Human Reproduction, Vol.25, No.9 pp. 2290–2297, 2010
Advanced Access publication on July 13, 2010 doi:10.1093/humrep/deq174

human reproduction

ORIGINAL ARTICLE *Infertility*

Prospective cohort study in high responder oocyte donors using two hormonal stimulation protocols: impact on embryo aneuploidy and development

Carmen Rubio[1,*], Amparo Mercader[1], Pilar Alamá[1], César Lizán[2], Lorena Rodrigo[1], Elena Labarta[1], Marco Melo[1], Antonio Pellicer[1], and José Remohí[1]

[1]Instituto Universitario-Instituto Valenciano de Infertilidad IVI-Valencia, Plaza Policía Local no. 3, 46015 Valencia, Spain [2]Instituto Valenciano de Infertilidad IVI-Alicante, Av. Dénia, 111, 03015, Alicante, Spain

*Correspondence address. E-mail: c.rubio@ivi.es

Submitted on January 12, 2010; resubmitted on June 8, 2010; accepted on June 11, 2010

BACKGROUND: Ovarian stimulation regimens for *in vitro* fertilization seem to have a deleterious effect on oocyte quality and embryo aneuploidy in a dose-dependent manner. This study aims to test the influence of gonadotrophin doses on embryo aneuploidy rates.

METHODS: A total of 32 young oocyte donors with a high response to ovarian stimulation, were included in the study. Two subsequent stimulation treatments were performed in each donor: first, a standard dose cycle using a 225 IU starting dose of recombinant FSH (r-FSH) and secondly, a reduced dose cycle with a starting dose of 150 IU r-FSH. In both cycles, GnRH agonist co-treatment was used for down-regulation. Ovarian response, embryo development and aneuploidy for chromosomes 13, 15, 16, 17, 18, 21, 22, X and Y were the main outcomes of the study.

RESULTS: A total of 22 donors completed both treatments with different gonadotrophin doses. In the remaining 10 donors, the reduced dose cycle was cancelled due to low ovarian response. In those donors who completed both regimens, significant increases in rates of fertilization and chromosomally normal blastocysts were observed in the reduced dose cycle. No differences were observed in pregnancy and implantation rates in recipients who received oocytes from standard and reduced doses cycles.

CONCLUSIONS: Despite the limited numbers in our study, we can conclude that in high responder donors, a decrease in the gonadotrophin dose could improve fertilization rates and embryo quality. However, due to the reduced oocyte numbers with lower doses, a similar reproductive outcome in terms of live births would be expected.
Clinical Trial.gov Identifier: nCT 00802295.

Key words: ovarian stimulation / aneuploidy / blastocyst / PGS / oocyte donation

Introduction

In recent years, there has been increasing controversy over the genetic risks of Assisted Reproductive Technology (ART), particularly related to *in vitro* fertilization (IVF) treatments. Preliminary studies suggest that aneuploidy rates in embryos may be altered by ovarian stimulation protocols employed in IVF, as well as patient estradiol (E_2) levels and the number of retrieved oocytes (Munne *et al.*, 1997, 2006; Soares *et al.*, 2003; Katz-Jaffe *et al.*, 2005). Previous reports from preimplantation genetic screening (PGS) programmes have shown contradictory results. For example, Gianaroli *et al.* (2000) reported a similar incidence of chromosomally abnormal embryos, independent of the number of retrieved oocytes, in both patients with advanced maternal age and recurrent implantation failure. However, polar body testing has shown a higher incidence of chromosome abnormalities in two groups of patients with high oocyte yield: women less than 35 years old and between 35 and 40 years old (Haaf *et al.*, 2009).

Several authors have hypothesized that mild stimulation protocols or natural cycles would reduce aneuploidy (Nargund *et al*., 2001; Pelinck *et al*., 2005). Baart *et al*. (2007) conducted a prospective randomized study comparing PGS results in conventional ovarian stimulation versus mild stimulation regimens. These authors considered a group of young IVF patients without additional indications for PGS, and found a lower frequency of chromosomal abnormalities with the mild stimulation protocol. More recently, to produce more 'friendly' IVF protocols and help to avoid the side effects of ovulation induction protocols, unstimulated IVF cycles were performed in conjunction with PGS. Aneuploidies were detected in 4 out of 11 embryos, demonstrating that aneuploidies were present in human embryos even in the absence of ovarian stimulation (Verpoest *et al*., 2008). In a previous report comparing embryo morphology in stimulated versus unstimulated cycles, no differences were found in embryo quality (Ziebe *et al*., 2004).

In a previous study (Soares *et al*., 2003), we described high aneuploidy rates (56.5%) in Day-3 embryos from young high responder donors with a mean number of 25.9 retrieved oocytes. If we compare this aneuploidy rate to 37.3% chromosomally abnormal embryos in young fertile normal responder patients undergoing PGS due to sex-linked diseases (where lower E_2 peak and significantly lower number of oocytes were retrieved than in the donors group), the increase in chromosomally abnormal embryos could be attributed to the high ovarian response to gonadotrophins. Munne *et al*. (2006) confirmed that a large oocyte cohort from normal oocyte donors could result in up to 50% chromosomally abnormal embryos. More recently, Keskintepe *et al*. (2007) in a comparison study using FISH and comparative genomic hybridization (CGH), noted even higher aneuploidy rates by CGH in young donors.

Because of our previous results and other authors' publications, we conducted this study to determine the impact of different gonadotrophin doses on the percentage of aneuploidy and the total number of chromosomally normal blastocysts as the primary outcome measures reflecting the reproductive efficiency of the subsequent treatment cycles.

Materials and Methods

Design

This is a crossover study in which PGS was performed on the embryos resulting from two stimulation regimens: in the first cycle (standard cycle), donors received a standard stimulation dose and in the second cycle (reduced dose cycle), the same donors received a 30% decreased dose from the previous dose, with an interval of at least 3 months. Of the 22 donors with two completed cycles (standard and reduced dose cycles), for 7 patients the interval between the two donations was 3 months, for 6 patients the interval was 6 months and for the remaining patients the interval between donations was more than 1 year.

Oocyte donors

The study included a total of 32 high responder donors from our oocyte donation programme. Inclusion criteria were donors with more than 20 oocytes or serum E_2 levels >3000 pg/ml on the day of hCG in a previous cycle with standard stimulation, without developing ovarian hyperstimulation syndrome. This study was approved by the Institutional Review Board on the use of human subjects in research at the Instituto Universitario-IVI, Valencia, Spain. All donors were included in our oocyte donation programme after being thoroughly informed and having fulfilled our inclusion criteria (Soares *et al*., 2005). Subjects were between 18 and 35 years old; we had access to their complete medical history, which included current or past exposure to radiation or hazardous chemical substances, intravenous drug use and reproductive history. All subjects were found to be normal in a physical and gynaecological examination, had no family history of hereditary or chromosomal diseases, had a normal karyotype and tested negative for Fragile X Syndrome. The donors admitted to the study had normal menstrual cycles of 26–34 days duration, normal weight (BMI of 18–28 Kg/m^2), no endocrine treatment (including gonadotrophins and oral contraception) in the 3 months preceding the study, and normal uterus and ovaries at transvaginal ultrasound (Garrido *et al*., 2002). Donors with a diagnosis of PCOS according to Rotterdam criteria were excluded (Rotterdam Consensus Workshop Group, 2004).

GnRH agonist protocols were used for controlled ovarian stimulation (COS) and patients started administration of 0.5 mg of leuprolide acetate (Procrin®; Abbott, Madrid, Spain) in the mid-luteal phase of the previous cycle, until a negative vaginal ultrasound defined ovarian quiescence. The dose of GnRH agonist was then decreased to 0.25 mg until the day of hCG administration (Melo *et al*., 2006).

Oocyte donors underwent two consecutive cycles with the following two different doses of gonadotrophins to perform COS:

- Standard dose cycle: the fixed starting dose of gonadotrophins was 225 IU/day of recombinant FSH (r-FSH) (Gonal-F®; Merck-Serono, Geneve, Switzerland or Puregon; MSD, NJ, USA) for the first 5 days. Then, a serum E_2 determination was performed for individual adjustments as follows: if E_2 was <400 pg/ml, the dose was increased to 300 IU/day; if E_2 was between 400 and 600 pg/ml, then the dose was maintained at 225 IU/day; if E_2 was >600 pg/ml, the dose was lowered to 150 IU/day.
- Reduced dose cycle: at least 3 months later all of the donors were enrolled in a new COS cycle with 150 IU/day of r-FSH (Gonal-F®; Merck-Serono, Geneve, Switzerland; or Puregon, MSD, NJ, USA). In this protocol the dose of r-FSH was never increased in spite of the response, but the r-FSH dose was decreased if E_2 was >600 pg/ml after 5 days of stimulation.

Initially, 32 donors underwent the standard dose cycle, but in the reduced dose cycle, 10 were cancelled due to an inadequate response for the purpose of oocyte donation in our clinical programme (defined as less than five follicles greater than 18 mm on day of hCG stimulation). Therefore, 22 donors successfully underwent both stimulation protocols (Fig. 1).

When at least five follicles reached 18 mm in diameter, recombinant hCG (Ovitrelle®, 250 μg, Merck-Serono, Geneva, Switzerland) was administered and oocyte retrieval by transvaginal ultrasound-guided puncture of follicles was scheduled 36 h later. Serum E_2 and *P* levels were measured on the morning of hCG administration.

Oocyte recipients

Oocyte recipients ($n = 60$) were admitted in our oocyte donation programme due to low response to stimulation (11%), endometriosis (11%), premature ovarian failure (17%), advanced maternal age (58%) or genetic or chromosomal disorders of maternal origin (3%).

In all cases, intracytoplasmic sperm injection (ICSI) was performed with motile and normal morphology ejaculated sperm. Cases with uterine pathology (submucous or larger than 2 cm intramural fibroids, polyps, adhesions, adenomyosis or müllerian defects), recurrent miscarriage, age >49 years old or severe male infertility (<5 million of fresh spermatozoa/ml, <5% normal forms and/or non-obstructive azoospermia) were not included in the present study.

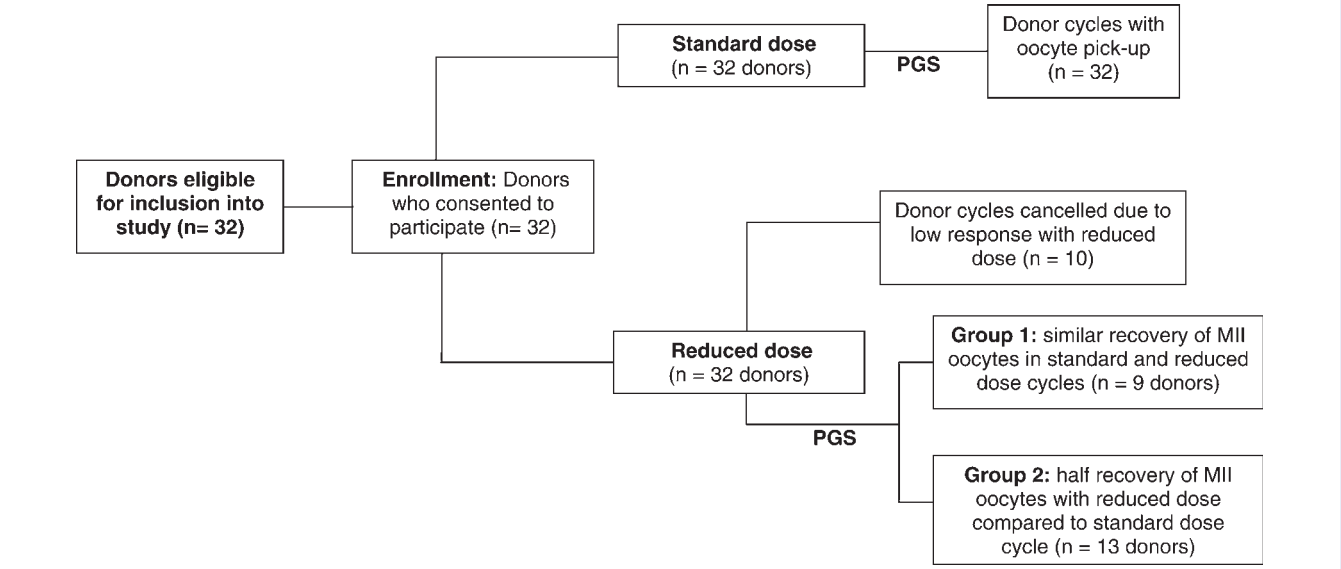

Figure 1 Flow of patients through the trial. PGS, preimplantation genetic screening; MII, metaphase II.

The protocol for hormonal replacement therapy (HRT) was described previously (Remohí *et al*., 1995). A baseline transvaginal scan was carried out prior to down-regulation to ensure that the uterus and ovaries were normal. For all recipients who were still cycling, down-regulation was performed using an IM dose of 3.75 mg of Triptorelin (Decapeptyl®; Ipsen Pharma; Barcelona, Spain) in the mid-luteal phase of the previous cycle. HRT was initiated on Day 1–3 of the following cycle, and doses of estradiol valerate (Progynova®; Schering Spain, Madrid, Spain) were increased as follows: 2 mg/day for the first 8 days of treatment, 4 mg/day for the following 3 days and a minimum of 6 mg/day until the pregnancy test. On Day 15, an ultrasound was performed to evaluate endometrial growth. On the day after the donation, 800 mg/day of micronized intravaginal progesterone (Progeffik®; Effik Laboratories, Madrid, Spain) was administered. After signing written consent, PGS for chromosomes 13, 15, 16, 17, 18, 21, 22, X and Y was performed in the resulting embryos and embryo transfer was carried out on Day 5 of development.

Embryo biopsy and culture conditions

After oocyte retrieval, IVF and embryo culture were performed as described previously (Mercader *et al*., 2006). Fertilization was assessed 17–20 h following microinjection and embryo cleavage was recorded every 24 h. Embryos were grown in IVF medium: CCM medium (1:1) (Vitrolife AB, Kungsbacka, Sweden) on Day 2 and subsequently cultured in CCM medium with a monolayer of endometrial epithelial cells from Day 2 until Day 5, when embryo transfer was performed.

Embryo biopsy was performed on Day 3. Embryos were placed on a droplet containing Ca^{2+} and Mg^{2+} free medium (G-PGD, Vitrolife) and the zona pellucida was perforated using laser technology (OCTAX, Herbron, Germany). Only embryos with $\geq$5 nucleated blastomeres and $\leq$25% of fragmentation degree were biopsied and one or two blastomeres were aspirated depending on the cell number on Day 3 (one blastomere was biopsied in embryos with 5–7 blastomeres and two blastomeres in embryos with $\geq$8 blastomeres). Individual blastomeres were fixed onto glass slides under an inverted microscope, using a slightly modified Tarkowski's protocol (Tarkowski *et al*., 1966).

FISH protocol for PGS

Chromosomes 13, 15, 16, 17, 18, 21, 22, X and Y were analysed by FISH in two consecutive rounds. In the first hybridization round, chromosomes 13, 16, 18, 21 and 22 were analysed using MultiVysion PB panel probe (Vysis Inc., Downers Grove, IL, USA). Co-denaturation of DNA from the nuclei and probes was performed in a thermoplate for 4 min at 73 $\pm$ 1°C, followed by 3 h of hybridization at 37°C. In a second hybridization round, chromosomes 15, 17, X and Y were analysed with Multi Vysion 4 Custom Color panel probe (Vysis Inc., Downers Grove) after co-denaturation and 1 h of hybridization at 42°C. Nuclei with non-conclusive signals (overlapping, fibre or split signals) or with absence of signals for any of the tested chromosomes were re-analysed using subtelomeric probes. An embryo was defined as normal when all blastomeres analysed from this embryo (one or two) showed two clear and separated dots for each of the tested autosomes and one or two dots for sex chromosomes. An embryo was considered as abnormal when all blastomeres analysed from this embryo (one or two) showed a different number of signals. Embryos considered as mosaic were those with two cells showing discordant signal numbers for one or more chromosomes.

Slides were analysed using an Olympus AX-70 epifluorescence microscope (Olympus Optical Co., Hamburg, Germany) equipped with a triple-bandpass filter for DAPI/Texas Red/FITC and single-bandpass filters for FITC, Texas Red, Gold, Aqua-Blue and Blue.

Outcome measures

Primary outcome measures were ovarian response, assessed by the number of retrieved oocytes, the percentage of aneuploid embryos and the total number of chromosomally normal blastocysts, defined in this paper as embryos diagnosed as normal on Day 3 after biopsy and that subsequently develop into blastocysts.

Secondary outcome measures were the fertilization rate, total gonadotrophin doses, estradiol levels on the day of hCG and pregnancy outcome in terms of implantation, pregnancy, miscarriage and live birth rates. Implantation rate was obtained by dividing the number of gestational sacs seen in ultrasound by the number of replaced embryos. Clinical pregnancy was considered when the embryonic sac(s) was/were seen by

vaginal ultrasound from the fifth week of pregnancy. Early miscarriage was defined as a loss before 12 weeks (according to the definition of the ESHRE special interest group of early pregnancy).

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences for Windows, version 15.0 (SPSS, Chicago, IL, USA) and MedCalc Software (Ghent, Mariakerke, Belgium). The means of the continuous variables analysed followed a normal distribution and were analysed with Student's *t*-test. Fisher's test was used to assess the significance of categorical parameters. A power analysis was performed for all variables statistically significant ($\alpha < 0.05$). Only those variables with a power higher than 80% were considered significant.

Results

A total of 32 donors started the standard dose cycle, but only 22 donors completed both cycles. For the remaining 10 donors, the reduced dose cycle was cancelled due to low ovarian response. Of the 22 donors who completed both treatments, in the standard dose cycle, none of the donors lowered the dose depending on the E_2 level in the first five stimulation days, but eight donors reduced the dose the day before HCG administration, whereas in the reduced dose cycle, initial doses did not change along the treatment. For 3 of the 10 uncompleted donors, doses were increased after 5 days of stimulation.

Table I shows the results of ovarian stimulation and oocyte donation in the 10 donors with only the standard dose cycle and the corresponding 10 recipients. In the 22 completed donors, comparisons between standard dose and reduced dose cycles were performed (Table II). The significant decrease in daily gonadotrophin administration ($P = 0.0086$) resulted in a significant decrease in E_2 levels measured on the day of hCG administration ($P = 0.0019$), and a significant decrease in the number of oocytes retrieved ($P < 0.0001$). A total of 50 women received donated oocytes from these 22 donors. Comparisons between the standard and reduced dose cycles showed a significant increase in rates of fertilization ($P = 0.0312$), blastocyst development (0.0441) and chromosomally normal blastocysts ($P = 0.0236$), with reduced doses. No significant differences were found in the percentage of chromosomally abnormal embryos with both treatments. In the recipients, the pregnancy outcome was similar with in two stimulation protocols, with a similar number of live births after the two treatments.

In the 22 donors with two completed cycles (standard and reduced dose cycles), the median of the difference between the number of MII oocytes retrieved with standard and reduced doses was 8. Donors were categorized in two different groups according to the difference in MII oocyte number between the two stimulation cycles: Group 1, 9 donors with differences below the median (similar numbers of MII oocytes recovered in standard and reduced dose cycles) and Group 2, 13 donors with differences above the median (half number of MII oocytes recovered in the reduced dose cycle compared with the standard dose cycle). Basal hormone levels, previous reproductive history and demographics were similar for each donor subgroup.

In Group 1 (Table III), reduced doses significantly improved fertilization rates ($P = 0.0269$), with an increased percentage of chromosomally normal blastocysts per MII oocyte, resulting in a higher mean number of normal blastocysts and a 2-fold increase in live births per donor. The percentages of chromosome abnormalities were 65.5% with the standard protocol and 50.9% with reduced doses, and the percentages of mosaic embryos when two cells per embryo were analysed were 43.5 and 27.3%, respectively (the differences were not statistically significant). In this subgroup, when we analysed abnormalities for each chromosome individually, we observed a significant increase in aneuploidies for chromosome 13 ($P = 0.0105$) and a trend towards higher aneuploidy rates with the standard protocol for most of the tested chromosomes (data not shown).

In Group 2 (Table IV), the decrease of gonadotrophin doses produced a significant decrease in both E_2 levels ($P = 0.0019$) and the number of MII oocytes recovered ($P < 0.0001$). However, no improvement was observed for any of the analysed parameters, and the number of live births was half of that achieved with standard doses. Chromosome abnormalities were similarly distributed in both stimulation regimens, without differences in either the total percentage of abnormalities (47.3 versus 49.4%) or abnormalities for each individual chromosome.

After categorization in these two groups, mean daily gonadotrophins doses, stimulation days and total gonadotrophin doses did

Table I Ovarian stimulation in 10 oocyte donors with only the standard dose cycle performed and the reduced dose cycle cancelled due to insufficient ovarian response.

Uncompleted donors	Standard dose
No. of donors (Mean age, SD)	10 (25.3, 3.1)
No. of recipients (Mean age, SD)	10 (41.0, 3.6)
Mean daily gonadotrophins (IU, SD)	245.0 (52.4)
Mean stimulation days (SD)	9.2 (0.8)
Mean total gonadotrophins (IU, SD)	2260 (537.9)
Mean E_2 day HCG (pg/ml, SD)	3034.7 (1174.3)
No. of retrieved oocytes (Mean, SD)	212 (21.2, 6.0)
No. of MII oocytes (Mean, SD)	183 (18.3, 4.,0)
No. of 2 PN (%)	132 (75.9)
No. of Day 3 biopsied embryos (%)	88 (66.7)
No. of abnormal/informative embryos (%)*	49/82 (59.7)
No. of mosaic embryos (%)**	20/48 (41.7)
No. of blastocysts/biopsied embryos (%)	66/88 (75.0)
No. of chromosomally normal blastocysts (% per MII)	29/183 (15.8)
Mean chromosomally normal blastocysts per donor (SD)	2.9 (1.2)
Mean embryos transferred/donor (SD)	1.8 (0.4)
No. of pregnancies/transfer (%)	9/11 (81.8)
No. of miscarriages (%)	2 (22.2)
Implantation rate (%)	9/20 (45.0)
No. of live births	7

*Informative embryos were defined as embryos with a conclusive result after FISH analysis.
**Discordant results between blastomeres when two cells were analysed.

Table II Ovarian stimulation in 22 donors with completion of the two stimulation cycles with standard and reduced doses.

Completed donors	Standard dose	Reduced dose	*P*-value
No. of donors (Mean age, SD)	22 (26.6, 3.9)	22 (26.6, 3.9)	–
No. of recipients (Mean age, SD)	27 (41.2, 4.9)	23 (40.5, 4.6)	Ns
Mean daily gonadotrophins (IU, SD)	228.6 (101.1)	144.9 (100.3)	$P = 0.0086$
Mean stimulation days (SD)	10.2 (1.7)	11.7 (1.7)	$P = 0.0055$
Mean total gonadotrophins (IU, SD)	2211.4 (1059.7)	1579.9 (1014.4)	$P = 0.0499$
Mean E_2 day HCG (pg/ml, SD)	3056.4 (1001.5)	2074.3 (959.3)	$P = 0.0019$
No. of retrieved oocytes (Mean, SD)	525 (23.9, 7.0)	324 (14.7, 7.0)[j]	$P < 0.0001$
No. of MII oocytes (Mean, SD)	428 (19.5, 4.7)	262 (11.9,3.3)	$P < 0.0001$
No. of 2 PN (%)	301 (70.3)	202 (77.1)	$P = 0.0312$
No. of Day 3 biopsied embryos (%)	208 (69.1)	152 (75.2)	Ns
No. of abnormal/informative embryos (%)*	107/204 (52.4)	74/148 (50.0)	Ns
No. of mosaic embryos (%)**	37/106 (34.9)	17/63 (26.9)	Ns
No. of blastocysts/biopsied embryos (%)	139 (66.8)	115 (75.6)	$P = 0.0441$
No. of chromosomally normal blastocysts (% per MII)	69 (16.1)	59 (22.5)	$P = 0.0236$
Mean chromosomally normal blastocysts per donor (SD)	3.1 (1.9)	2.7 (1.9)	Ns
Mean embryos transferred/donor (SD)	1.7 (1.0)	1.6 (1.0)	Ns
No. of pregnancies/transfer (%)	14/25 (56.0)	12/23 (52.2)	Ns
No. of miscarriages (%)	4 (28.6)	3 (25.0)	Ns
Implantation rate (%)	16/49 (32.6)	15/44 (34.1)	Ns
No. of live births	13	11	—

*Informative embryos were defined as embryos with a conclusive result after FISH analysis.
**Discordant results between blastomeres when two cells were analysed.
Comparisons between standard dose and reduced dose were performed in recipients with two completed cycles using Student's *t*-test and Fisher's exact test.

Table III Cycle outcome in the subgroup of donors with two completed treatments that resulted in similar recovery of MII oocytes with both stimulation regimens (Group 1 = 9 donors).

	Standard dose	Reduced dose	*P*-value
No. of donors (Mean age, SD)	9 (27.4, 4.1)	9 (27.4, 4.1)	–
No. of recipients (Mean age, SD)	9 (39.9, 5.3)	9 (41.3, 4.3)	Ns
Mean E_2 day HCG (pg/ml, SD)	2575.4 (994.3)	2112.7 (1004.7)	<0.0001
No. of MII oocytes retrieved (Mean, SD)	140 (15.5, 5.4)	119 (13.2, 5.3)	Ns
No. of 2 PN (%)	100 (71.4)	98 (82.3)	0.0269
No. of abnormal/informative embryos (%)*	38/58 (65.5)	29/57 (50.9)	Ns
No. of mosaic embryos (%)**	10/23 (43.5)	6/22 (27.3)	Ns
No. of blastocysts/biopsied embryos (%)	36/58 (62.1)	42/58 (72.4)	Ns
No. of chromosomally normal blastocysts (% per MII)	16 (11.4)	24 (20.2)	Ns
Mean chromosomally normal blastocysts per donor (SD)	1.8 (1.5)	2.7 (1.5)	Ns
Mean embryos transferred/donor (SD)	1.6 (0.9)	1.5 (0.9)	Ns
No. of pregnancies/transfer (%)	4/8 (50.0)	6/10 (60)	Ns
No. of miscarriages (%)	2 (50.0)	1 (16.7)	Ns
Implantation rate (%)	4/14 (28.6)	8/19 (42.1)	Ns
No. of live births	3	6	–

*Informative embryos were defined as embryos with a conclusive result after FISH analysis.
**Discordant results between blastomeres when two cells were analysed.
Comparisons between standard dose and reduced dose were performed in recipients with two completed cycles using Student's *t*-test and Fisher's exact test.

Table IV Cycle outcome in the subgroup of donors with two completed treatments that resulted in recovery of half the number of MII oocytes with decreased doses (Group 2: 13 donors).

	Standard dose	Reduced dose	*P*-value
No. of donors (Mean age, SD)	13 (26.0, 4.0)	13 (26.0, 4.0)	–
No. of recipients (Mean age, SD)	18 (41.9, 4.5)	14 (40.0, 4.7)	Ns
Mean E_2 day HCG (pg/ml, SD)	3389.3 (1001.5)	2047.8 (959.3)	0.0019
No. of MII oocytes retrieved (Mean, SD)	288 (22.1, 5.8)	143 (11.0, 5.6)	<0.0001
No. of 2 PN (%)	201 (69.8)	104 (72.7)	Ns
No. of abnormal/informative embryos (%)*	69/146 (47.3)	45/91 (49.4)	Ns
No. of mosaic embryos (%)**	27/83 (32.5)	11/41 (26.8)	Ns
No. of blastocysts/biopsied embryos (%)	103/150 (68.7)	73/94 (77.6)	Ns
No. of chromosomally normal blastocysts (% per MII)	53 (18.4)	35 (24.5)	Ns
Mean chromosomally normal blastocysts per donor (SD)	4.1 (1.9)	2.7 (1.9)	Ns
Mean embryos transferred/donor (SD)	1.6 (1.0)	1.5 (0.9)	Ns
No. of pregnancies/transfer (%)	10/17 (58.8)	6/13 (46.1)	Ns
No. of miscarriages (%)	2 (20.0)	2 (33.3)	Ns
Implantation rate (%)	12/35 (34.3)	7/25 (28.0)	Ns
No. of live births	10	5	–

*Informative embryos were defined as embryos with a conclusive result after FISH analysis.
**Discordant results between blastomeres when two cells were analysed.
Comparisons between standard dose and reduced dose were performed in recipients with two completed cycles using Student's *t*-test and Fisher's exact test.

not show statistical differences either for the standard protocol or for the reduced dose protocol when comparing between Groups 1 and 2.

Discussion

In this study we have described a significant increase in fertilization, blastocyst and chromosomally normal blastocyst development rates in high responder donors after receiving a reduced dose of gonadotrophins in a second cycle. The improvement in these variables compensated for the decrease in oocyte number following the reduced dose protocol. Therefore, the efficiency of oocyte donation cycles was similar with the two stimulation therapies in terms of the reproductive outcome of the recipients and the total number of live births.

A number of other studies support the findings we report here, namely that of high embryo aneuploidy rates (~50%) in high responder donors with high E_2 levels during ovarian stimulation, and an overall improvement of blastocyst development rates with the decreased gonadotrophin doses. In women undergoing IVF, there appears to be no increase in the incidence of chromosome abnormalities in aborted fetuses when compared with natural conceptions (Plachot, 1989; Ma *et al*., 2006; Martínez *et al*., 2010). However, in IVF cycles with a high response or even hyperstimulation syndrome, an increased risk of miscarriage and fetal aneuploidy has been reported (Nasseri *et al*., 1999; Raziel *et al*., 2002; O'Brien *et al*., 2009). Several studies in oocyte donation programmes have described high incidences of chromosome abnormalities on Day-3 embryos from oocyte donors with high oocyte yield recovery (Soares *et al*., 2003; Nelson *et al*., 2005; Munne *et al*., 2006). Additionally, our group showed that culture of mouse embryos with increasing E_2 levels impaired blastocyst formation and embryo adhesion (Valbuena *et al*., 2001). Taken together, these studies suggest that stimulation protocols and the type of response would have a direct effect on embryo quality, the number of blastocyst and aneuploidy.

In our study, the second treatment was started at least after 3 months, since it has been stated that this is the time needed for renewing the follicular pool (Gougeon hyphotesis). Moreover, our group performed a study to evaluate the effect of repeated COS in donors and the oocyte retrieval rate was maintained through consecutive cycles. No adverse effects of repeated stimulations were found in the quantity or the quality of the retrieved oocytes (Caligara *et al*., 2001). Therefore, the differences observed in embryo number and quality cannot be attributed to repeated stimulations and/or the interval between cycles in the same donors.

In recent years, many authors have proposed more physiological stimulation protocols to avoid the adverse effects of ovarian stimulation. Mild stimulation protocols aim to induce only a subtle interference in the physiological process of follicle domination (Fauser *et al*., 1999; Hohmann *et al*., 2003; Heijnen *et al*., 2007; Polinder *et al*., 2008; Verberg *et al*., 2009), assuming that these regimens will select the healthiest, chromosomally normal oocytes with the additional benefits of lower costs and less patient discomfort; but still, mild stimulation would not guarantee a complete selection against chromosomally abnormal embryos, since a moderate incidence of aneuploidy has still been reported in IVF embryos from mild stimulation protocols (Baart *et al*., 2007).

Ovarian stimulation in IVF/ICSI treatments induces the development and growth of multiple follicles, resulting in high numbers of oocytes for retrieval. In natural cycles, around the mid-follicular phase, the most mature follicle gains dominance over other cohort follicles. This dominant follicle continues its growth despite decremented FSH concentrations, whereas the remaining follicles from the recruited cohort enter atresia due to insufficient stimulation by FSH

(Fauser et al., 1993). An FSH 'window' may exist, whereby an upper FSH limit does not increase the follicle recruitment when such a limit is exceeded, while a lower 'threshold' prohibits the follicle development if it is not reached. An interesting observation of our study is that responses to the same reduced doses of gonadotrophins vary, as seen when dividing the patients into two response groups. In Group 1, both treatments resulted in a similar recovery of MII oocytes, suggesting that the standard dose had exceeded the FSH 'ceiling'. This excessive dose led to a decreased percentage of chromosomally normal blastocysts and a trend towards decreased implantation rates with the standard stimulation protocol. In Group 2, however, the reduced dose resulted in the recovery of 50% fewer MII oocytes, suggesting that both regimens appear between the limits of the FSH 'window'. Subsequently, similar rates of development of chromosomally normal blastocyst and implantation were obtained in Group 2. Yet a third pattern also emerged in 'uncompleted donors', in which a drastic reduction in the follicular development forced cancellation due to a very low response. These patients are likely to have a very narrow FSH window. Munne et al. (2006) also reported high variability in the incidence of chromosome abnormalities in embryos from young oocyte donors (average 57%, range 0–100%), and they associated these findings with limited success rates from some donors.

Regarding implantation, multiple factors can play a role, not just embryo aneuploidy. In this study, we have focused on the percentage of donors' euploid embryos that reached the blastocyst stage as a measure for embryo quality, and uterine factors in the recipients have not been ruled out. We are aware that PGS does not assess the whole chromosomal status and limitations of this approach included the limited number of chromosomes and blastomeres analysed. Newer approaches such as CGH and CGH arrays will allow a more comprehensive analysis of the embryo chromosomal status (Hellani et al., 2008; Fishel et al., 2010; Johnson et al., 2010). Regardless the technical limitations of our study, our group and other authors have shown that FISH analysis on Day-3 using additional rounds with subtelomeric probes increases the accuracy of the technique and the results are representative of the chromosomal status of the corresponding Day-5 embryo (Colls et al., 2007; Mir et al., 2010).

In conclusion, despite the limited number of donors included in our study, we suggest that, in high responders, ovarian stimulation protocols affect embryo quality following different patterns and therefore they should be individualized. We should find the 'minimal effective dose' for an optimal IVF outcome in each patient and donor. While a universal protocol will always be more cost-effective, this may not always be the best option to offer.

Authors' roles

C.R. had roles in design of the study, embryo biopsy and FISH analysis of embryos, data analysis and interpretation and writing of the manuscript. She is the corresponding author for the reviewing procedure. A.M. performed IVF, embryo biopsy and data analysis. P.A. participated in patients' recruitment, care during the medical treatment and writing of the manuscript. C.L. participated in patients' recruitment, care during the medical treatment and writing of the manuscript. L.R. performed FISH analysis of sperm and embryos. E.L. participated in patients' recruitment and care during the medical treatment. M.M. participated in patients'recruitment and care during the medical treatment. A.P. participated in patients'recruitment and care during the medical treatment. J.R. contributed to the conception, design, analysis and interpretation of data. He reviewed the intellectual content of the manuscript.

Acknowledgements

The authors wish to thank the clinicians, IVF embryologist and technicians and PGD teams of IVI-Valencia and IVI-Alicante clinics for their cooperation in the development of this study. Special thanks to all the staff involved in our oocyte donation programmes. We are very grateful to Dr Marcos Messeguer and Dr Nicolás Garrido for statistical support.

Funding

This study was partially supported by IZASA S.A., CH-Werfen Company, Spain.

References

Baart EB, Martini E, Eijkemans MJ, Van Opstal D, Beckers NG, Verhoeff A, Macklon NS, Fauser BC. Milder ovarian stimulation for *in vitro* fertilization reduces aneuploidy in the human preimplantation embryo: a randomized controlled trial. *Hum Reprod* 2007;**22**:980–988.

Caligara C, Navarro J, Vargas G, Simón C, Pellicer A, Remohí J. The effect of repeated controlled ovarian stimulation in donors. *Hum Reprod* 2001; **16**:2320–2323.

Colls P, Escudero T, Cekleniak N, Sadowy S, Cohen J, Munné S. Increased efficiency of preimplantation genetic diagnosis for infertility using 'no result rescue'. *Fertil Steril* 2007;**88**:53–61.

Fauser BC, Donderwinkel P, Schoot DC. The step-down principle in gonadotrophin treatment and the role of GnRH analogues. *Bailliere Clin Obstet Gynaecol* 1993;**7**:309–330.

Fauser BC, Devroey P, Yen SS, Gosden R, Crowley WF Jr, Baird DT, Bouchard P. Minimal ovarian stimulation for IVF: appraisal of potential benefits and drawbacks. *Hum Reprod* 1999;**14**:2681–2686.

Fishel S, Gordon A, Lynch C, Dowell K, Ndukwe G, Kelada E, Thornton S, Jenner L, Cater E, Brown A *et al.* Live birth after polar body array comparative genomic hybridization prediction of embryo ploidy-the future of IVF? *Fertil Steril* 2010;**93**:1006.e7–1006.

Garrido N, Zuzuarregui JL, Meseguer M, Simon C, Remohi J, Pellicer A. Sperm and oocyte selection and management: experience of a 10 year follow-up of more than 2100 candidates. *Hum Reprod* 2002; **17**:3142–3147.

Gianaroli L, Magli MC, Ferraretti AP, Fortini D, Tabanelli C, Gergolet M. Gonadal activity and chromosomal constitution of *in vitro* generated embryos. *Mol Cell Endocrinol* 2000;**161**:111–116.

Haaf T, Hahn A, Lambrecht A, Grossmann B, Schwaab E, Khanaga O, Hahn T, Tresch A, Schorsch M. A high oocyte yield for intracytoplasmic sperm injection treatment is associated with an increased chromosome error rate. *Fertil Steril* 2009;**91**:733–738.

Heijnen EM, Eijkemans MJ, De Klerk C, Polinder S, Beckers NG, Klinkert ER, Broekmans FJ, Passchier J, Te Velde ER, Macklon NS *et al.* A mild treatment strategy for *in vitro* fertilisation: a randomised non-inferiority trial randomized trial. *Lancet* 2007;**369**:743–749.

Hellani A, Abu-Amero K, Azouri J, El-Akoum S. Successful pregnancies after application of array-comparative genomic hybridization in PGS-aneuploidy screening. *Reprod Biomed Online* 2008;**17**:841–847.

Hohmann FP, Macklon NS, Fauser BC. A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist co-treatment for *in vitro* fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol. *J Clin Endocrinol Metab* 2003; **88**:166–173.

Johnson DS, Gemelos G, Baner J, Ryan A, Cinnioglu C, Banjevic M, Ross R, Alper M, Barrett B, Frederick J *et al*. Preclinical validation of a microarray method for full molecular karyotyping of blastomeres in a 24-h protocol. *Hum Reprod* 2010;**25**:1066–1075.

Katz-Jaffe MG, Trounson AO, Cram DS. Chromosome 21 mosaic human preimplantation embryos predominantly arise from diploid conceptions. *Fertil Steril* 2005;**84**:634–643.

Keskintepe L, Sher G, Keskintepe M. Reproductive oocyte/embryo genetic analysis: comparison between fluorescence *in situ* hybridization and comparative genomic hybridization. *Reprod Biomed Online* 2007; **15**:303–309.

Ma S, Philipp T, Zhao Y, Stetten G, Robinson WP, Kalousek D. Frequency of chromosomal abnormalities in spontaneous abortions derived from intracytoplasmic sperm injection compared with those from *in vitro* fertilization. *Fertil Steril* 2006;**85**:236–239.

Martínez MC, Méndez C, Ferro J, Nicolás M, Serra V, Landeras J. Cytogenetic analysis of early non-viable pregnancies after assisted reproduction treatment. *Fertil Steril* 2010;**93**:289–292.

Melo MAB, Meseguer M, Garrido N, Bosch E, Pellicer A, Remohi J. The significance of premature luteinization in an oocyte-donation programme. *Hum Reprod* 2006;**21**:1503–1507.

Mercader A, Valbuena D, Simón C. Human embryo culture. *Methods Enzymol* 2006;**420**:3–18.

Mir P, Rodrigo L, Mateu E, Peinado V, Milán M, Mercader A, Buendía P, Delgado A, Pellicer A, Remohí J *et al*. Improving FISH diagnosis for preimplantationgenetic aneuploidy screening. *Hum Reprod* 2010; May 19 [Epub ahead of print] PubMed PMID: 20488802.

Munne S, Magli C, Adler A, Wright G, de Boer K, Mortimer D, Tucker M, Cohen J, Gianaroli L. Treatment-related chromosome abnormalities in human embryos. *Hum Reprod* 1997;**12**:780–784.

Munne S, Ary J, Zouves C, Escudero T, Barnes F, Cinioglu C, Ary B, Cohen J. Wide range of chromosome abnormalities in the embryos of young egg donors. *Reprod Biomed Online* 2006; **12**:340–346.

Nargund G, Waterstone J, Bland J, Philips Z, Parsons J, Campbell S. Cumulative conception and live birth rates in natural (unstimulated) IVF cycles. *Hum Reprod* 2001;**16**:259–262.

Nasseri A, Mukherjee T, Grifo JA, Noyes N, Krey L, Copperman AB. Elevated day 3 serum follicle stimulating hormone and/or estradiol may predict fetal aneuploidy. *Fertil Steril* 1999;**71**:715–718.

Nelson JR, Potter DA II, Wilcox JG, Frederick JL, Kolb BA, Behr BR. Preimplantation genetic diagnosis in embryos created from oocytes donation. *Fertil Steril* 2005;**84**(Suppl. 1):S328. P-492.

O'Brien K, Lazar E, Athanassiou A, Ravnikar V. Ovarian hyperstimulation syndrome associated with fetal trisomy 21. *J Perinatol* 2009;**29**:388–390.

Pelinck MJ, Vogel NE, Hoek A, Arts EG, Simons AH, Heineman MJ. Minimal stimulation IVF with late follicular phase administration of the GnRH antagonist cetrorelix and concomitant substitution with recombinant FSH: a pilot study. *Hum Reprod* 2005;**20**:642–648.

Plachot M. Chromosome analysis of spontaneous abortions after IVF. A European survey. *Hum Reprod* 1989;**4**:425–429.

Polinder S, Heijnen EM, Macklon NS, Habbema JD, Fauser BJ, Eijkemans MJ. Cost-effectiveness of a mild compared with a standard strategy for IVF: a randomized comparison using cumulative term live birth as the primary endpoint. *Hum Reprod* 2008;**23**:316–323.

Raziel A, Friedler S, Schachter M, Strassburger D, Mordechai E, Ron-El R. Increased early pregnancy loss in IVF patients with severe ovarian hyperstimulation syndrome. *Hum Reprod* 2002;**17**:107–110.

Remohí J, Gutiérrez A, Cano F, Ruiz A, Simon C, Pellicer A. Long estradiol replacement in an oocyte donation programme. *Hum Reprod* 1995; **10**:1387–1391.

Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertil Steril* 2004;**81**:19–25.

Soares SR, Rubio C, Rodrigo L, Simón C, Remohí J, Pellicer A. High frequency of chromosomal abnormalities in embryos obtained from oocyte donation cycles. *Fertil Steril* 2003;**80**:656–657.

Soares SR, Troncoso C, Bosch E, Serra V, Simón C, Remohí J, Pellicer A. Age and uterine receptiveness: predicting the outcome of oocyte donation cycles. *J Clin Endocrinol Metab* 2005;**90**:4399–4404.

Tarkowski AK. An air drying method for chromosome preparations from mouse eggs. *Cytogenetics* 1966;**5**:394–400.

Valbuena D, Martin J, de Pablo JL, Remohí J, Pellicer A, Simón C. Increasing levels of estradiol are deleterious to embryonic implantation because they directly affect the embryo. *Fertil Steril* 2001;**76**:962–968.

Verberg MFG, Eijkemans MJC, Macklon NS, Heijnen EMEW, Baart EB, Hohmann FP, Fauser BCJM, Broekmans FJ. The clinical significance of the retrieval of a low number of oocytes following mild ovarian stimulation for IVF: a meta-analysis. *Hum Reprod Update* 2009;**15**:5–11.

Verpoest W, Fauser BC, Papanikolaou E, Staessen C, Van Landuyt L, Donoso P, Tournaye H, Liebaers I, Devroey P. Chromosomal aneuploidy in embryos conceived with unstimulated cycle IVF. *Hum Reprod* 2008;**23**:2369–2371.

Ziebe S, Bangsboll S, Schmidt KLT, Loft A, Lindhard A, Nyboe Andersen A. Embryo quality in natural versus stimulated cycles. *Hum Reprod* 2004;**19**:1457–1460.