

Selection of the most common chromosome abnormalities in oocytes prior to ICSI

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So far, all preimplantation genetic diagnosis (PGD) protocols in use produce results after the eggs have been fertilized. However, these approaches are not acceptable for patients with moral objections to the generation and discard of supernumerary zygotes or embryos. In these circumstances, only those oocytes to be replaced may be inseminated. The purpose of this study was to develop a PGD protocol to diagnose first polar bodies (PBs) prior to Intracytoplasmic Sperm Injection (ICSI) in order to inseminate only those oocytes found to be chromosomally normal.

PB biopsy was performed 1 hour after ovum pick up, and after fixation, the PBs were analysed by FISH and the eggs inseminated by ICSI no later than 7 hours after retrieval. One third (33.3%) of the PBs were aneuploid. Fifty-four normal and 12 non-resolved oocytes were injected by ICSI, of which 65% became 2-PN zygotes. Embryo transfer on day 2 was possible in all 10 patients (average maternal age 35.2 ± 3.2 , range 29–39 years), of which 6 became pregnant with 8 fetuses (28.6% or 8/28 transferred embryos). The results indicate that PB analysis of some common chromosome abnormalities is feasible within time limits imposed by ICSI insemination (6 hours or less). Copyright © 2000 John Wiley & Sons, Ltd.

KEY WORDS: preimplantation genetic diagnosis (PGD); aneuploidy; polar bodies; oocyte selection

INTRODUCTION

It is well known that the risk of aneuploidy increases with maternal age in oocytes, embryos, fetuses and live offspring (Hassold and Chiu, 1985; Warburton *et al.*, 1986; Antonorakis *et al.*, 1991; Fisher *et al.*, 1995; Munné *et al.*, 1995a; Dailey *et al.*, 1996) while decreasing implantation rates after *in vitro* fertilization (Navot *et al.*, 1994). We hypothesized that selection of chromosomally normal embryos could reverse the decreased implantation rates in women of advanced maternal age (Munné *et al.*, 1993). Preimplantation genetic diagnosis (PGD) of common aneuploidies using fluorescence *in situ* hybridization (FISH) has been applied either in human blastomeres and polar bodies (PBs) with the aim of selecting against aneuploidy and other chromosome abnormalities before embryo replacement (Munné *et al.*, 1993, 1995a,b, 1998a,b; Verlinsky *et al.*, 1995, 1996; Verlinsky and Kuliev, 1996; Manor *et al.*, 1996; Gianaroli *et al.*, 1997). More than a thousand cycles have been performed using this technique resulting in several hundred chromosomally normal babies. However, increased implantation or a decrease in abnormal offspring has not been demonstrated until recently. Recent reports indicate a decrease in spontaneous abortions and take-home baby rate (Munné *et al.*, 1999), or an increased implantation rate (Gianaroli *et al.*, 1999) after embryo biopsy and PGD of

chromosome abnormalities in women of advanced maternal age.

All the protocol used in the above studies produced results after fertilization, thus chromosomally abnormal embryos or zygotes already existed; and would be discarded to avoid undesirable trisomic offspring, or to improve embryo implantation in women of advanced maternal age. However, these approaches are not acceptable for patients with moral objections to the generation and discard of zygotes or embryos, or under certain legislations. The purpose of this study was to develop a PGD protocol to diagnose first polar bodies in a short enough time period so ICSI can be performed only in chromosomally normal oocytes and within the time period appropriate for insemination.

The chromosome constitution of the first PB is a mirror image of the one found in the M-II oocyte because during meiosis-I, the two sets of univalent chromosomes (with two joint chromatids per chromosome) segregate one to the first PB and the other to the oocyte. Therefore, any error during meiosis-I will be detected in the first PB. For instance, if an extra chromosome 21 is found in the first PB, the oocyte must have a missing chromosome 21. Therefore, the present test can detect chromosome abnormalities produced during maternal meiosis-I, that is >80% of aneuploidies (Hassold and Chiu, 1985; Warburton *et al.*, 1986; Antonorakis *et al.*, 1991; Fisher *et al.*, 1995).

MATERIALS AND METHODS

Ten patients underwent PGD for aneuploidy at Clínica Las Condes for different reasons. Six had

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advanced maternal age while the others had multiple failed attempts of assisted reproduction procedures or had previous spontaneous abortions or trisomic conceptions. The average maternal age was 35.2 ± 3.2 (range 29–39) and the mean number of previously failed attempts was 2.3.

These PGD procedures were performed in accordance with guidelines approved by the Internal Ethical Review Board of Clinica las Condes, including individual written informed consent.

Ovarian stimulation was performed as previously described (Zegers-Hochschild *et al.*, 1995). Briefly, it consisted of subcutaneous leuprolide acetate from the preceding luteal phase, followed by daily injections of 150–300 IU of hMG starting on cycle day 2. Once follicles had reached 18–20 mm in diameter and the ratio of oestradiol per follicle bigger than 16 mm was found to be at least at 900 pmol/l, then 10 000 IU of hCG was administered. All the patients received their hCG injections from a registered nurse at the Unit of Reproductive Medicine, Clinica Las Condes. Follicle aspirations were performed ultrasonographically 35 to 36 hours later and at least one clean sample of follicular fluid was obtained from each patient and stored at -20°C for retrospective hormone analysis.

PB biopsy was performed 1 hour after ovum pick-up and the polar body was fixed immediately. PB biopsy and fixation were performed as previously described (Munné *et al.*, 1998c). After fixation, the PBs were analyzed by fluorescence *in situ* hybridization (FISH) using probes for 13, 16, 18, 21 and 22 labelled respectively in spectrumRed, SpectrumAqua, SpectrumBlue, SpectrumGreen and SpectrumGold (Vysis Inc., Downers Grove, IL). The slide with the fixed PBs and the probes were co-denatured on a hotplate at 73°C for 5 minutes and then hybridized at 37°C for 3 hours. The excess probe was washed for 5 minutes at 73°C in $0.7\times\text{SSC}$ solution, and then the slides were mounted in antifade solution. The slides were observed with a fluorescence microscope with filters for the respective fluorochromes. After FISH analysis, the eggs were inseminated by ICSI. The aim was to perform ICSI within 6 hours after oocyte retrieval.

Because the time for analysis was limited, only one round of hybridization was possible and other chromosomes found to be less involved in abnormalities in oocytes and cleavage-stage embryos, such as chromosome X (Munné *et al.*, 1998a, b; Bahçe *et al.*, 1999) were not used.

As previously reported, PBs quickly degenerate in culture, most showing degenerated nuclei after 10 hours or more, but if they are biopsied 3–6 hours after retrieval they are mostly at metaphase (Munné *et al.*, 1995b). In the present study we decided to measure PB degeneration not from the time of retrieval but from the time hCG was given. We compared PBs from eggs retrieved 35 hours after hCG with those obtained later.

A chi-square test was applied to the comparison of aneuploidy frequencies between age groups.

RESULTS

Out of 135 retrieved oocytes, 19 oocytes were immature and 116 were mature M-II. In cases where the retrieval was performed 35 hours post-hCG, slightly more immature eggs were obtained (12/71) than when it was performed later than 35 hours post-hCG (7/64).

The 116 mature oocytes were biopsied, but some PBs were lost during biopsy or fixation, and only 93 PBs were fixed. After fixation, it was observed that polar bodies obtained from retrievals performed later than 35 hours post-hCG produced 39% (18/46) degenerated PBs compared with only 13% (6/47) of PBs from retrievals performed 35 hours post-hCG ($p < 0.005$).

Of the 93 fixed PBs, we obtained FISH results in 87, of which 29/87 (33.3%) were aneuploid (Table 1). The frequency of aneuploid events, including missing and extra chromatids and non-disjunction of mono-valent chromosomes (two chromatids) was 10.3% for chromosome 13, 3.5% for chromosome 16, 8.0% for chromosome 18, 10.3% for chromosome 21, and 9.2% for chromosome 22. There was a similar number of non-disjunction events ($n=19$) to pre-division of chromatids ($n=17$). However, more non-disjunction events ($n=17$) were observed in women >35 years than in younger ones ($n=2$) ($p < 0.005$), while the number of extra or missing chromatids was similar between those age groups (8 and 9, respectively).

There was a similar number of missing chromosomes and chromatids ($n=16$) to extra chromosomes and chromatids ($n=20$). Figure 1 shows a PB with an extra chromatid and a normal PB. An excess of missing chromosomes or chromatids may have indicated procedural loss during fixation.

Fifty-four normal and 12 non-resolved oocytes were injected by ICSI, of which 43 became 2-PN zygotes (65.2%) and five 1-PN zygotes; the others did not fertilize. Of these 66 eggs, 26 were inseminated within 6 hours after retrieval, producing 18 (69.2%) 2-PN zygotes and one 1-PN zygotes (3.8%), while 40 were inseminated within 6.5–7 hours after retrieval producing 25 (62.5%) 2-PN zygotes and four 1-PN zygotes (10%). The average percentage of 1-PN in this clinic is 5% in non-PGD cases.

Embryo transfer on day 2 was possible in all 10 patients with an average of 2.8 embryos per patient. Six patients (ages 29, 31, 32, 36, 37 and 38) became pregnant (60%) with 8 gestational sacs with heartbeat. Implantation rate was 28.6% (8 intra-uterine gestations/28 embryos transferred). So far, two pregnancies have aborted spontaneously. One twin pregnancy had premature rupture of membranes at 14 weeks; cytogenetic analysis revealed two normal embryos (46XY and 46XX). The other pregnancy (singleton) aborted at 20 weeks of gestation and cytogenetic analysis revealed a 45XO karyotype. The other four pregnancies are ongoing.

Table 1—PGD results

Patient	Age	Normal PBs	Aneuploid PBs	PB abnormality
1	36	7	1	Missing chromosome 22
2	34	1	1	Extra chromatid 13 and 21
3	31	9	2	Extra chromatid 13
4	39	7	5	Extra chromatid 13 and 16
				Extra chromatid 13
				Missing chromatid 22
				Extra chromosome 13
				Missing chromosome 16
5	38	5	3	Missing chromosome 16 and extra chromatid 18
				Extra chromosome 22
				Missing chromosome 21
				Extra chromatid 18 and extra chromosome 21
6	32	5	2	Missing chromosome 21
				Extra chromatid 18 and missing chromosome 21
7	37	5	6	Missing chromosome 13
				Missing chromosome 22
				Extra chromosome 21
				Extra chromosome 22
				Extra chromosome 22
				Extra chromatid 13 and missing chromosome 22
8	29	5	3	Missing chromatid 18
				Missing chromatid 21
				Extra chromatid 21
9	37	11	3	Missing chromatid 18
				Extra chromatid 18
				Extra chromatid 22
10	38	3	3	Missing chromosome 13
				Missing chromosome 18
				Missing chromosome 13 and extra chromosome 21
Total		58	29	

CONCLUSIONS

The results indicate that PB analysis of some common chromosome abnormalities is feasible within the time-period appropriate to ICSI insemination (6 hours or less). Shorter hybridization times may be possible in the future, possibly allowing a second round of hybridization and the analysis of more chromosomes (i.e. chromosome X).

As previously reported, PBs quickly degenerate in culture (Munné *et al.*, 1995b). In the present study we decided to measure PB degeneration not from the time of retrieval, as before, but from when hCG was given. We compared PBs from eggs retrieved 35 hours after hCG with those obtained later; in the first group we found significantly more PBs at metaphase stage, and not degenerated. Although aneuploidy detection can still be performed in aged oocytes (Verlinsky *et al.*, 1995, 1996), some chromatids separate and signals become fuzzier, making it increasingly more difficult to differentiate single chromatids from monovalent chromosomes (with two chromatids) (Munné *et al.*, 1995b). However, by retrieving earlier we retrieved more immature non-usable oocytes.

As previously reported, ICSI should be no later than 9 hours after retrieval (Yanagida *et al.*, 1998), and, according to other groups, ideally between 5–6 hours after retrieval (Jacques Cohen, personal communica-

tion), otherwise the oocytes could age *in vitro*. To avoid producing PGD results later than 6 hours, the biopsy procedure could be expedited by using double-tool holding micromanipulators or by using laser biopsy; either method would reduce by half the time used for biopsy, and thereby also reduce the potential damage to the oocytes due to the shorter time outside the incubator. Also, when more than one case of PGD is to be performed in a single day, the retrievals could be separated each by about 3 hours; which would allow better timing of the ICSI procedures thereafter. These modest changes would easily permit ICSI within 6 hours of retrieval.

The aneuploidy results support previous observations indicating a maternal age effect for non-disjunction (Dailey *et al.*, 1996), but the underlying factor producing predivision of chromatids remains unknown, since no relation to maternal age was found in this or Dailey's study, nor to the number of previous IVF attempt cycles (present study). However, Angell (1997) has stated that pre-division of chromatids is the primary mechanism producing maternal-origin aneuploidy and also that she has found a relationship to maternal age for those abnormalities. Angell's data are also supported by the PGD results on first and second polar bodies of Verlinsky's group (Verlinsky *et al.*, 1995, 1996; 1998; Verlinsky and Kuliev, 1996). The differences between studies might

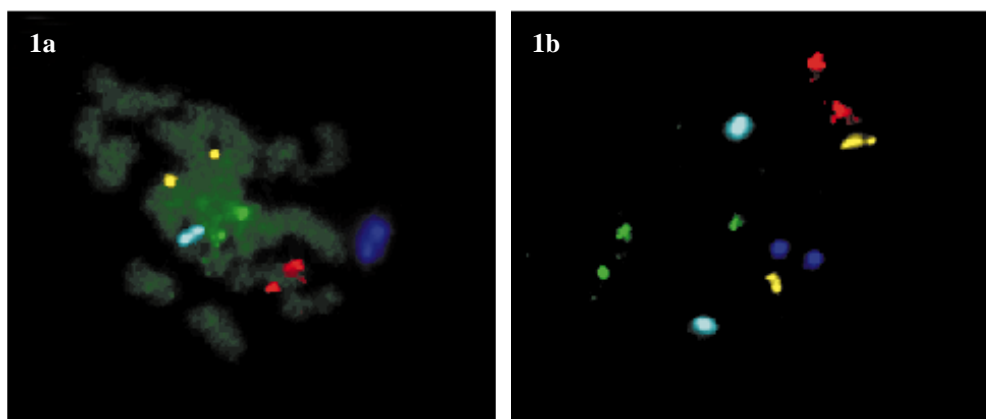


Figure 1—Two first PBs analysed with probes for chromosomes 13 (red), 16 (deep blue), 18 (light blue), 21 (green), and 22 (yellow). Figure 1a shows a PB normal for these chromosomes and Figure 1b a PB with an extra chromatid 21. In addition, the chromosomes analysed with alpha-satellite probes (16 and 18) show paired chromatids in Figure 1a, indicating that the univalent chromosomes are intact, but in Figure 1b chromosome 18 has separated chromatids, indicating predivision of chromatids

be because in this and Dailey's study, only unbalanced pre-divisions of chromatids were counted as true abnormalities (one chromatid or 2+1 chromatids); while Angell also included two separated chromatids as abnormalities. Our studies did not count two separated chromatids as abnormalities because they could be the result of PB and egg degeneration through *in vitro* ageing (Munné *et al.*, 1995b). In either case, both mechanisms seem to produce aneuploidy but the importance of the maternal age over both aneuploidy mechanisms remains to be clarified in freshly collected oocytes, where artefactual pre-division of chromatids produced by *in vitro* ageing would be less common.

Even without the modifications suggested herein, a high pregnancy rate was obtained. In conclusion, PGD for chromosome abnormalities can be successfully applied to the selection of oocytes prior to ICSI in those situations in which, the patients, or legislation, have objections about discarding chromosomally abnormal zygotes or embryos.

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