Article

Relationship between embryo quality and aneuploidies

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Abstract

Many high-grade embryos selected for transfer according to their morphological evaluation were detected to have chromosomal abnormalities after aneuploidy screening for infertility by preimplantation genetic diagnosis (PGD). The aim of this study was to detect if there is any correlation between embryo quality and genetic status. The chromosomal status of the day three embryos was studied by multicolour fluorescence in-situ hybridization for chromosomes 13, 18, 21, X and Y. PGD was performed on 132 patients for 1107 embryos. The correlation between embryo quality and aneuploidy was analysed. The analysis showed that a large proportion of normal embryos (50.7%, $n = 280$) were grade I. In addition, a considerably high proportion of aneuploid embryos (36.1%, $n = 83$) were evaluated as grade I. There was a significant relationship between PGD results and embryo grades ($P = 0.001$). Of the 69 polyploid embryos, 21.7% were grade I and 37.8% were grade II. Of the 83 haploid embryos, 27.8% were grade I and 34.9% were grade II. Euploidy was positively related to morphological grade of embryo ($P = 0.001$). It was also possible for chromosomally abnormal embryos to have a good developmental potential, and they could be selected for embryo transfer unless the PGD procedure was applied.

Keywords: *aneuploidy, chromosomal abnormalities, embryo quality, preimplantation genetic diagnosis*

Introduction

With the introduction of fluorescence in-situ hybridization (FISH), preimplantation genetic diagnosis (PGD) has become an important tool in reproductive medicine. It has been previously shown that chromosome analysis of human embryos indicated higher rates of aneuploidy than those reported for prenatal testing (Warburton *et al*., 1986; Eiben *et al*., 1994; Munné *et al*., 1995; Márquez *et al*., 2000; Sandalinas *et al*., 2001; Clouston *et al*., 2002), revealing that many chromosomally abnormal embryos are eliminated early during development. There is a natural selection against chromosomal abnormalities, leading to a decrease in the incidence of chromosomal abnormalities over the duration of pregnancy, which is ~6% in stillborns (Machine and Crolla, 1974) and 0.6% in live births (Nielsen, 1975). Thus, chromosomal abnormality is one of the major causes of embryonic loss (Sandalinas *et al*., 2001; Los *et al*., 2004). The most common reason for spontaneous abortions are thought to be some de-novo numerical abnormalities, especially autosomal trisomies for chromosomes 13, 14, 15, 16, 21 and 22, followed by monosomy X (Strom *et al*., 1992; Stephenson *et al*., 2002).

It has been reported and suggested that instead of using cleavage stages, culturing embryos to the blastocyst stage increases the implantation rate (Gardner *et al*., 1998; Coskun *et al*., 2000; Levitas *et al*., 2004). Blastocyst formation is a good indicator of the developmental potential of a preimplantation embryo. Analyses have been made to reveal if any correlation between chromosomally abnormal embryos and embryo development exists, and to see whether blastocyst formation is an effective tool for selecting against chromosomally abnormal embryos (Munné *et al*., 1995; Márquez *et al*., 2000; Sandalinas *et al*., 2001). It was also concluded that not all chromosomally affected embryos were morphologically abnormal and failed to form blastocyst or vice versa (Sandalinas *et al*., 2001). In

a preliminary study by Rubio *et al*. (2003), the effect of the different chromosomal abnormalities on embryo development was analysed. It was revealed that a considerable proportion of aneuploid embryos have the ability to reach the blastocyst stage, and among aneuploidies trisomies were more likely to develop into blastocysts when compared with autosomal monosomies.

The focus of this study was to determine if there was any relationship between cleavage-stage embryo quality and chromosomal aneuploidies, in order to evaluate whether or not morphological evaluation, *per se*, is adequate for embryo selection before embryo transfer.

Materials and methods

This retrospective study was performed on day three embryos using a multicolour FISH technique, which enabled selection of embryos for transfer that were chromosomally normal for the chromosomes analysed.

Patients

Patient characteristics and PGD cycles were summarized in **Table 1**. Embryos were obtained from 132 patients undergoing PGD for infertility. Patients underwent ovarian stimulation and requested PGD for infertility (advanced maternal age, recurrent IVF failure and spontaneous abortions) in the GenArt Woman Health and Reproductive Biotechnology Clinic. Patients were classified into three groups according to their age: ≤33 years (n = 53), 34–39 years (n = 44) and ≥40 years (n = 35). The mean age was 35.45 ± 5.6 years.

Stimulation protocol and oocyte recovery

Patients were treated with short gonadotrophin-releasing hormone (GnRH) agonist starting on the second day of the menses to assist the flare-up effect, along with gonadotrophin stimulation if vaginal ultrasonography revealed a thin endometrium and normal ovarian echogenity. Tryptorelin acetate (Decapeptyl; Ferring GmbH, Kiel, Germany) 0.1 mg subcutaneous daily or nafarelin acetate (Synarel; Searle Pharmaceuticals, France) 200 μg intranasally three times a day was administered for each patient. Stimulation was initiated with 300 IU follitropin alpha i.m. (Gonal-F; Serono, Geneva, Switzerland), except in patients with known poor response, where 450 IU or 600 IU was used for better recruitment along with dexamethasone 0.5 mg p.o. daily. Human chorionic gonadotrophin (HCG) (Profasi; Serono) was administered 10,000 IU i.m. exactly 36 h before oocyte retrieval when at least three mature follicles with a diameter of 18 × 18 mm were encountered during vaginal sonography. Oocyte retrieval was accomplished by ultrasound-guided follicular puncture 36 h after the HCG administration. In-vitro oocyte culture and preparation for intracytoplasmic sperm injection (ICSI) have been described elsewhere (Emiliani *et al.*, 1999). Fertilization was performed in all cases by the ICSI technique.

Embryo morphology classification

Biopsied embryos were also evaluated morphologically and monitored daily. The chromosomally normal embryos with good morphology were selected to transfer on day 4, whereas the chromosomally abnormal ones and those without results were discarded. The embryos were scored according to grading criteria that were modified from those of Veeck *et al*. (1998). This scoring system contains five main embryo morphological grades: (i) an embryo with blastomeres of equal size and no cytoplasmic fragmentation was classified as a high-quality embryo; Grade I; (ii) an embryo with blastomeres of equal size and minor cytoplasmic fragmentation covering ≤10% of the embryo surface was classified as a qualified embryo; Grade II; (iii) an embryo with blastomeres of equal or unequal size with significant cytoplasmic fragmentation covering >10% of the embryo surface was classified as an insufficient embryo; Grade III; (iv) an embryo with few blastomeres of any size and severe fragmentation covering ≥50% of the embryo surface was classified as Grade IV; (v) an embryo with few blastomeres of any size with severe fragmentation covering ≥50% of the embryo surface was classified as Grade V. Grade V embryos were excluded from blastomere biopsy and further processes.

Blastomere biopsy

Embryo biopsy was performed on day three embryos, which were at the 5–12-cell stage of development. Embryos were placed in droplets containing Ca^{2+}-Mg^{2+}-free medium (G-PDG Scandinavian IVF, Gothenburg, Sweden) for 10 min, and the zona pellucida was dissected with a mechanical method. A blastomere was gently removed with an aspiration pipette. Each blastomere was then transferred to a hypotonic solution (1% sodium citrate, 0.6 mg/ml human serum albumin-HSA). The nuclei were fixed on a glass slide using a fixative solution containing Tween 20 (0.1% in 1 M HCl).

FISH analysis

Hybridization after the fixation technique was accomplished using the fluorescent labelled probes for chromosomes X, Y, 13, 18 and 21 (Vysis Inc., Downers Grove, IL, USA) in a multicolour FISH protocol. The slides were rinsed twice in 2× SSC (sodium chloride and sodium citrate) and dehydrated through an ethanol series (70, 85, 90%) and then air dried. Dehydrated samples were then denatured at 73°C on the hot plate for 5 min. The probe mix was also denatured in a water bath at 73°C, then 1.5 μl of the mixture was added on the fixed blastomere and covered with a 1 cm round cover slip. The slide was left to hybridization for 5 h at 37°C. All probes were directly labelled with fluorophores and obtained commercially (Vysis Inc.). The post-hybridization wash was performed in 0.4 × SSC/0.3 Nonidet P40 at 42°C for 2 min. Counterstaining was done by using antifade-II. The slides were examined using a Chroma Technology filter set; (Santa Clara, CA, USA) allowing the visualization of red, green, aqua, blue and gold (chromosomes 13, 21, 18, X and Y respectively), and images were captured with an Applied Imaging system (Cytovision version 2.7; Newcastle, England).

Embryo chromosome pattern classification

Embryos were classified according to criteria previously proposed by Munné *et al*. (2003) as follows: '(i) when the cell

had two copies of each chromosome analysed, the embryo was classified as presumptively normal; (ii) when the cell had three or more copies of each chromosome, the embryo was classified as polyploid; (iii) when the cell had one or fewer copies of each chromosome, the embryo was classified as haploid; (iv) when the cell had one or two chromosomes with an abnormal number of copies, the embryo was classified as aneuploid.' Embryos were also observed containing more than one abnormal chromosome pair, and they were classified as complex abnormalities. Embryos were also classified as unevaluated if they had anuclear blastomeres, blastomeres with fragmented nucleus, or were fixation or FISH failures. Embryos with one or more multinuclear blastomeres indicating different genetic features were classified as chaotic.

Statistical analysis

Statistical analysis was performed using SPSS for Windows (Statistical Package for Social Science, version 11.5, SPSS Inc., Chicago, IL, USA). To compare embryo chromosome pattern classification among high-quality or low-quality embryos, the chi-squared test was used. Differences were considered statistically significant if $P \leq 0.05$.

Results

The demographic information for the patients is shown in **Table 1**. A total of 132 patients undergoing PGD with their IVF cycles were included in this study; 1107 embryos were analysed via PGD–FISH to reveal the correlation of embryo quality with aneuploidy results. The mean number of biopsied embryos per patient was 7.89 standard deviation (SD) = 3.9. Of the 132 patients, 11 had no transfer due to the lack of chromosomally normal embryos. Out of 347 chromosomally normal embryos transferred [mean (SD) per patient = 2.69 (1.3)], a 32.2% pregnancy rate was achieved per embryo transfer, and 29.5% per oocyte retrieval. The implantation and spontaneous abortion rates were found to be 9.2% and 3.3% respectively.

Taking age into account, there was no significant difference found in the distribution of age compared with PGD results, indicating that homogeneity existed among the patients. When the aneuploid embryos were analysed according to age, no statistical difference was found between the age groups; 18.3% (≤33 years), 22.2% (34–39 years) and 25% (≥40 years) (**Figure 1**).

Of the 1107 embryos, 131 had no results after PGD, either because of chaotic ($n = 48$) or non-analysable ($n = 83$) results, which might be due to the fixation method producing small nuclei. The remaining 976 embryos were analysable, and after PGD 552 (56.6%) were classified as normal, 230 (23.6%) were aneuploid, 42 (4.3%) were complex abnormal, 69 (7.1%) were polyploid, and 83 (8.5%) were haploid. The proportions of grade I, II, III and IV embryos were 42.7% ($n = 417$), 30.1% ($n = 294$), 17.9% ($n = 175$) and 9.2% ($n = 90$) respectively. There was a statistically significant relationship between PGD results and embryo grades ($P < 0.05$).

The distribution of PGD results in relation to embryo grades is shown in **Table 2**. A larger proportion of normal embryos (50.7%, $n = 280$) was grade I. In addition, a considerably high proportion of aneuploid embryos (20%, $n = 83$) was evaluated as grade I. When the complex abnormalities were considered, 3.8% ($n = 16$) were classified as grade I. Of the 69 polyploid embryos, 21.7% ($n = 15$) were grade I and 37.8% ($n = 26$) were grade II. Of the 83 haploid embryos, 27.8% ($n = 23$) were grade I and 34.9% ($n = 29$) were grade II.

When the grade III and grade IV embryos were examined, higher aneuploidy rates were obtained (25.1% and 37.8% respectively) compared with grade I and grade II embryos (20% and 23.5% respectively). Of the 48 chaotic embryos, 29.5% were grade I, 34.1% were grade II, 13.6% were grade III and 22.7% were grade IV.

Higher embryo quality occurred in the normal compared with aneuploid embryos, and this difference was statistically significant ($P = 0.001$). This relationship is also shown in **Figure 1**.

Table 1. Demographics of all patients ($n = 132$) in IVF–PGD cycles.

Parameter	*Total no.*	*Mean (SD) per patient*	*Median (range)*
Maternal age (years)	132	35.45 (5.6)	36 (24–48)
Aspirated oocytes	2081	15.80 (9.0)	15 (1–43)
Mature oocytes	1777	13.46 (8.4)	12 (1–41)
Fertilized oocytes	1177[a]	8.92 (6.3)	7.5 (1–32)
Cleaved embryos	1266	9.59 (6.1)	8 (1–27)
Biopsied embryos	1041	7.89 (3.9)	8 (1–14)
Transferred embryos	347	2.69 (1.3)	3 (0–5)

[a]Number of pronucleated oocytes on day one (16–18 h after microinjection).

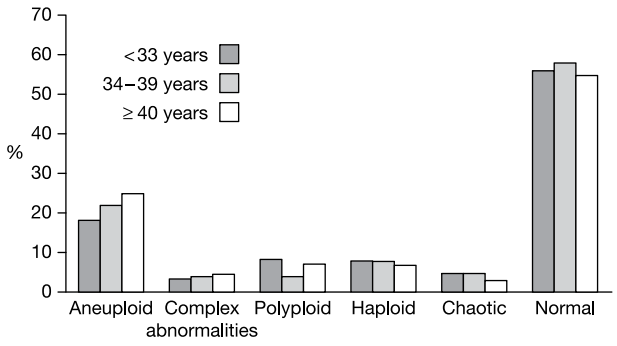

Figure 1. Distribution of preimplantation genetic diagnosis (PGD) results on the basis of maternal age, where patients were grouped into three as: ≤33 years, 34–39 years and ≥40 years.

Table 2. Distribution of PGD results relative to embryo grades.

PGD results	*Embryo grades*				*Total*
	Grade I	*Grade II*	*Grade III*	*Grade IV*	
Aneuploid					
Frequency	83	69	44	34	230
% within PGD results	36.1	30	19.1	14.8	100.0
% within embryo grades	20	23.5	25.1	37.8	23.6
% of total	8.5	7.1	4.5	3.5	23.6
Complex abnormalities					
Frequency	16	13	8	5	42
% within PGD results	38.1	31	19	11.9	100.0
% within embryo grades	3.8	4.4	4.6	5.5	4.3
% of total	1.6	1.3	0.8	0.5	4.3
Polyploid					
Frequency	15	26	17	11	69
% within PGD results	21.7	37.8	24.6	15.9	100
% within embryo grades	3.6	8.8	9.7	12.2	7.1
% of total	1.5	2.7	1.7	1.1	7.0
Haploid					
Frequency	23	29	25	6	83
% within PGD results	27.8	34.9	30.1	7.2	100.0
% within embryo grades	5.5	9.9.	14.3	6.7	8.5
% of total	2.4	3.0	2.6	0.6	8.5
Normal					
Frequency	280	157	81	34	552
% within PGD results	50.7	28.4	14.7	6.2	100.0
% within embryo grades	67.1	53.4	46.3	37.8	56.5
% of total	28.7	16.1	8.3	3.5	56.6
Total					
Frequency	417	294	175	90	976
% within PGD results	42.8	30.1	17.9	9.2	100.0
% within embryo grades	100.0	100.0	100.0	100.0	100.0
% of total	42.7	30.1	17.9	9.2	100.0

Discussion

PGD has become an important diagnostic and therapeutic tool in reproductive medicine, due to the frequent association between genetic factors and infertility. PGD is being used to improve IVF outcomes in patient groups with advanced maternal age (≥38 years), recurrent miscarriage, repeated IVF failure (Caglar *et al*., 2005; Gianaroli *et al*., 2005; Rubio *et al*., 2005; Taranissi *et al*., 2005). Despite the restricted chromosome number analysed per single cell, increased implantation rates and decreased spontaneous abortion rates have been achieved (Gianaroli *et al*., 1999; Munné *et al*., 1999, 2003).

When embryo selection for transfer is performed based on morphological criteria, no improvement in reproductive outcome can be achieved in poor prognosis IVF patients (Verlinsky *et al*., 2005). The present study shows that chromosomally abnormal embryos can have a good developmental potential and be selected for embryo transfer if the PGD procedure is not applied. In this study, embryo quality at the cleavage stage was correlated with chromosomal abnormalities, showing that the morphological assessment might not reflect the chromosomal status of the embryo. Among the aneuploid embryos, a considerably high proportion (66.1%) were of good quality (Grade I and Grade II). For this reason, selecting and transferring embryos according to morphological assessment *per se* may result in a low implantation rate, high miscarriage rate and aneuploid offspring. Nevertheless chromosome abnormalities increased with increasing dysmorphism and developmental incompetence; thus our study supports previous ones reporting that chromosome abnormality occurring post meiotically, but not aneuploidy, increased with decreasing morphology and development (Munné *et al*., 1995; Márquez *et al*., 2000).

In earlier studies aneuploidy significantly increased with maternal age, suggesting that aneuploidy may be the main reason contributing to implantation failure in older women (Munné *et al*., 1995; Márquez *et al*., 2000). In the present study, the distribution of maternal age according to PGD results was examined in order to check whether there was any age had any effect on the results related to the incidence of chromosomal abnormalities. As expected, an increase in the aneuploidy rates was observed with maternal age; however no statistically significant difference was found. Such an unexpected result probably arose because of insufficient numbers of women aged 40 years or older (n = 35), and a restricted number of retrieved oocytes and biopsied embryos.

About 26–65% embryos are able to reach the blastocyst stage, depending on culture methods and medium composition (Veeck *et al*., 2003). It was previously reported that 39% of all embryos reached the blastocyst stage on day 5; 47% of grade I and grade II embryos (good quality), in contrast to 21% of grade III and Grade IV embryos (poor quality) (Rijnders and Jansen, 1998). Another study by Magli *et al*. (2000) also showed that 34% of euploid embryos developed to the blastocyst stage, compared with 22% for aneuploid embryos. Embryos with serious chromosomal abnormalities were evaluated as not compatible with blastocyst development (Munné *et al*., 1994; Evsikov and Verlinsky, 1998; Veiga *et al*., 1999; Márquez *et al*., 2000; Sandalinas *et al*., 2001). According to Hardarson *et al*. (2003), the chromosomal imbalance in blastocysts is reduced compared with embryos at cleavage stage. However there still remains the problem of selecting the blastocysts capable of giving rise to a healthy individual, especially if single embryo transfer is preferred. Even though there is a strong natural selection against chromosomally abnormal embryos when progressing to the blastocyst stage, extended culture to day 5–6 cannot be used as a reliable tool to select against clinically relevant chromosomal abnormalities (Sandalinas *et al*., 2001).

Many IVF clinics do prefer day three embryo transfers. Therefore, we wanted to evaluate PGD results in comparison to day three embryo grades. In summary, an expected relationship was detected between embryo quality and chromosomal abnormalities, but many chromosomally normal embryos seemed to have higher embryo grades. This study demonstrated that a considerable number of chromosomally abnormal embryos with good development potential can be selected for embryo transfer without being identified as abnormal. Although it is standard to perform morphological assessment before the transfer, it seems that it might not always be sufficient alone, and there still remains the risk of choosing an unhealthy embryo. This reveals the importance and necessity of PGD, which provides increased ongoing pregnancy rates by reducing the risk of early fetal loss. Whilst this is not a novel finding, it contributes to the data pool on this topic and will add credibility to statistical data providing a valuable resource for other workers in this field.

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