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Symposium: Embryo implantation failure and recurrent miscarriage

Embryo aneuploidy screening for repeated implantation failure and unexplained recurrent miscarriage

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Abstract

Among other factors, chromosomal abnormalities that originate from gametogenesis and preimplantation embryonic development are thought to be one of the major contributing factors for early embryonic death and failure of pregnancy. However, so far, no non-invasive technique exists that allows the detection of the chromosomal complement of an oocyte or a developing embryo as a whole. Rather, by removing polar bodies/blastomeres, recent developments on preimplantation genetic diagnosis for aneuploidy screening (PGD-AS) have paved the way to detect and possibly eliminate the majority of chromosomally abnormal embryos, thereby increasing the chance of a healthy pregnancy. This article summarizes the origin and impact of chromosomal abnormalities on human reproduction in cases with repeated implantation failure (RIF) and unexplained recurrent miscarriage. It also discusses recent advances regarding the possible benefits of PGD-AS in such cases.

Keywords: *aneuploidy screening, chromosomal abnormality, PGD, recurrent miscarriage, repeated implantation failure*

Chromosomal abnormalities in human gametes and embryos

Nearly 50% of the cases with early pregnancy loss contain chromosomal abnormalities and it has recently been reported that the most common cause of spontaneous abortions is de-novo numerical abnormalities, particularly in chromosomes 13, 14, 15, 16, 18, 21 and 22, followed by monosomy X (Hassold *et al*., 1980; Chandley, 1984; Zenzes and Casper, 1992; Jacobs and Hassold, 1995; Jobanputra *et al*., 2002; Stephenson *et al*., 2002). However, the incidence of chromosomal abnormalities decreases over the course of pregnancy in such a way that in stillbirths it is ~6% and in live births it further drops to 0.6% (Machin and Crolla, 1974; Nielsen, 1975). From this picture, it becomes evident that, starting at early gametogenesis and through the pregnancies that survive to term, chromosomal abnormality is the leading factor for early embryonic losses.

It is now very well known that chromosomal abnormalities originate predominantly from female meiosis and the rate of these abnormalities increases with the increasing female age (Sherman *et al*., 1994; Hassold *et al*., 1995). However, before entering meiosis, the precursor cells have to undergo numerous mitotic divisions, each of which is also at risk of chromosomal error. The successful segregation of homologous chromosomes at the first meiotic division warrants certain key events that have to be tightly regulated and controlled, such as the maintenance of physical connections between homologues until anaphase I and a mechanism that directs sister chromatids for the attachment to the same spindle pole. The former is achieved by the chiasmata, a complex organization at the sites of recombination. It was suggested that the age-related increase of common trisomies is probably determined by the age-related alterations of meiotic

recombination, resulting in premature separation of bivalents and chromosomal non-disjunction in both meiosis I and II (Lamb *et al*., 1996).

Among several studies reporting chromosomal abnormalities by conventional karyotyping, the most recent large scale karyotyping study of 1397 oocytes from 792 patients of mean age 33.7 years found aneuploidy in 10.8% of samples and this rate is independent of IVF indications (Pellestor *et al*., 2002). However, Dailey *et al*. used fluorescence in-situ hybridization (FISH) for chromosomes 18 and X on 383 oocytes from 107 IVF patients, and found that, compared with women <35 years of age, woman aged at least 40 years displayed significant increase in non-disjunction of whole chromosomes (Dailey *et al*., 1996). It has also been reported that anomalies, instead of being distributed randomly, existed in a way such that smaller chromosomes (13, 16, 18, 21, 22) and X were preferentially involved in alterations. On the other hand no anomalies were detected for chromosomes 1, 9 and 12 (Mahmood *et al*., 2000; Cupisti *et al*., 2003). In their large series of 6733 ooyctes that were tested by first and second polar bodies, Kuliev *et al*. found that 52.1% of the oocytes studied contained chromosomal abnormalites originated at meiosis I (41.8%), meiosis II (30.7%) or both (27.6%). Furthermore, 45.1% of the abnormal oocytes had complex errors (Kuliev *et al*., 2002). Overall, it becomes clear that about 10–50% of human oocytes carry abnormalities in at least one of the chromosomes studied.

Spermatogenesis also requires very tightly scheduled events such as multiple mitotic and subsequent meiotic divisions, as well as gross changes in overall morphology. Any deviations during this process may cause impairments in normal sperm function and disturbances in the regulation of chromosome segragation during division processess. Recent studies have shown an increased incidence of sperm chromosomal abnormality, and the value seems to increase with increasing degree of impairment (Gianaroli *et al*., 2000; Silber *et al*., 2003). Moreover, since the evaluation of sperm chromosome aberrations in patients with teratozoospermia as a sole indication has shown a similar incidence with patients having oligoasthenoteratozoospermia (OAT), abnormal sperm morphology may in fact be the critical parameter (Calogero *et al*., 2003). Unfortunately, the cases studied are scarce and the only direct link with increased sperm chromosomal aneuploidy has been shown with large-headed spermatozoa, namely macrocephalic spermatozoa in several case studies (Weissenberg *et al*., 1998; Viville *et al*., 2000; Devillard *et al*., 2002; Vicari *et al*., 2003). However, compared with human oocytes, the average chromosomal abnormality rate for human spermatozoa is around 2.5–10% (Shi and Martin, 2000; Szczgiet and Kurpisz, 2001). Among clinically recognized abortions, the contribution of paternally derived trisomies of chromosomes 13, 18 and 21 is found to be around 8–12% (Nicolaidis and Petersen, 1998).

Since direct karyotyping of human embryos is technically challenging, recent information on the chromosomal status of human embryos has mainly been derived from the outcome of preimplantation genetic diagnosis for aneuploidy screening (PGD-AS) studies. The incidence of chromosomal abnormalities in human embryos is in fact comparably high and recent studies reported that selection of embryos according to morphology-based methods does not eliminate an existing chromosomal defect (Magli *et al*., 2000; Sandalinas *et al*., 2001). When both scoring criteria are combined, recent studies indicated certain subgroups of gametes/embryos showing correlations in morphological and chromosomal outcome (Munné *et al*., 1995; Kahraman *et al*., 2000b, 2002, 2004b; Hardarson *et al*., 2001; Balaban *et al*., 2004; Findikli *et al*., 2004). Kahraman *et al*. have shown that, although a variety of different oocyte/sperm morphologies exist in a given cohort, patients with distinct types of abnormalities, such as oocytes with central granularity or spermatozoa with enlarged heads, show a high rate of chromosomal abnormality, which can lead to a lower implantation rate and a higher rate of abortion. Application of PGD-AS in these cases in fact significantly improved the cycle outcome (Kahraman *et al*., 2000b, 2004b).

The data obtained from the above studies complement those obtained from research studies on human oocytes, since the majority of trisomic conceptions are of maternal origin (Munné *et al*., 2004). In the latter study, chromosomes that are most frequently involved in aneuploidy were found to be 22, 16, 21 and 15. Moreover, an excess rate of monosomies was found, further confirming the previous studies in that mechanisms other than non-disjunction are possibly involved in the genesis of chromosomal abnormalities in human gametogenesis and preimplantation stage embryo development. Besides constitutional abnormalities mainly originated from gamete cells, embryos of younger women seem to have a high risk of carrying 'mosaicism', consisting of tetraploid/polyploid, aneuploid and chaotic cells. Interphase FISH detection with up to nine chromosomes has revealed that mosaicism can affect more than half of the embryos in the same cohort (Delhanty *et al*., 1993, 1997; Harper *et al*., 1995; Munné *et al*., 1998a). Although the exact mechanism is largely unknown, recent arguments state that the extend of mosaicism could be related to reduced expression of certain cell-cycle checkpoint genes during preimplantation development, or defective or immature centrosome structures, as seen in cases with severe male infertility (Delhanty and Handyside, 1995; Silber *et al*., 2003).

So far, studies have also indicated that the mechanisms leading to abnormalities during gametogenesis and embryogenesis appear to have similarities. Although the underlying mechanisms may be different, in both events non-disjunction is common and loss of chromosomal material exceeds gain. During oogenesis, the latter is clearly related to univalent formation (which in turn is tied to a decrease in the number of chiasmata) whereas post-zygotic loss may be due to lack of specific gene products (Delhanty, 2005).

Aneuploid cells may arise through non-disjunction and chromosome loss, chaotic cells through multipolar spindles, and polyploid cells through failure in cytokinesis/karyokinesis. Shi and King (2005), however, suggested that cytokinesis is inhibited in cells that spontaneously missegregate chromosomes during the preceding mitosis. Those authors reported that single non-disjunction events in cell lines are tightly coupled with regression of the cleavage furrow and inhibition of cytokinesis, such that it results in the formation of one binucleate cell rather than two aneuploid cells (Shi and King, 2005). Chatzimeletiou *et al*. (2005a) proposed a path leading to mosaicism, which occurs either by formation of binucleate blastomeres with bipolar spindle and division to two tetraploid blastomeres, or alternatively to a multipolar spindle, which gives rise to a chaotic embryo (Chatzimeletiou *et al*., 2005a).

Preimplantation genetic diagnosis for aneuploidy screening

Based on evidence that some infertile patients are more inclined to chromosomal errors in their embryos, preimplantation genetic diagnosis has been performed in many centres worldwide, with the aim of improving the prognosis for pregnancy. Removal of a blastomere from a cleavage-stage human embryo and subsequent analysis of its genetic content has first been initiated in the late 1980s as a research activity in the UK (Handyside *et al*., 1989). After the first pregnancy was achieved, PGD-AS became a useful diagnostic, as well as a therapeutic, tool in a clinical IVF setting (Handyside *et al*., 1990). So far, applications of PGD for aneuploidy screening to a large extent involved poor prognosis indications including, for example, advanced maternal age (AMA), repeated implantation failures (RIF) and recurrent miscarriage (RM) (Gianaroli *et al*., 2001; Munné *et al*., 2003; Kahraman *et al*., 2004a; Verlinsky *et al*., 2004, 2005; Rubio *et al*., 2005).

Currently, selection of genetically or chromosomally normal embryos can only be possible through oocyte and/or embryo biopsy following a subsequent FISH or DNA analysis. There are mainly three biopsy products that can be analysed via PGD: polar bodies, cleavage-stage blastomeres and trophoblast cells obtained at blastocyst stage. First and second polar bodies of either an oocyte or fertilized zygote can be analysed for a given chromosomal or DNA-sequence-based genetic defect (Verlinsky *et al*., 1996). However, results obtained constitute only the maternal profile and do not give information regarding paternal contribution. Cleavage-stage blastomere biopsy or blastocyst-stage biopsy on the other hand, can reveal genetic information that is inherited from both parents. Some centres use both polar body and blastomere biopsy in order to increase the accuracy of the results (Magli *et al*., 2004; Verlinsky *et al*., 2005).

ESHRE PGD Consortium and the Preimplantation Genetic Diagnosis International Society (PGDIS) have recently published 'guidelines' that summarize the important steps and precautions that should be taken during clinical PGD applications (PGDIS, 2004; Thornhill *et al*., 2005).

Impact of PGD on repeated implantation failure

After endocrinological, uterine and immunological factors are excluded, chromosomal abnormalities are thought to be the major responsible factor for repeated implantation failure (RIF). RIF is generally defined as the failure of pregnancy establishment after three or more failed IVF attempts, or repeated transfer of >10 morphologically good embryos to a recipient uterus. Although there is still no optimum management strategy for these couples, selective elimination of abnormal embryos before embryo transfer via PGD has been offered as a treatment option in many clinics worldwide. The fact that RIF can originate from existing chromosomal abnormalities in embryos was first stated by Munné and his colleagues, and earlier studies indicated that evaluation of chromosomal status of a developing day 3 embryo could increase the chance of pregnancy in this group of patients (Munné *et al*., 1993; Gianaroli *et al*., 1999). When the data regarding the number of previous unsuccessful trials are compared, several groups have reported similar rates of chromosomally abnormal embryos but emphasizing the increased rate of mosaicism, while other studies have observed an increased rate of abnormality as the number of trials increase (Gianaroli *et al*., 1997; Munné *et al*., 2003; Pehlivan *et al*., 2003; Kahraman *et al*., 2004a).

Although studies performed so far have differences in the study design, presence/absence of a control group, number of chromosomes analysed as well as differences in the cut-off values, they all report similar implantation rates compared with controls, indicating that there is still no direct evidence that these couples benefit from PGD-AS (**Table 1**). However, very few studies have evaluated the impact of PGD in young patients associated with RIF and, in fact, PGD-AS could not only be a diagnostic tool to delineate the reasons for IVF failure but it might also be beneficial for young patients (Gianaroli *et al*., 1999; Kahraman *et al*., 2000a; Munné *et al*., 2003; Caglar *et al*., 2005). In these studies, variable, albeit higher, implantation rates (17–30%) were obtained among young patients; however in the older patients (≥35 or ≥37 years of age), PGD-AS seems to be of limited benefit. In a more recent study, Tarranissi and his colleagues retrospectively analysed 116 couples with a history of RIF undergoing 130 cycles of PGD-AS (Tarranissi *et al*., 2005). When chromosomal status of developing embryos and cycle outcome were analysed according to female age (<40 and ≥40 years of age), they found that the younger age group had a significantly higher proportion of euploid oocytes/embryos, cycles reaching embryo transfer, pregnancy (43 versus 25%), clinical pregnancy (36.1 versus 16.6%) and ongoing delivery (32 versus 12.5%) rates per transfer. These results, confirm the previous studies in that PGD-AS for recurrent IVF implantation failure using FISH probes is associated with improved outcome in younger women, but has a high cancellation rate and low cycle outcome in women ≥40 years of age (Tarranissi *et al*., 2005).

Impact of PGD in unexplained recurrent miscarriage

Recurrent miscarriage (RM), which is observed in 2–5% of couples, is usually defined as three or more consecutive miscarriages prior to 20 weeks of gestation. The occurrence rate in general population is somewhat higher than the expected rate of 0.3%, indicating that there exist complex underlying conditions (Coulam, 1991; Stephenson, 1996). Recent reports also indicated that 50–60% of the abortuses are chromosomally abnormal in women with two or more miscarriages (Stern *et al*., 1996; Osagawa *et al*., 2000; Carp *et al*., 2001; Ferro *et al*., 2003). Usually, cytogenetic analysis of the couples are the first action to take in most centres and even if parental karyotypes are normal, couples may still be at increased risk for aneuploidy as a result of gonadal mosaicism (Robinson *et al*., 2001; Simpson and Elias, 2003). Despite the presence of several well-defined causes such as uterine, genetic, endocrinological and immunological factors, almost 50% of the RM cases are termed as unexplained. However, the chance of a normal term pregnancy after three or six consecutive miscarriages is still 60 and 40% respectively (Stirrat, 1990; Clifford *et al*., 1997).

Based on the above facts, PGD for RM has recently been introduced in the clinical setting with the rationale that de-

Table 1. Outcomes of studies with over 70 PGD-AS cycles performed between 1998 and 2005.

Study	*No. patients*	*No. cycles*	*Maternal age*	*PGD-AS indication*	*Embryos analysed by FISH*	*Found abnormal %*	*ET cycles*	*CPR/ET%*	*IR%*
Gianaroli, 1999	*	73	39.2	AMA	432	64	57	39	25.8
	*	27	32.2	RIF	138	54	20	25	17.3
Munné, 2003	138	138	39.8	Poor prognosis	1071	70.3	119	*	21.3
Rubio, 2003	51	63	33.2	RM	426	70.7	49	38.8	30.8
	20	23	38.4	RM ± AMA	133	70.7	18	22.2	21.3
Kahraman, 2004	276	282	35.2	Poor prognosis	1147	40.9	278	31.6	*
Verlinsky, 2004	*	3747	39	Poor prognosis	*	*	3099	23.3	*
Verlinsky, 2005	1493	2176	38.5	Poor prognosis	8213	*	1744	26	14.7
Gianaroli, 2005	740	1029	37.3	Poor prognosis	5115	67	699	30	21.3
Sermon, 2005	*	1012	37	Poor prognosis	5079	*	716	24	*
Platteau, 2005	279	394	39.9	AMA	2097	65.3	267	16.4	10.7
Munné, 2005	21	25	32.6	RM	241	57	23	57	38
	37	44	39.5	RM ± AMA	409	67	37	46	31
Taranissi, 2005	78	86	36.3	RIF	838	70.1	84	36.1	24
	38	44	42.0	RIF ± AMA	†	†	55	16.6	12

*Not mentioned in the article. †Included in value given in cell above.
AMA = advanced maternal age; CPR = cumulative pregnancy rate; ET = embryo transfer; FISH = fluorescence in-situ hybridisation; IR = implantation rate; PGD-AS = preimplantation genetic diagnosis for aneuploidy screening; RIF = repeated implantation failure; RM = recurrent miscarriage.

novo abnormalities arising from random errors produced during gamete or embryo development may be an important underlying aetiology of miscarriage. If this is true, elimination of chromosomally abnormal embryos from transfer cohort can increase the clinical outcome by increasing implantation and decreasing abortions (Simon *et al.*, 1998; Vidal *et al.*, 1998; Pellicer *et al.*, 1999).

At the embryo level, several studies have already demonstrated variable chromosomal abnormality rates of 32.1–70.7% (Pellicer *et al.*, 1999; Rubio *et al.*, 2003, 2005; Werlin *et al.*, 2003; Kahraman *et al.*, 2004a; Wilding *et al.*, 2004; Christiansen *et al.*, 2005; Munné *et al.*, 2005; Platteau *et al.*, 2005a,b). Rubio and her colleagues have reported their results regarding PGD in women with two or more miscarriages. A total of 71 women undergoing 86 PGD cycles were included in the study and in 67 of these cycles, transfer of euploid embryos resulted in 23 (34.3%) pregnancies. Of them, 10 resulted in live births, and nine were ongoing at the time of publication. One pregnancy was detected as ectopic and the remaining three pregnancies ended up as miscarriages (13.0%). Overall, an 83% live birth rate was obtained in this study (Rubio *et al.*, 2003). The same group have recently expanded their series to a total of 241 PGD cycles. Similarly, elimination of chromosomally abnormal embryos resulted in a 36.5% pregnancy and 12.8% miscarriage rates. In both reports, they indicated a consistently high chromosomal abnormality rate (66.1–70.7%) however the pregnancy and miscarriage rates failed to show any significant difference when compared with a control group (Rubio *et al.*, 2005). It has been shown in a recent controlled clinical study that, with the application of PGD in cases with three or more miscarriages, the rate of pregnancy loss was reduced to 16.7%, which was found to be statistically significant when compared with an expected rate of 37% (Munné *et al.*, 2005).

The current clinical data are still scarce, and all of the above reports pointed out that well-designed prospective (if possible, randomized) studies are needed to document the subset of patients who would mostly benefit from such treatment and a possible beneficial effect of PGD, if they exist, in RM cases. Three such studies, albeit with very low numbers of patients have recently been reported for RM. In the first study, Werlin and his colleagues analysed 19 RM patients who were randomly allocated as the study and the control group, and concluded that PGD may be beneficial in patients with two or more miscarriages (Werlin *et al.*, 2003). In the second, Winding *et al.* analysed 48 couples with two or more abortions and obtained 35.5% pregnancy and 21.1% implantation rates. Out of 58 gestational sacs with fetal heartbeats, 54 resulted in healthy live births. A more recent study involved 49 RM patients, previously screened for all other known RM pathological conditions, but the results failed to show any beneficial effect of PGD (Platteau *et al.*, 2005). Hence, more studies with larger sampling sizes are warranted to document the possible benefits of PGD-AS in RM cases.

Effect of maternal age

While decreased endometrial receptivity has also been proposed as one of the limiting factors in such cases, recent results with increased implantation rates in donation cycles have implied that oocyte-related factors, especially aneuploidy are more pronounced in AMA (Abdalla *et al.*, 1997; Kuliev and Verlinsky, 2003). It has now been well established that maternal ageing is not only associated with diminishing ovarian reserve and fertility potential, but also is associated with an increased rate of chromosomal aberrations or cytoplasmic abnormalities leading to defective meiosis. Although a recent study by a Belgium group indicated the lack of benefit when PGD is

combined with blastocyst stage embryo transfer, selection and subsequent elimination of chromosomally abnormal embryos has now been shown to increase the assisted reproduction outcome in women with advanced (>38 years) age in several other recent reports (Gianaroli *et al.*, 1999; Kahraman *et al.*, 2000a; Kuliev and Verlinsky, 2003; Munné *et al.*, 2003; Staessen *et al.*, 2004; Platteau *et al.*, 2005; Rubio *et al.*, 2005; Verlinsky *et al.*, 2005).

Carp *et al.* (2001) reported that when compared with the 30–40 age group in which the incidence of fetal chromosomal abnormalities was found to be 23%, in women above the age of 40, this rate increased up to 63.6% (Carp *et al.*, 2001). Similarly, the results of Spandorfer and his colleagues showed that the incidence of fetal loss with a chromosomal abnormality was 65 and 82% for the women aged <40 and ≥40 years respectively (Spandorfer *et al.*, 2004). Although there is a tendency of decreasing the miscarriage rates in the overall group, when cycle outcome was analysed according to maternal age (<35 and ≥35 years of age) and compared with a predefined expected pregnancy loss rate, Munné *et al.* found a significant reduction in spontaneous abortions and increased implantation rates after PGD was observed in the latter group only. From this finding, PGD is recommended to RM patients who are 35 years and older (Munné *et al.*, 2005). In contrast, in the prospective work done by the Belgium group, cycle outcome was documented and analysed according to maternal age with a cut-off value of 37 years, but both younger and older group seemed to get no clear therapeutic benefit from PGD.

Besides inconclusive effects of selecting euploid embryos through PGD in the latter case, the current data favour that, for cases in which repeated chromosomally abnormal miscarriages or cases in which the maternal age is ≥37, PGD seems to be the treatment of choice (Carp *et al.*, 2004; Munné *et al.*, 2005; Rubio *et al.*, 2005). However, in general, only a minority of the patients can fulfil these criteria; in most cases, results regarding abortus materials in RM are not available therefore it becomes very difficult to draw a treatment strategy towards this direction. Furthermore, younger couples with three or more conceptions with the same trisomy are good candidates for gonadal mosaicism (Cozzi *et al.*, 1999; Somprasit *et al.*, 2004).

RIF and RM due to structural chromosomal abnormalities

Due to their increased risk of producing unbalanced gamete cells, carriers of structural abnormalities such as inversions and translocations are also among other PGD candidates for RIF and RM. The frequency of chromosomal translocations is significantly higher among infertile couples compared with the normal population (0.6 versus 0.2%; Testart *et al.*, 1996). Moreover, the study of De Braekeleer has shown that 4.7% of the RM cases were associated with structural chromosomal defects (De Braekeleer and Dao, 1990). In RIF cases, this incidence was found to be around 2.5% (Stern *et al.*, 1999).

As an alternative to prenatal diagnosis and pregnancy termination of unbalanced fetuses, PGD has been offered to carriers of balanced translocations in several centres worldwide. (Conn *et al.*, 1998; Munné *et al.*, 1998b; Scriven *et al.*, 1998, 2000; Gianaroli *et al.*, 2002; Kuliev and Verlinsky, 2002; Munné, 2002; Findikli *et al.*, 2003). Apart from the cases with normal karyotypes, PGD-AS clearly improved clinical outcome with decreased early abortions after selection of abnormal embryos in couples with structural chromosomal rearrangements. However, to date, there exist no case–control studies that compare miscarriage rates between natural pregnancies and PGD in such cases.

Limitations of the current applications and future perspectives

Although the application of PGD becomes an invaluable tool for assisted reproduction and clinical genetics, in order to evaluate its efficiency in the current clinical practice of RIF and RM cases, several parameters and limitations should also be considered.

Although aneuploidies, to a great extent, originate through female meiosis and analysis of polar bodies is very informative for cases involving advanced maternal age, this strategy clearly overlooks the contribution of spermatozoa. However, in some countries such as Italy and Germany, it becomes the only available option, since in these countries further manipulation of embryos is strictly prohibited by law. From the aspect of embryo development, controversy still exists regarding the feasibility of removing one (or more) blastomere from a developing cleavage stage human embryo. This parameter is of extreme importance because: (i) removal of one blastomere does not necessarily represent the whole embryo, since mosaicism can exist; and (ii) its removal could proportionally affect the overall cellular mass in the late preimplantation development. A recent study comparing the development of human biopsied embryos drilled with non-contact infrared laser or acid Tyrode's based on blastomere viability, cytoskeletal analysis and molecular cytogenetics revealed that, although no significant difference was noted between the proportion of laser and acid Tyrode's biopsied embryos that develop to the blastocyst stage by day 6, blastocyst formation was evident earlier (day 5) in the laser drilled group. Furthermore, cell numbers at the blastocyst stage were not significantly different among biopsied embryos from each group that reached the blastocyst stage on day 5, but were significantly lower than controls on day 6 and 7. In addition, on day 6, there was a significant reduction in the cell numbers of acid Tyrode's biopsied embryos compared with those biopsied following drilling with the infrared laser. This reduction may be attributed to the delayed cavitation experienced by the acid Tyrode's biopsied embryos (Chatzimeletiou *et al.*, 2005b). However, current results can also show that acceptable implantation rates are obtained after transfer of biopsied embryos in more than 5000 PGD cycles (Verlinsky *et al.*, 2004). Another technical limitation regarding biopsy is the low survival rate after freezing/thawing of biopsied embryos. Several groups reported limited success upon thawing with slow freezing protocols, which, according to a recent study, can be increased by vitrification (Joris *et al.*, 1999; Magli *et al.*, 1999; Ciotti *et al.*, 2000; Zheng *et al.*, 2005).

For genetic aspects, the first limitation is the number of chromosomes suitable for analysis, since only a few chromosomes can be simultaneously analysed in a single biopsied cell. Although selected chromosomes can be

representatives of nearly 70% of the abnormalities, in order to determine an actual chromosomal abnormality, one would expect to identify every chromosome in a single blastomere (Munné *et al*., 1999). To date, the majority of data presented by several groups include mainly chromosomes 13, 18, 21, X and Y. However, involvement of other chromosomes (15, 16, 17 and 22) has also increased the sensitivity and clinical outcome (Munné *et al*., 2003). Currently, many clinics now adopt their routine analysis protocols in such a way that two consecutive rounds of hybridization (five to six chromosomes in the first, three to four chromosomes in the second round) are performed, allowing the analysis of chromosomes that are thought to be susceptible for alterations in the above indications. Interphase FISH also fails to determine whether the analysed arrangement is normal or balanced in the case of structural chromosomal abnormalities.

In order to increase the efficiency, several recent improvements have also been achieved with the technique itself. First, application of nucleus conversion technique, which involves the fusion of a biopsied sample with a mouse zygote, has recently been reported on 94 cycles, giving a 30.3% pregnancy rate in one centre (Verlinsky, 2002). So far, this technique has not been applied in cases other than structural chromosomal abnormalities and its real impact on PGD needs to be determined with more studies. Secondly, comparative genomic hybridization (CGH) has been proposed as an alternative to interphase FISH. Wilton *et al*. have analysed embryos of 20 RIF patients with CGH and conventional FISH technique and concluded that the proportion of abnormal blastomeres incorrectly diagnosed as normal by FISH is 60% for five chromosomes and 40% for nine chromosomes (Wilton *et al*., 2003). Another study in which CGH was the method of analysis showed that besides the chromosomes that are commonly applied in PGD-AS, other chromosomes were also involved in the abnormalities in RIF cases (Voullaire *et al*., 2002). These results, although they have to be confirmed by others, would possibly explain the limited clinical improvement of PGD-AS in cases with repeated implantation failures. Although the time required for the analysis requires cleavage stage embryos to be cryopreserved hence is not suitable for current clinical procedures at the moment, successful pregnancies have already been reported by CGH indicating that in the near future, improvements in the protocols, either shortening the time required for CGH or cryopreservation will create an alternative protocol for analysing the whole set of chromosomes in a given embryo (Wilton *et al*., 2001, 2003; Wells *et al*., 2002; Gutierrez-Mateo *et al*., 2004). Also, an improved version of CGH, named array CGH that does not need a metaphase plate for hybridization, has already been adapted in a clinical setting in order to assay single cells (Handyside *et al*., 2004; Hellani *et al*., 2004).

Another approach, which utilizes polymerase chain reaction and sequencing-based methods, hence named DNA fingerprinting, has been developed and tested for the most common chromosomal abnormalities such as trisomy 21 (Katz *et al*., 2002). This technique initially included markers for five chromosomes. However, it needs to be determined whether this number can be sufficiently increased and be a powerful alternative to conventional FISH analysis. Recent developments in microarray technology have been another powerful tool in reproductive medicine. The first impact seems to be the analysis of gene expression or mutation profiles on oocytes and embryos of different developmental stages, providing potential targets for diagnosis (Wells *et al*., 2005). Development of customized microarrays, in which aneuploidy testing for all chromosomes could be possible, would boost the efficiency and eliminate the use of conventional FISH techniques.

Currently, successful results are obtained in >90% of the blastomeres analysed with conventional FISH analysis for selected chromosomes and the improvements above will eventually let us analyse all the chromosomes in a given blastomere. However, the presence of mosaicism is of major concern in PGD-AS cycles and, unlike amniocentesis or chorionic villus sampling in which hundreds of cells could be analysed, analysis of a single blastomere leaves little margin for error. It has been reported that a certain rate of mosaicism is present in preimplantation embryos and this rate is even higher in certain cases such as patients with severe sperm defects and advanced maternal age (Magli *et al*., 2000; Bialenska *et al*., 2002; Munné *et al*., 2002; Silber *et al*., 2003; Baart *et al*., 2005; Wilton, 2005). Also, with current technology, one would be able to detect the majority of the chromosomal aberrations; there exist also some genetic/cytogenetic factors, such as skewed X chromosome inactivation and cryptic structural abnormalities, that cannot be detected by contemporary cytogenetics workup (Lanasa *et al*., 2001; Uehara *et al*., 2001; Benkhalifa *et al*., 2005). The latter group has recently applied Array CGH on 26 spontaneous abortion samples that failed to grow in culture and found that 57.7% of the samples contained abnormalities and, interestingly, this new approach was able to detect novel abnormalities that cannot be detected by conventional cytogenetics.

Overall, accumulated data on PGD-AS with the above indications clearly show that the prevalence of chromosomal abnormalities in oocytes, as well as at cleavage stages, can be very high. Elimination of such embryos can prevent the birth of a trisomic child, decrease the rates of abortion as well as high order pregnancy rates and has a positive impact on implantation, creating a beneficial approach of selecting euploid embryos for embryo transfer (Munné *et al*., 2003; Kuliev and Verlinsky, 2004; Verlinsky *et al*., 2004). Moreover, two groups have recently documented their results regarding the pregnancy outcome after PGD-AS cases (Gianaroli *et al*., 2005; Verlinsky *et al*., 2005). They concluded that, although randomized controlled studies could still be useful to further show the clinical impact of selecting euploid embryos, results on reproductive outcome in the same group of patients (PGD-AS cycle and previous cycles) provide strong evidence for an improvement in reproductive outcome.

Conclusion

Cumulative analysis of more than 6000 PGD-AS cycles performed to date on patients with poor prognosis (AMA, RIF and recurrent spontaneous abortion) show that application of PGD: (i) reduces the risk of high order pregnancies as well as repeated early abortions, especially in couples with structural chromosomal abnormalities; and (ii) improves the assisted reproduction outcome by eliminating the number of chromosomally abnormal embryos transferred (thereby decreasing the high order pregnancies and increasing the implantation rates). On the other hand, more studies (prospective as well as retrospective) are required to delineate the subgroup

of patients who would most probably get benefit from PGD-AS. Studies have also shown that PGD-AS procedure is safe and reliable; it can be used not only as a diagnostic but also a therapeutic tool, provided that several limitations are overcome with the technical protocols.

Results of the accumulated clinical data on PGD-AS are encouraging. As a result, PGD facilities started to become an integrated part of numerous ART clinics worldwide. With increasing and expanding experience on gamete and embryo manipulation detailed and careful patient selection, as well as novel approaches such as CGH and DNA microarray technologies, are likely to make PGD-AS a premium embryo selection tool in RIF and RM cases in the near future.

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