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Improvement of delivery and live birth rates after ICSI in women aged >40 years by ovarian co-stimulation with growth hormone

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BACKGROUND: Growth hormone (GH) is required for ovarian follicular development, and its administration during ovarian stimulation improves pregnancy rate in cow and sheep. Data on the use of exogenous GH in human assisted reproduction treatment are inconsistent. This prospective randomized study evaluates the usefulness of GH administration in women of >40 years undergoing ovarian stimulation for assisted reproduction treatment. METHODS: One hundred women of >40 years undergoing assisted reproduction treatment were randomized between a GH treatment group and a placebo group. Assisted reproduction treatment outcomes were evaluated. RESULTS: In patients of the GH treatment group, a similar number of oocytes, embryos and pregnancies was achieved as compared with the placebo group. However, the patients treated with GH suffered fewer pregnancy losses, resulting in higher delivery and live birth rates. These patients also showed higher peak serum estradiol concentration and higher concentrations of GH and estradiol in pre-ovulatory follicular fluid as compared with the placebo group. CONCLUSIONS: Administration of GH during ovarian stimulation alleviates age-related decrease in assisted reproduction treatment efficiency. This effect appears to be mainly due to an improvement of oocyte developmental potential, but GH action on the uterus cannot be excluded.

Key words: ovarian stimulation/growth hormone/ovarian ageing/delivery rate/birth rate

Introduction

The chance of live birth in assisted reproduction treatment decreases with increasing female age and drops markedly after 40 years of age (Edwards and Steptoe, 1983; Edwards *et al.*, 1984; Gosden, 1984; Wood *et al.*, 1985; Gindoff and Jewelewicz, 1986; Piette *et al.*, 1990; Hull *et al.*, 1996; Janny and Ménézo, 1996). No similar age-related decline was observed in oocyte recipients participating in oocyte donation programmes (Levran *et al.*, 1991; King and Kovacs, 1992; Abdalla *et al.*, 1993; Sauer *et al.*, 1993; Balmaceda *et al.*, 1994; Navot *et al.*, 1994) although some authors noted a moderate decrease in pregnancy and implantation rates in older recipients (Flamigni *et al.*, 1993). These observations suggest that a decrease in oocyte quality is a determining factor of assisted reproduction treatment failure in women approaching the end of their reproductive period.

Several studies have suggested that chromosomal abnormalities, mainly arising during the first meiotic division (Angell *et al.*, 1993; Angell, 1997; Dailey *et al.*, 1996), are essentially responsible for the age-related decline in oocyte developmental potential (Hassold *et al.*, 1984; Hassold and Chiu, 1985; Kornafel and Sauer, 1994). Interestingly, an impairment of meiotic spindle assembly has been reported in oocytes of older (40–45 year old) women (Battaglia *et al.*, 1996). It is thus possible that the age-related decline of oocyte developmental potential may be at least partly caused by oocyte cytoplasmic abnormalities during final phases of intrafollicular development which, in their turn, may be related to an abnormal content of essential follicular fluid components.

We have shown previously that the ability of human oocytes to form morphologically normal and implantation-competent embryos is related to the concentration of different hormones in follicular fluid (Mendoza *et al.*, 1999, 2002). Among the hormones studied, growth hormone (GH) showed the most consistent relationship with different parameters of embryo quality, and higher concentrations of GH in follicular fluid were associated with rapid cleavage, good cleaving embryo morphology and a high embryo implantation potential (Mendoza *et al.*, 1999, 2002). In a preliminary study (unpublished) we noted a decrease in follicular fluid GH concentration in women aged >40 years as compared with young women. The question then arises whether exogenous GH administration during ovarian stimulation can counteract the age-related decline of oocyte quality.

Exogenous GH has been shown to be beneficial in bovine superovulation protocols where it decreases the proportion of unfertilized oocytes and degenerate embryos (Cushman *et al.*, 2001; Moreira *et al.*, 2002), although another recent study failed to confirm these observations (Hasler *et al.*, 2003). A beneficial effect of exogenous GH on the number of transferable embryos and birth rate was also described in superovulated ewes (Folch *et al.*, 2001).

Studies evaluating the benefits of co-treatment with GH during controlled ovarian stimulation for human assisted reproduction treatment have reported controversial findings. A randomized trial showed an improvement of ovarian response to gonadotrophins by GH co-treatment in women with polycystic ovaries (Owen *et al.*, 1991), but another randomized study failed to confirm this effect (Homburg *et al.*, 1995). In patients with low pituitary GH reserve (clonidine-negative), but not in those with normal pituitary GH reserve (clonidine-positive), GH co-treatment reduced gonadotrophin requirement and increased pregnancy rate when combined with hMG for ovarian stimulation (Blumenfeld *et al.*, 1994). In another small series of 12 patients, a better fertilization and pregnancy rate was reported with GH co-treatment as compared to a previous attempt without GH (Wu *et al.*, 1996). An enhancement of *in vitro* maturation and fertilization of human germinal vesicle oocytes retrieved from small antral follicles by *in vivo* administration of GH has also been described (Hassan *et al.*, 2001). On the other hand, no effect of GH on the number of oocytes, embryos and pregnancies was obtained in two small series (seven and 22 patients respectively) of women with a poor ovarian response to gonadotrophins (Levron *et al.*, 1993; Suikkari *et al.*, 1996). A larger double-blind prospective study (21 patients in both the GH treatment and placebo arms) failed to show significant effects of GH on ovarian stimulation cycle characteristics and the number of oocytes collected, but pregnancy rate has not been evaluated (Hughes *et al.*, 1994).

It has to be remembered that both the positive and the negative data concerning the use of GH in ovarian stimulation were generated in small studies. Moreover, the target patient population was not always defined clearly, and the outcome measures did not always include delivery and live birth rates which are the most relevant parameters of assisted reproduction treatment success. In a recently updated Cochrane review including all randomized controlled trials employing GH in the ovarian stimulation, no effect of GH was noted in normal responders, but a significant improvement in live birth rate was found in poor responders, although this result was only just significant (Harper *et al.*, 2003).

In this study we tested the usefulness of GH co-stimulation in a group of patients defined by advanced female age (>40 years). Delivery and live birth rate were used as the main outcome measures.

Materials and methods

Patients

This study involved 100 couples with female age of >40 years (41–44 years) entering an assisted reproduction programme. Couples with azoospermia requiring testicular sperm extraction were excluded. After informed consent, the couples were randomly allocated to two groups, one receiving GH co-treatment and the other placebo co-treatment during ovarian stimulation. Randomization was done by using computer-generated random numbers concealed in opaque envelopes. The randomization process and distribution of medication were coordinated by a nurse, while the patients, the clinicians and the biologists involved in the study were blinded to the treatment allocation. Basic patients' demographic characteristics (Table I) did not differ between the two groups.

Table I. Basic patients' characteristics

Characteristic	Placebo group	GH treatment group
Age (years)	42.3 ± 1.0	42.2 ± 1.1
No. of previous attempts	2.8 ± 2.1	2.9 ± 2.3
Body mass index (kg/m^2)	24.3 ± 2.4	24.1 ± 2.3
Cycle length (days)	28.2 ± 1.2	28.0 ± 1.1
Baseline hormone levels (day 2–3)		
FSH (IU/l)	10.1 ± 1.8	10.2 ± 1.9
LH (IU/l)	4.4 ± 1.8	4.7 ± 2.0
Estradiol (pg/ml)	38 ± 12	40 ± 13
Progesterone (ng/ml)	2.4 ± 1.2	2.5 ± 1.1

Values are mean ± SD.

Design

Patients in the GH co-treatment group were given a daily subcutaneous injection of 8 IU of GH (Saizen; Serono, Geneva, Switzerland) from day 7 of exogenous gonadotrophin administration till the day following the ovulation-triggering injection of hCG (see 'Controlled ovarian stimulation' below). Patients in the placebo group received solvent only at the same time.

To compare assisted reproduction outcomes in women co-stimulated with GH with those in a placebo group, delivery rate (percentage of women under treatment who delivered a living newborn) and live birth rate (percentage of embryos transferred that gave rise to a living newborn) were chosen as the main outcome measures. In our previous series of attempts (non-prospective, non-randomized) the delivery rate in women of >40 years of age was 5%, and it reached 25% after co-stimulation with GH. It was calculated that some 50 attempts were needed to be performed in both the treatment and the control group to detect a difference of 20% (from 5 to 25%) with 80% power and 5% significance level (two-tailed test with alpha = 0.05).

Controlled ovarian stimulation

All patients included in this study adhered to the same protocol consisting of pituitary down-regulation with triptorelin (Decapeptyl; Ipsen, Slough, UK) started in the mid-luteal phase at a dose of 0.1 mg/day. The dose was reduced to 0.05 mg/day from the day of the subsequent vaginal bleed. This reduced daily dose was administered until the day of ovulation-inducing hCG injection.

The starting dose of gonadotrophins was 450 IU of recombinant human FSH (rFSH, Puregon; Organon, Oss, The Netherlands) and 150 IU of hMG (Menopur; Ferring, Geneva, Switzerland), and this treatment was continued during the first 4 days of stimulation. From day 5 of stimulation the dose of gonadotrophins was adapted according to changes in the number and size of ovarian follicles and serum concentrations of estradiol and LH. These determinations were performed on day 5 and then every other day until the day of ovulation-triggering injection of hCG. According to the study design (see previous section), daily injections of 8 IU of GH or placebo were administered from day 7 of stimulation till the day following

hCG administration. The chosen dose of 8 IU of GH daily was determined as an intermediate between the lowest dose (4 IU daily; Suikkari *et al.*, 1996) and the highest dose (12 IU daily; Hughes *et al.*, 1994) reported in the literature. When at least one ovarian follicle had reached a diameter of 18 mm, ovulation was induced with 250 μg recombinant hCG (Ovitrelle; Serono, Geneva, Switzerland).

Assisted reproduction techniques

Oocyte recovery was performed by ultrasound-guided follicle aspiration under general anaesthesia. Oocyte–cumulus complexes were incubated in Gamete medium (Vitrolife, Göteborg, Sweden) for 2–4 h. The cumulus oophorus and the corona radiata were subsequently removed from the oocytes by a brief incubation in hyaluronidase solution (Hyase, Vitrolife) followed by repeated aspiration with plastic stripper tips (Mid Atlantic Diagnostics, Marlton, New Jersey, USA). After this procedure oocyte maturity was assessed, and all mature (metaphase II) oocytes were inseminated by ICSI which was performed within 30–60 min after the cumulus oophorus and corona radiata removal. ICSI was carried out with the previously described technique and instruments (Tesarik and Mendoza, 2002).

After ICSI the oocytes were cultured at 37°C in IVF medium (Vitrolife) equilibrated with 5% CO_2 in air for 14–16 h. After this period, fertilization results were assessed. Normally fertilized oocytes (two pronuclei and two polar bodies) were separated and incubated in fresh medium of the same composition for an additional 22–26 h when they were put to fresh culture medium again. All cultures were performed in 4-well culture dishes (Nunc, Roskilde, Denmark). All embryos (1–7) of each couple were grouped together in a single well containing 0.5 ml medium. Three days after ICSI, all living embryos developing from normally fertilized oocytes (1–5) were transferred to the patient's uterine cavity.

Hormone assays

Hormone assays used in this study were applied to both blood serum (estradiol, LH and GH) and follicular fluid (estradiol and GH) samples. Estradiol and LH were determined by using commercial enzyme immunoassay kits (Boehringer Mannheim, Mannheim, Germany). The intra- and inter-assay variabilities were 2.7 and 5.0% for estradiol, and 1.8 and 5.1% for LH. Direct radioimmunoassay was used for the determination of GH (Sorin Biomedica, Vercelli, Italy). The intra-and inter-assay variability for GH determination was 1.9 and 4.5% respectively.

Zygote and cleaving embryo evaluation

Zygotes were evaluated according to a previously described scoring system (Tesarik and Greco, 1999) simplified by grouping together all abnormal patterns (Tesarik *et al.*, 2000). Cleaving embryos were evaluated twice, first at the time of medium change 2 days after ICSI, and second 10–30 min before transfer to the uterus 3 days after ICSI. Cleavage speed and embryo morphology were evaluated as described (Tesarik and Greco, 1999). Embryos that had >3 cells 2 days after ICSI and >6 cells 3 days after ICSI and that showed $<20\%$ of space occupied by cell fragments were considered to have good morphology.

Statistical analysis

Differences between groups were assessed by two-tailed χ^2-test with Yates' correction or Fisher's exact test for categorical variables, and by Mann–Whitney *U*-test for continuous variables. All analyses were performed using the Statistica 5.0 package (Statsoft Version 5.1, Hamburg, Germany).

Results

Patient flow through the trial

The trial was performed according to Consolidated Standards of Reporting Trials (CONSORT) guidelines. Patients continued to be enrolled into the study until 50 attempts were analysed in either arm. A total of 134 patients of >40 years of age asking for an assisted reproduction attempt by ICSI were assessed for eligibility. Thirty-four patients were excluded because they did not meet criteria demanded for successful ovarian stimulation. The patients excluded were those whose day 3 serum concentration of FSH was >14 IU/l or whose day 3 inhibin B concentration was <30 pg/ml. The remaining 100 patients were randomized between the GH co-treatment group ($n = 50$) and the placebo group ($n = 50$). All of these patients received the allocated intervention, all of them had oocytes retrieved and ≥ 1 embryo transferrred. All of these cases were included in the analysis (Figure 1).

Effect of GH on ovarian stimulation cycle characteristics

Ovarian stimulation cycles of the patients enrolled in the GH co-treatment and in the placebo group did not differ as to the duration, the total dose of rFSH and hMG administered and the number of total and metaphase II oocytes recovered (Table II). However, the peak values of serum estradiol achieved during the stimulation as well as GH and estradiol concentrations in follicular fuid aspirated at oocyte recovery were significantly higher in the GH co-stimulation group as compared with the placebo group (Table II).

Effects of GH co-stimulation on fertilization and post-fertilization development

With similar numbers of oocytes injected in the GH co-treatment and the placebo group, no significant differences were found between the two groups in the number of normal and abnormal zygotes and embryos, although there was a trend towards a higher number of rapidly cleaving and good morphology embryos in the GH co-treatment group (Table III).

Effects of GH co-stimulation on implantation and pregnancy outcomes

Patients in the GH co-treatment group received slightly more embryos per transfer as compared with the placebo group (4.2 versus 3.5; $P > 0.05$) (Table IV). This was due to the availability of more transferrable embryos in the GH co-treatment group and to the policy of transferring up to 5 fresh embryos in the ovarian stimulation cycle. Similar total numbers of pregnancies were achieved in both groups (16 and 10 respectively; $P > 0.05$), but the patients in the GH co-treatment group had fewer biochemical pregnancies, more clinical pregnancies, and more gestational sacs with heart beat as compared with the placebo group, resulting in a higher clinical pregnancy rate (26 versus 6%; $P < 0.05$) and clinical implantation rate (6.2 versus 1.7%; $P < 0.05$) (Table IV). Pregnancy outcomes were also better in the GH co-treatment group in which more deliveries occurred and more babies were born, which was reflected in a higher delivery rate

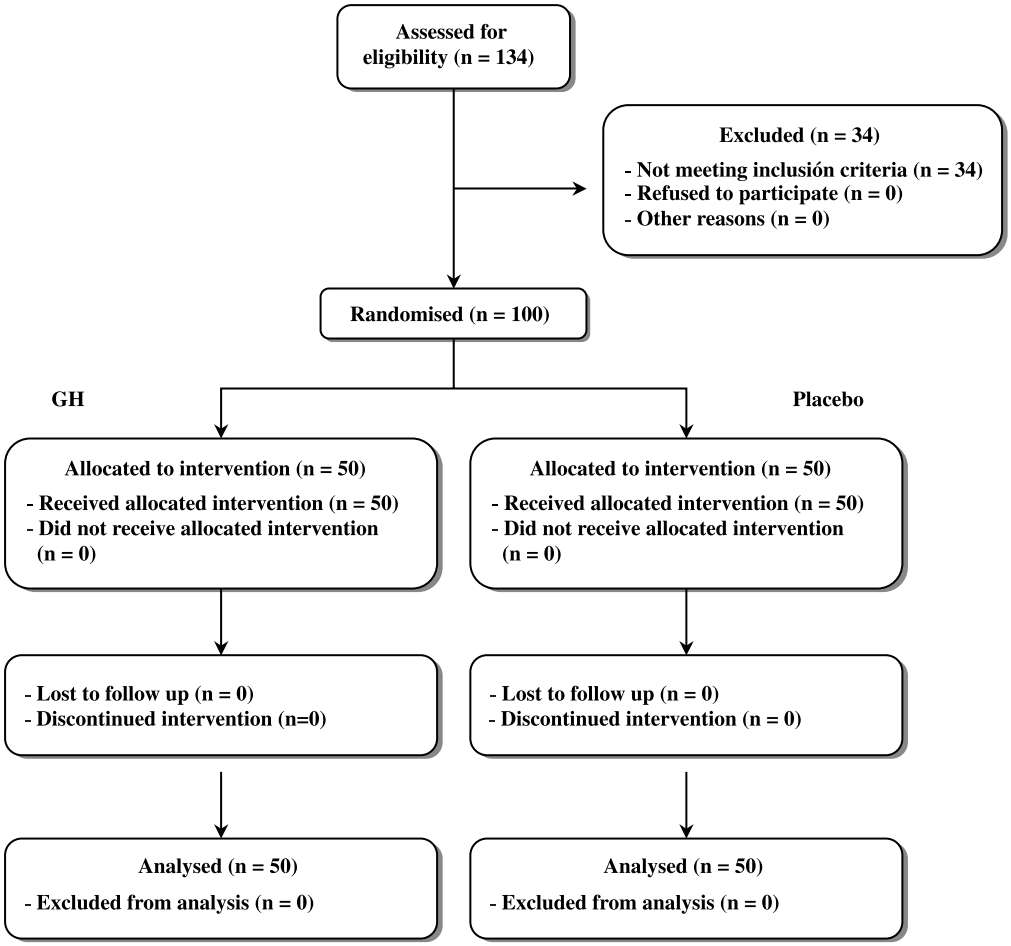

Figure 1. CONSORT flow diagram of the progress of participants through each state of the randomized trial.

(22 versus 4%; $P < 0.05$) and live birth rate (5.2 versus 1.1%; $P < 0.05$) as compared with the placebo group (Table IV).

Discussion

The main observation of this study was a significant improvement of delivery and live birth rates in women of >40 years treated by administration of exogenous GH during ovarian stimulation in an ICSI programme. To the best of our knowledge this is the largest series of women of this age category in whom the effect of GH co-stimulation was evaluated in a prospective randomized study. These observations are in agreement with the conclusions of a recent Cochrane review analysing data from three small trials and showing a significant improvement in live birth rate in previous poor responders (Harper *et al.*, 2003). In the same meta-analysis, however, no effect of GH co-treatment was found in women with no history of poor response to ovarian stimulation (Harper *et al.*, 2003). Since poor response to ovarian stimulation becomes more frequent with increasing female age, it remains to be determined whether it is not the female age, rather than the poor response by itself, which can characterize the female population who would benefit from GH co-stimulation.

It has to be noted that GH concentration in follicular fluid aspirated from women in the placebo group was low. Similar low GH concentrations in follicular fluid have previously been shown to be associated with assisted reproduction treatment failure (Mendoza *et al.*, 2002). In contrast, in the GH co-treatment group the concentration of GH in follicular fluid was markedly higher. Taking into consideration the recent findings showing a requirement for GH to support ovarian follicular development in rat and mouse (Bachelot *et al.*, 2002; Zhao *et al.*, 2002) and the impact of GH insufficiency

Table II. Ovarian stimulation cycle characteristics

Characteristic	Placebo group	GH treatment group
Duration of stimulation (days)	11.8 ± 0.9	12.1 ± 1.0
Total rFSH administered (IU)	4126 ± 385	4019 ± 368
Total hMG administered (IU)	1230 ± 115	1208 ± 104
Peak serum E_2 concentration (pg/ml)	912 ± 129	1523 ± 208[a]
E_2 concentration in FF (pg/ml)	578 ± 85	921 ± 98[a]
GH concentration in FF (ng/ml)	1.7 ± 0.3	3.7 ± 0.4[a]
No. of oocytes recovered[b]	5.6 ± 1.9	5.8 ± 2.0
No. of MII oocytes recovered[b]	4.7 ± 1.7	4.6 ± 1.8

Values are mean ± SD.
[a]Significantly different from the placebo group ($P < 0.01$).
[b]Per stimulated cycle.
E_2 = estradiol; FF = follicular fluid; GH = growth hormone; MII = metaphase II.

Table III. Fertilization and post-fertilization development[a]

Variable per patient	Placebo group	GH treatment group
Oocytes injected	4.7 ± 1.7	4.6 ± 1.8
Total zygotes	4.1 ± 1.5	4.2 ± 1.5
Normal zygotes[b]	3.6 ± 1.3	4.9 ± 1.4
Rapidly cleaving embryos[c]	1.4 ± 0.9	2.6 ± 1.1
Good morphology embryos[d]	1.9 ± 1.0	2.6 ± 1.2

[a]Values are mean ± SD. Differences between the control and growth hormone group were assessed by the Mann–Whitney *U*-test. Differences between the GH treatment and placebo group were not significant ($P > 0.05$) for any of the variables.
[b]Zygotes with two equal-sized pronuclei detected 12–14 h after ICSI.
[c]Embryos with >6 cells 3 days after ICSI.
[d]Embryos with equal-sized cells and <10% of volume occupied by anucleate fragments.

on ovarian function in women (Spiliotis, 2003), the improvement of delivery and live birth rate by GH co-stimulation observed in the present study may be related to the correction of an age-related decrease in endogenous intrafollicular GH concentration during the final phase of oocyte meiotic maturation. Interestingly, a previous randomized, double-blind and placebo-controlled study reported an increase in follicular fluid GH and in pregnancy rate in low responders co-treated with pyridostigmine, an acetylcholinesterase inhibitor, during controlled ovarian stimulation (Kim *et al.*, 1999).

As to the secondary outcome measures, this study did not find any significant difference in the duration of ovarian stimulation, the total dose of gonadotrophins used, and the number of total and metaphase II oocytes recovered. These observations are in agreement with the conclusions of a Cochrane review compiling data of six small studies (Kotarba *et al.*, 2000), but they differ from those of a European and Australian multicentre study (1995) in which GH co-stimulation led to a reduction of the gonadotrophin dose and shortening of the ovarian stimulation duration (European and Australian Multicenter Study, 1995). Here again, this difference can be explained by the different character of the patient populations enrolled in both studies, namely women suffering from hypogonadotrophic hypogonadism (European and Australian Multicenter Study, 1995) and normogonadotrophic or slightly hypergonadotrophic women with advanced age (this study).

On the other hand, peak serum estradiol concentration achieved during ovarian stimulation was higher in women co-stimulated with GH as compared with controls in this study. With the same numbers of large antral follicles and oocytes recovered by follicular aspiration in both groups, this observation means that there was more estradiol per large antral follicle in the GH co-treatment group. In fact, follicular fluid aspirates from women co-stimulated with GH contained more estradiol than those from controls. Since higher concentrations of estradiol in pre-ovulatory follicular fluid predict a higher chance of pregnancy (Mendoza *et al.*, 2002), this observation suggests that the effect of GH on intrafollicular estradiol production may mediate the beneficial effect of GH on oocyte quality. Stimulating action of GH on the production of estradiol by ovarian follicular cells has previously been described (Mason *et al.*, 1990; Lanzone *et al.*, 1996). Further study is needed to determine whether effects of GH on intrafollicular estradiol concentration is the only mechanism involved in the improvement of oocyte quality in women of >40 years or whether other mechanisms also come into play.

Unlike sheep (Folch *et al.*, 2001) and cattle (Moreira *et al.*, 2002), where co-stimulation with GH was reported to improve fertilization and preimplantation embryo development, we have not found any difference in fertilization rate and embryo morphology between the GH co-stimulation and the control group. Even though a decline in oocyte quality is known to be the principal cause of assisted reproduction treatment failure in ageing females (Schramm *et al.*, 2002), the contribution of GH effects on uterine receptivity, similar to those suggested by a study using GH in cattle reproduction (Moreira *et al.*, 2003), cannot be excluded.

In conclusion, this prospective randomized study shows that women aged >40 years undergoing assisted reproduction treatment and co-stimulated with GH achieve more ongoing pregnancies and suffer less pregnancy wastage, resulting in more deliveries and live births, as compared with women of the same age category stimulated with gonadotrophins alone. Further study is needed to determine the mechanism of this effect.

Table IV. Embryo implantation, pregnancy and pregnancy outcomes[a]

Variable per group	Placebo group	GH treatment group
Transfer procedures	50	50
Embryos transferred per patient (mean)	3.5	4.2
Total pregnancies	10	16
Biochemical pregnancies[b]	7	3
Clinical pregnancies[c]	3	13
Deliveries	2	11
Births	2	11
Pregnancy rate (%)[d]	20	32
Clinical pregnancy rate (%)[e]	6	26[f]
Clinical implantation rate (%)[g]	1.7	6.2[f]
Delivery rate (%)[h]	4	22[f]
Birth rate (%)[i]	1.1	5.2[f]

[a]Differences between the control and growth hormone group were assessed by two-tailed χ^2 test with Yates' correction and Fisher's exact test.
[b]Pregnancies detected by determination of serum β-hCG that ended before the detection of a gestational sac with heartbeat.
[c]Pregnancies in which a gestational sac with heartbeat was detected.
[d]Percentage of treatment attempts that resulted in a pregnancy.
[e]Percentage of treatment attempts that resulted in a clinical pregnancy.
[f]Significantly different from the placebo group ($P < 0.05$).
[g]Percentage of embryos transferred that formed an embryo with heartbeat.
[h]Percentage of treatment attempts that resulted in a delivery.
[i]Percentage of embryos transferred that gave rise to a childbirth.

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