rates in preimplantation embryos: long protocol stimulation. *Human Reproduction* **23**, 499–503.

Westergaard LG, Laursen SB, Andersen CY 2000 Increased risk of early pregnancy loss by profound suppression of luteinizing hormone during ovarian stimulation in normogonadotrophic women undergoing assisted reproduction. *Human Reproduction* **15**, 1003–1008.

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