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Milder ovarian stimulation for *in-vitro* fertilization reduces aneuploidy in the human preimplantation embryo: a randomized controlled trial

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BACKGROUND: To test whether ovarian stimulation for *in-vitro* fertilization (IVF) affects oocyte quality and thus chromosome segregation behaviour during meiosis and early embryo development, preimplantation genetic screening of embryos was employed in a prospective, randomized controlled trial, comparing two ovarian stimulation regimens. METHODS: Infertile patients under 38 years of age were randomly assigned to undergo a mild stimulation regimen using gonadotrophin-releasing hormone (GnRH) antagonist co-treatment (67 patients), which does not disrupt secondary follicle recruitment, or a conventional high-dose exogenous gonadotrophin regimen and GnRH agonist co-treatment (44 patients). Following IVF, embryos were biopsied at the eight-cell stage and the copy number of 10 chromosomes was analysed in 1 or 2 blastomeres. RESULTS: The study was terminated prematurely, after an unplanned interim analysis (which included 61% of the planned number of patients) found a lower embryo aneuploidy rate following mild stimulation. Compared with conventional stimulation, significantly fewer oocytes and embryos were obtained following mild stimulation ($P < 0.01$ and <0.05, respectively). Consequently, both regimens generated on average a similar number (1.8) of chromosomally normal embryos. Differences in rates of mosaic embryos suggest an effect of ovarian stimulation on mitotic segregation errors. CONCLUSIONS: Future ovarian stimulation strategies should avoid maximizing oocyte yield, but aim at generating a sufficient number of chromosomally normal embryos by reduced interference with ovarian physiology.

Key words: aneuploidy/embryo quality/*in-vitro* fertilization/ovarian stimulation/preimplantation genetic screening

Introduction

Human reproduction is a relatively inefficient process (Norwitz *et al.*, 2001). The chance of achieving a spontaneous pregnancy after timed intercourse is 20–30% (Evers, 2002; Taylor, 2003), significantly lower than ~70% in the rhesus monkey (Ghosh *et al.*, 1997), 80% in captive baboons (Stevens, 1997) or 90% in rodents and rabbits (Foote and Carney, 1988). Moreover, up to 30% of early human embryos fail to develop into viable fetuses (Wilcox *et al.*, 1988), largely due to chromosomal abnormalities (Boué *et al.*, 1975; Vorsanova *et al.*, 2005). The incidence of embryo aneuploidy increases with maternal age (Hassold and Hunt, 2001).

In-vitro fertilization (IVF) is the major treatment strategy for infertility, employing complex and costly ovarian stimulation protocols to generate multiple embryos (Fauser *et al.*, 2005; Macklon *et al.*, 2006). After ovarian stimulation and IVF, the best quality embryos are selected for transfer into the uterine cavity. Although embryo morphology is widely used to evaluate embryo quality, this subjective method provides only limited information concerning the chromosomal constitution (Munné, 2006). The introduction of fluorescence *in-situ* hybridization (FISH) on interphase nuclei allowed the screening of embryos for chromosomal aneuploidies, a procedure referred to as preimplantation genetic screening (PGS) (Thornhill *et al.*, 2005). Clinically, PGS is being advocated for older women (Munné *et al.*, 2003; Staessen *et al.*, 2004) and for patients with recurrent spontaneous abortion or repeated implantation failure (Gianaroli *et al.*, 2003; Pehlivan *et al.*, 2003; Platteau *et al.*, 2005). High rates of aneuploidy have been reported in these women. Moreover, in studies where the entire embryo was analysed, a high incidence of chromosomal mosaicism has been observed (Delhanty *et al.*, 1993; Bielanska *et al.*, 2002). The frequent occurrence of mosaicism,

resulting from mitotic segregation errors (Delhanty, 1997), is also reflected in the high incidence of discordant FISH results when two blastomeres are analysed by PGS (Baart *et al.*, 2004b, 2006).

The mechanisms underlying aneuploidy are still poorly understood. However, recent observations suggest that inaccuracies of the chromosome segregation machinery in oocytes are often involved, and this process is influenced by maternal age (Hassold and Hunt, 2001; Champion and Hawley, 2002). Preliminary observations suggest that aneuploidy in embryos may also be affected by ovarian stimulation regimens employed in IVF (Munné *et al.*, 1997; Katz-Jaffe *et al.*, 2005). Conventional IVF regimens routinely use a gonadotrophin-releasing hormone (GnRH) agonist long protocol co-treatment to prevent a premature luteinizing hormone (LH) rise. Down-regulation of pituitary function takes around 2 weeks, after which high doses of exogenous FSH are administered to induce multiple follicle growth. The recent availability of GnRH antagonists has enabled the development of milder approaches in ovarian stimulation. To prevent an LH rise, GnRH antagonist administration can be limited to the mid-to-late follicular phase (Fauser and Devroey, 2005), allowing the endogenous inter-cycle, FSH rise to be utilized for follicle stimulation. Cyclic follicle recruitment and initial stages of dominant follicle selection can proceed within the natural cycle and the use of exogenous FSH for inducing multiple follicle development can be restricted to the mid-late follicular phase (Fauser and Van Heusden, 1997; Fauser *et al.*, 1999; Hohmann *et al.*, 2003).

To test whether the conventional ovarian stimulation protocol and a mild stimulation approach differentially affect the competence of oocytes and embryos for proper chromosome segregation, PGS was employed in a prospective randomized controlled trial in a group of IVF patients younger than 38 years of age.

Materials and methods

Study design

All patients were recruited from the outpatient clinic at the Erasmus Medical Center and the Medical Center 'Rijnmond Zuid' from December 2002 to August 2005. Patients were randomly assigned to undergo a mild ovarian stimulation regimen using GnRH antagonist co-treatment or a conventional high-dose gonadotrophin regimen and GnRH agonist long protocol co-treatment. A schematic representation of the study is outlined in Figures 1 and 2. A population of infertile couples was targeted, who were not at an *a priori* increased risk for chromosomally abnormal embryos. Only women below 38 years of age, with a regular indication for IVF and with a partner with a sperm count >5 million progressively motile sperm per millilitre (prior to capacitation) were invited to participate. Additional inclusion criteria were: history of regular menstrual cycles (ranging from 25 to 35 days), body mass index between 19 and 29 $kg\,m^{-2}$, no known chromosomal abnormalities, no relevant systemic disease or uterine and ovarian abnormalities, no history of recurrent miscarriage, and no previous IVF cycles not resulting in an embryo transfer. Couples could participate in the study for one cycle only. Prior to commencing the study, ethical approval was received from the Dutch Central Committee on Research Involving Human Subjects (CCMO) and the local institutional Ethics Committee. Written informed consent was obtained from each couple.

A higher cancellation rate before oocyte retrieval and less embryos was expected following mild ovarian stimulation (Hohmann *et al.*, 2003). Therefore, randomization to one of the two treatment groups was performed according to a computer-generated randomization schedule in a ratio of 4 : 6 (conventional group: mild group; see statistical paragraph), assigned via numbered sealed envelopes. After the patient agreed to participate, the next available numbered envelope on entry into the study was opened by the treating physician during the preparatory IVF consultation. Blood samples were drawn from each patient on cycle Day 3 or 4 before the start of stimulation, to assess baseline FSH and inhibin B levels.

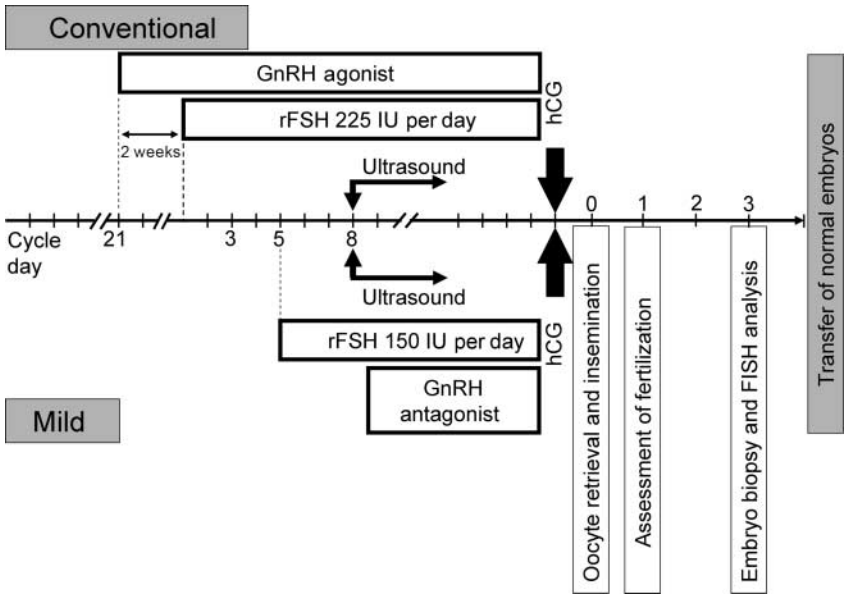

Figure 1. Schematic representation of the two ovarian stimulation protocols for IVF and laboratory procedures for preimplantation genetic screening (PGS).

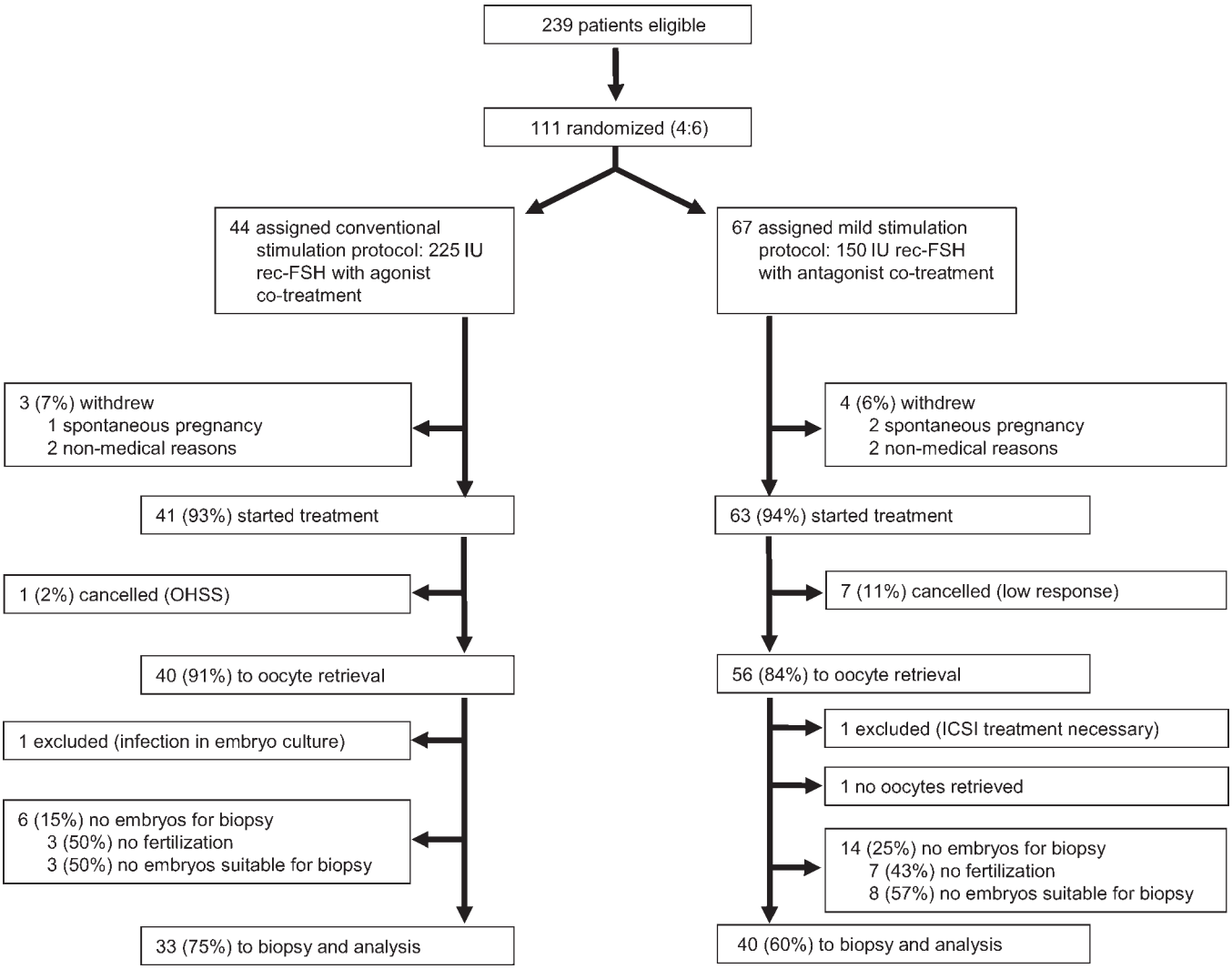

Figure 2. Trial profile and flow of patients. Ovarian hyperstimulation syndrome (OHSS), stimulation was discontinued because of signs of an imminent OHSS; low response, only one growing follicle observed at ultrasound; ICSI, Intracytoplasmic sperm injection necessary due to unexpected poor semen quality on the day of oocyte retrieval.

Multifollicular ovarian stimulation

Patients randomized to undergo conventional ovarian stimulation were treated for at least 2 weeks with the GnRH agonist Triptorelin (Decapeptyl®, Ferring BV, Hoofddorp, The Netherlands) 0.1 mg per day s.c., starting 1 week before the expected menses. Following pituitary down regulation, patients received a fixed daily dose of 225 IU s.c. recombinant FSH (Puregon®, NV Organon, Oss, The Netherlands). Preferably, FSH treatment was started on Mondays or Tuesdays to reduce chances for a biopsy procedure on weekends. Patients randomized to the mild stimulation protocol were treated with a fixed dose of 150 IU s.c. recombinant FSH (Puregon®) starting on cycle Day 5. GnRH antagonist co-treatment (Orgalutran®, NV Organon) at 0.25 mg per day s.c. was initiated on the day the leading follicle reached a diameter of 14 mm (Hohmann *et al.*, 2003). To induce final oocyte maturation, a single dose of 10 000 IU s.c. hCG (Pregnyl®, NV Organon Oss, The Netherlands) was administrated as soon as the leading follicle had reached a diameter of 18 mm and at least one additional follicle had reached a diameter of 15 mm. Oocyte retrieval was carried out 35 h after hCG injection by transvaginal ultrasound-guided puncture of follicles.

In-vitro fertilization, embryo culture and biopsy

After oocyte retrieval, IVF and embryo culture were performed as described previously (Huisman *et al.*, 2000; Hohmann *et al.*, 2003). On day 3 after oocyte retrieval, embryos resulting from normally fertilized oocytes (as evidenced by two visible pronuclei) were scored according to previously published morphological criteria (Hohmann *et al.*, 2003), blinded to the stimulation protocol. These included cell number, regularity of blastomeres, fragmentation and morphological aspects including granulation. Normal morphology was defined as embryos with timely development, $<20\%$ fragmentation, about equal sized blastomeres and small or no irregularities observed in the cytoplasm. Biopsy was performed on embryos with more than five blastomeres. Two cells were removed unless the embryo consisted of only six blastomeres. Embryo biopsy and fixation of biopsied cells were performed as described elsewhere (Baart *et al.*, 2004a).

FISH analysis and diagnosis

FISH analysis was performed to determine the copy number of nine chromosome pairs (1, 7, 13, 15, 16, 18, 21, 22, X and Y), as previously described (Baart *et al.*, 2004, 2004b). FISH results were interpreted by

two independent observers, blinded to the stimulation protocol. For enumeration of the signals on single blastomere nuclei, we used previously published scoring criteria (Munné *et al.*, 1998). A nucleus was considered normal if it showed the normal (diploid) amount of signals for the chromosomes investigated and abnormal if one or more of the chromosomes investigated showed an increased or decreased number of signals. In case two cells were available, embryos were classified as normal (both nuclei normal FISH results), uniformly abnormal (both nuclei showing the same abnormality) or mosaic (one normal and one abnormal nucleus or two abnormal nuclei with each nucleus showing different chromosome abnormalities). No more than two normal embryos were transferred to the patient.

As a result of chromosomal mosaicism, the definition of an abnormal embryo is different if one cell is available for analysis when compared with two available cells. Also, embryos where only one cell could be biopsied differ developmentally from embryos where a two-cell biopsy was possible. To obtain uniformity for statistical analysis, we used two approaches. First, all embryos were classified in retrospect as either normal or abnormal on the basis of the FISH results obtained from the first biopsied blastomere, even if two cells were available. Second, the analysis was repeated for only those embryos with a PGS diagnosis based on two cells.

Outcome measures

Primary outcome measures were ovarian response, as assessed by the number of oocytes obtained and the proportion of chromosomally abnormal embryos per patient. This was expressed as the ratio of abnormal embryos on the number of embryos diagnosed per patient. Secondary outcome measures were the proportion of fertilized oocytes, the proportion of embryos with normal morphology and the proportion of embryos biopsied and diagnosed. All proportions were first calculated per patient and then averaged for each treatment group. As women were randomly assigned to two different ovarian stimulation protocols to detect possible differences in chromosomal abnormality rates of embryos generated, this is the correct unit of statistical analysis.

Statistical analysis

Before commencing the study, the sample size was determined. We assumed a reduction in the aneuploidy rate from 30% after conventional ovarian stimulation to 20% after mild ovarian stimulation. We calculated that 293 embryos in each group would achieve an 80% power to detect this 10% difference at an alpha level of 0.05 with the use of a two-sided *t*-test. With an expected average of six embryos following conventional and four following mild ovarian stimulation and an expected drop-out rate of one-third of the patients from each group, the total number of subjects to be included was 73 patients in the conventional group and 109 patients in the mild group. However, due to slow patient inclusion and an increasing concern regarding the safety of a two-cell biopsy with respect to the implantation potential of the embryo (Cohen and Munné, 2005), an unplanned interim analysis was performed after the inclusion of 111 patients. The proportion of chromosomally abnormal embryos per patient was found to be significantly reduced after mild ovarian stimulation [$P = 0.02$, which is below the Pocock critical bound of 0.0354 for a single interim analysis after 61% (111 of 181) of patients had been included (Pocock, 1977)] and the study was terminated.

A χ^2 test was used to test for differences between the two groups in the percentage of patients with oocyte retrieval and embryo biopsy. A *t*-test was used to test for differences in continuous variables and parameters that were, per patient, averaged over oocytes or over embryos, e.g. the average morphology score of the embryos or the percentages of abnormal embryos. Pearson's correlation coefficient was used to test for assocation between parameters of ovarian response and the proportion of abnormal embryos. To see whether these associations differed between the two groups, a test for interaction in analysis of variance (ANOVA) was used. *P*-values <0.05 were considered statistically significant, except for the proportion of chromosomally abnormal embryos per patient, the primary outcome measure, where $P < 0.0354$ was considered significant, according to Pocock's method for interim analysis (see above).

Results

Patient and study characteristics

One-hundred and eleven patients were included. Initial screening characteristics (median and range) for both groups are presented in Table I. There were no significant differences between the groups in demographic variables or initial screening parameters. In 73 (66%) patients, IVF treatment resulted in the availability of embryos for PGS. Reasons for patient drop-out and exclusion from analysis are given in Figure 2.

Table II presents the number of oocytes retrieved and successfully fertilized and the biopsy results. The number and results of embryos successfully analysed on one or two blastomeres are given. To obtain uniformity for statistical analysis, the embryos were classified in retrospect as either normal or abnormal on the basis of the FISH results obtained from the first biopsied blastomere, even if two cells were available. This resulted in 61/159 (38%) chromosomally normal embryos in the conventional stimulation group and 71/143 (50%) normal embryos in the mild stimulation group. The proportion of normal embryos was subsequently calculated per patient, as were the other primary and secondary outcome measures.

Table I. Baseline characteristics for IVF patients in the two different treatment groups for ovarian stimulation

	Conventional stimulation ($n = 44$)	Mild stimulation ($n = 67$)
Female age (years)	34.1 (28–37)	33.2 (22–37)
FSH level on cycle Day 3 or 4 (IU l^{-1})	8.1 (4.4–13.8)	7.6 (5.5–18.4)
Inhibin B level on cycle Day 3 or 4 (ng l^{-1})	86 (2–1056)	88 (15–593)
No. of previous IVF cycles, *n* (%)		
0	32 (73)	55 (82)
1	3 (7)	3 (4)
2	6 (14)	4 (6)
3	3 (7)	5 (7)

Data are expressed as median values and range, unless otherwise stated.

Table II. Number of embryos biopsied and analysed by Fluorescent *in-situ* hybridization (FISH) on one or two blastomeres after conventional or mild stimulation

	Conventional stimulation	Mild stimulation
No. of oocytes obtained	484	459
No. of embryos (2pn) obtained	271	260
No. of embryos suitable for biopsy	184 (68)	157 (60)
No. of embryos diagnosed	159 (86)	143 (91)
No. of embryos diagnosed based on two cells	98 (62)	96 (67)
Normal	27 (28)	37 (39)
Abnormal	12 (12)	14 (15)
Abnormal/normal mosaic	32 (33)	20 (21)
Abnormal/abnormal mosaic	27 (28)	25 (26)
No. of embryos diagnosed based on one cell	61 (38)	47 (33)
Normal	20 (33)	16 (34)
Abnormal	41 (67)	31 (66)

Values between parentheses are percentages.

Chromosomal competency of embryos correlates with ovarian response after mild stimulation

The distribution of the number of oocytes retrieved per patient was different following conventional and mild ovarian stimulation, with skewing of the curve following mild stimulation towards fewer oocytes (Figure 3a and b). For each stimulation protocol, differences in the proportion of abnormal embryos based on one-cell diagnosis were correlated to ovarian response per patient (Figure 3c and d). Within the mild group, a significant positive correlation (Pearson correlation $= 0.4$; $P = 0.006$) was observed between the number of oocytes obtained and the proportion of abnormal embryos. In the conventional stimulation group, no correlation was observed (Pearson correlation $= -0.08$; $P = 0.679$). The distribution found after mild stimulation was significantly different from the one found after conventional stimulation ($P = 0.016$; test for interaction in ANOVA).

Mild ovarian stimulation results in a reduced proportion of abnormal and mosaic embryos

Table III summarizes outcome measures and clinical results per patient. Although more oocytes were obtained per patient following conventional ovarian stimulation (12.1 versus 8.2, $P = 0.001$), no differences were observed in fertilization rates or percentage of embryos biopsied and diagnosed between the groups. The proportion of embryos with normal morphology was higher after mild, when compared with conventional ovarian, stimulation (51 versus 35%; $P = 0.04$).

On the basis of the first cell biopsied, the proportion of chromosomally abnormal embryos per patient was significantly decreased following mild stimulation (Table III). The percentage of abnormal embryos relative to the number of embryos diagnosed was 45% following mild stimulation (40 patients) compared with 63% following conventional stimulation (33 patients; $P = 0.02$). Mild stimulation resulted in significantly less oocytes and embryos, but there was no difference between the two study groups in the average number of chromosomally normal embryos (1.8) obtained per patient (Figure 4).

By analysing the group of embryos in which two cells were available for diagnosis, insight into chromosomal mosaicism could be obtained (Table III). In this group, the diagnosis could be normal, abnormal or mosaic. Overall abnormality rates (abnormal and mosaic embryos) were 55% following mild (38 patients) and 73% following conventional ovarian stimulation (30 patients; $P = 0.046$), confirming the difference in abnormality rates observed after single-cell diagnosis. However, the proportion of mosaic embryos per patient was more significantly increased following conventional ovarian stimulation (65 versus 37%; $P = 0.004$). This observation indicates that the increase in abnormal embryos is mainly due to an increase in mitotic segregation errors in early embryonic cleavage divisions.

Patient selection does not explain observed differences in aneuploidy rate

Although not significant (χ^2; $P = 0.097$), a trend was observed following mild stimulation towards a higher rate of drop out before PGS analysis, since 27 out of 67 (40%) patients were either lost before oocyte retrieval, fertilization or embryo biopsy (Figure 2). After conventional stimulation, 11 out of 44 (25%) patients did not reach PGS analysis. The retrieval of only a few oocytes after conventional stimulation has been attributed to ovarian ageing (Beckers *et al.*, 2002; de Boer *et al.*, 2002), and an age-dependent increase in chromosomal abnormalities in oocytes has been reported (Hassold and Hunt, 2001). It is possible that women with more advanced ovarian aging undergoing mild stimulation were less likely to meet the criteria for oocyte retrieval, thus creating a selection bias for women with a reduced incidence of aneuploid embryos. To exclude such a potential selection bias, female age and two distinct markers for ovarian ageing (early follicular phase FSH and inhibin B levels) (Groome *et al.*, 1996; Creus *et al.*, 2000) were retrospectively compared between the patients who did and those patients who did not reach PGS following mild stimulation. No differences were observed in age (33.2 ± 3.2 versus 32.3 ± 3.4 years; $P = 0.31$), baseline serum levels of FSH (7.8 ± 2.2 IU l^{-1} versus 7.7 ± 3.3 IU l^{-1}; $P = 0.93$) or inhibin B (110 ± 75 ng l^{-1} versus 108 ± 129 ng l^{-1}; $P = 0.96$). Therefore, we find no indications that women with more advanced ovarian ageing showed a higher drop-out rate after mild ovarian stimulation. However, it cannot be excluded that other mechanisms for patient selection may be involved.

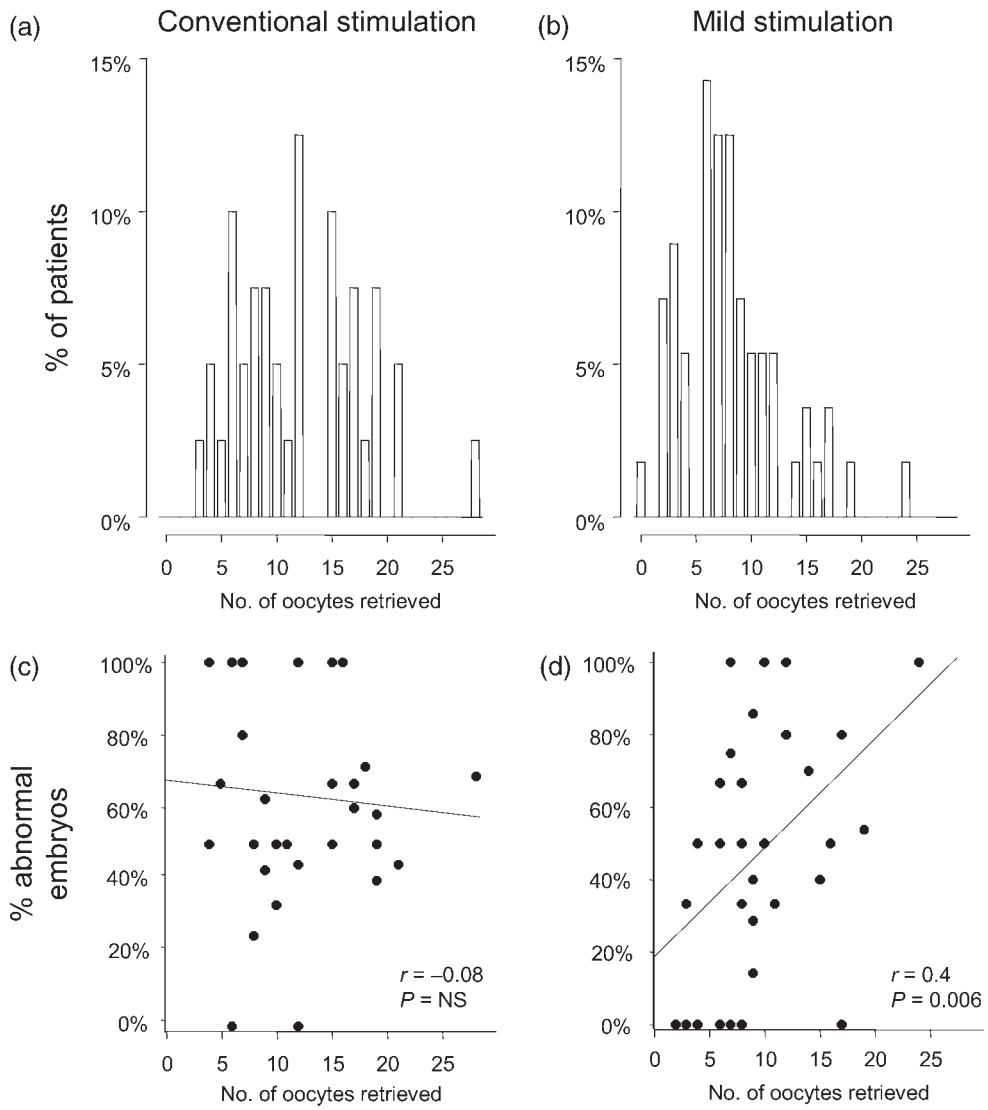

Figure 3. Distribution of number of oocytes retrieved per patient and relationship between oocyte number and percentage of abnormal embryos generated following conventional (**a** and **c**) and mild ovarian stimulation for IVF (**b** and **d**).

Discussion

The introduction of GnRH antagonists allows ovarian stimulation for IVF without disrupting early follicular phase dynamics. In the present randomized trial, we compare the effect of a mild stimulation approach to a conventional stimulation regimen by assessing chromosomal competence of embryos. We found that mild stimulation is associated with a reduction in the number of oocytes retrieved and embryos generated. However, the proportion of chromosomally normal embryos is significantly increased. Consequently, the number of chromosomally competent embryos obtained per woman is similar (around two), despite a significant reduction in the total number of embryos in the mild stimulation group. In addition, analysis of two cells per embryo suggests that the increase in chromosomal abnormalities observed after conventional stimulation, is mainly due to an increased incidence of chromosomal mosaicism.

In the mild stimulation group, patients received lower doses of exogenous FSH. Since no down-regulation of endogenous FSH production has taken place, serum FSH concentrations on cycle Day 8 were shown to be equivalent to those observed in conventional stimulation with a high dose of exogenous FSH (Hohmann *et al.*, 2003). The difference between the two stimulation protocols involves both follicle recruitment and selection. In the natural cycle, a synchronous cohort of follicles gains gonadotrophin dependence due to the intercycle rise in endogenous FSH and continues its development. The dominant follicle is selected around the mid-follicular phase from this pool of 20–30 antral follicles. Decreasing FSH concentrations are crucial for single dominant follicle selection (Zeleznik and Hillier, 1984; Fauser and Van Heusden, 1997). In addition, the dominant follicle suppresses subdominant follicles through intraovarian mechanisms (Baker and Spears, 1999). In mild stimulation, interference with decreasing FSH gives rise to the development of multiple dominant follicles, whereas follicle recruitment and the initial stages of selection remain unaffected. In contrast, during conventional ovarian stimulation, including pituitary down-regulation by GnRH agonist

Table III. Outcomes after IVF and preimplantation genetic screening diagnosis following conventional or mild ovarian stimulation

	Conventional stimulation	Mild stimulation	*P**	Difference (95% CI)
IVF characteristics				
No. of patients	40	55[a]		
Oocytes retrieved (*n*)	12.1 ± 5.7	8.3 ± 4.7	<0.01	3.7 (1.6–5.9)
Fertilization rate (%)	57 ± 28	55 ± 30	0.81	1.5 (−10–13)
Embryos (2pn)	6.8 ± 5.0	4.7 ± 3.9	0.03	2.0 (0.2–3.9)
Good quality embryo rate[b] (%)	35 ± 29	51 ± 40	0.04	−17 (−32–1)
Diagnosis based on first cell biopsied[c]				
No. of patients	33	40		
Embryos diagnosed	4.8 ± 3.5	3.6 ± 2.7	0.10	1.2 (−0.2–2.7)
Percentage of embryos diagnosed (%)	40 ± 22	45 ± 23	0.38	−5 (−15–6)
Abnormal embryos/embryos diagnosed (%)	63 ± 28	45 ± 35	0.016	19 (4–34)
Diagnosis based on two cells[d]				
No. of patients	30	38		
Abnormal embryos/embryos diagnosed (%)	73 ± 33	55 ± 42	0.046	19 (0.3–36)
Mosaic embryos/embryos diagnosed (%)	65 ± 37	37 ± 39	0.004	28 (10–47)
Clinical outcome measures				
Embryos/transfer	1.45 ± 0.51	1.46 ± 0.51		
Ongoing pregnancy rate/started cycle (%)	7/41 (17)	12/63 (19)		
Ongoing pregnancy rate/transfer (%)	7/31 (23)	12/35 (34)		

Data are expressed on a per patient basis and are presented as mean and SD, unless otherwise stated.
* *P*-values are from a two-sample t-test.
[a]One patient out of the 56 undergoing oocyte retrieval yielded no oocytes.
[b]Embryos with normal morphology were defined as embryos with timely development, <20% fragmentation, equally sized blastomeres and small or no irregularities observed in the cytoplasm.
[c]Diagnosis of normal or abnormal embryos was based on the FISH results of one cell. If two cells were available, the first cell biopsied was determined in retrospect and used for diagnosis. Rates were calculated first per patient and then averaged.
[d]Only embryos where two cells were available for diagnosis were taken into account. An embryo was considered abnormal if at least one of the two cells showed an abnormal result.

co-treatment, natural follicle recruitment and selection is completely overruled, allowing the non-discriminate growth of many follicles at different developmental stages.

Following recruitment into the growing pool, the oocyte expands from 35 to 120 μm in diameter, which represents a 100-fold increase in volume over a period of several months (Gosden and Bownes, 1995). Oocyte growth and maturation is interlinked with follicle development, and bi-directional signalling occurs between oocytes and granulosa cells (Eppig, 2001). Oocytes have to achieve both nuclear and cytoplasmic maturity in order to sustain the early stages of embryonic development (Albertini *et al.*, 2003). Recently, experimental evidence in mice showed that disturbances in the complex interplay of signals regulating folliculogenesis may alter the late stages of oocyte growth, increasing the risk for chromosome malsegregation in subsequent meiotic divisions (Hodges *et al.*, 2002). These findings offer a rationale for our findings of an increased proportion of chromosomally normal embryos after mild ovarian stimulation. However, the possibility that the different GnRH analogues directly influence the chromosomal constitution of the embryos in this study cannot be ruled out.

Interestingly, our results suggests that the increase in the proportion of abnormal embryos was mainly due to an increase in mitotic segregation errors, leading to mosaic embryos. The embryonic genome does not become active until the eight cell stage (Braude *et al.*, 1988), until then the cell cycle machinery is dependent on the protein and mRNA content of the oocyte. Recently, a direct link has been established between defects in the oocyte and an increased incidence in mitotic segregation errors. An experimental mouse model with an inactivated protein subunit of the meiotic synaptonemal complex (SYCP3) revealed not only an increased level of segregation errors at the first meiotic division but also showed a substantial increase in mitotic segregation errors during the first embryo cleavage divisions (Yuan *et al.*, 2002; Lightfoot *et al.*, 2006). More research into the developmental potential of embryos with mitotic segregation errors is needed to understand the significance of mosaicism in human embryos. However, there are indications that the implantation potential of embryos mosaic for trisomy 21 is reduced (Katz-Jaffe *et al*., 2004).

Within the mild stimulation group, we also found that a low oocyte yield is associated with a decrease in the proportion of aneuploid embryos. A previous study showed mild stimulation to result in high-quality embryos for transfer, as indicated by good embryo morphology, and pregnancy rates comparable to those following conventional ovarian stimulation (Hohmann *et al.*, 2003). Moreover, although no pregnancies were obtained in women who had produced four or less oocytes following the conventional protocol, the majority of pregnancies obtained following mild ovarian stimulation occurred in women where four or less oocytes were retrieved. A low number of oocytes retrieved after stimulation may, therefore, represent an appropriate response to mild stimulation. In contrast, a similar low response occurring after conventional ovarian stimulation is indeed indicative of ovarian ageing (Beckers *et al.*, 2002; de Boer *et al.*, 2002). Although few pregnancies were achieved, the pregnancy rates we observed after PGS are within the range reported by the ESHRE PGD consortium (Harper *et al.*, 2006).

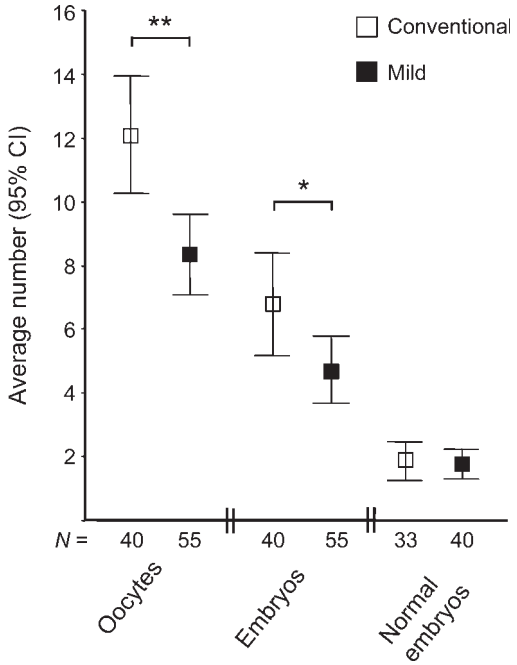

Figure 4. Oocyte and embryo yield and embryos successfully biopsied and diagnosed by fluorescent *in-situ* hybridization (FISH) as chromosomally normal on the basis of FISH results from one cell following conventional and mild stimulation. Values are expressed on a per patient basis and are represented as mean. Bars indicate the 95% confidence interval. $*P < 0.05$, $**P < 0.01$.

Although implantation, ongoing pregnancy and ultimately live birth are the most meaningful outcome measures, they are only partially influenced by embryo quality and can only be determined for the embryos transferred. In the current study, PGS was used as a parameter for assessing embryo quality. It revealed a significant effect of the ovarian stimulation regimen on the chromosome segregation ability of the resulting embryos. This observation supports the hypothesis that only the follicle with the most competent oocyte is selected during the natural cycle in the mono-ovulatory human species. The present mild stimulation protocol represents less interference with ovarian physiology, which may give rise to a higher proportion of developmentally competent oocytes. This concept is also consistent with an extensive analysis of historical data showing no significant improvement of the pregnancy rate per oocytes retrieved using ovarian stimulation when compared with IVF results in the early 1980s, when IVF was performed without ovarian stimulation (Inge *et al.*, 2005).

In conclusion, the present study shows, for the first time, that mild ovarian stimulation results in fewer oocytes and a decreased proportion of aneuploid and mosaic embryos. Obviously, our findings need to be confirmed by other groups, as both treatment strategies and PGS methodologies vary largely between centres. However, based on the current findings, we would like to propose that future ovarian stimulation strategies should not focus on obtaining as many oocytes as possible. Instead, strategies should aim at less interference with ovarian physiology, thus minimizing embryo aneuploidy rate and facilitating selection of the best quality embryo for transfer.

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