Iranian Journal of Reproductive Medicine Vol. 2. No.1 pp1-8, 2004

Current Status of Preimplantation Genetic Diagnosis

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Since its first clinical application in early 90s, preimplantation genetic diagnosis (PGD) has became a powerful diagnostic procedure in clinical practice for avoiding the birth of an affected child as well as increasing the assisted reproductive technologies (ART) outcome . The technique involves the screening of preimplantation embryos for chromosomal abnormalities in certain indications such as advanced maternal age, repeated abortions and translocations, or for single gene defects, the majority of which are cystic fibrosis and thalassaemias. In this context, it becomes an alternative option for traditional prenatal diagnosis. So far, more than 1000 unaffected babies have been born after PGD, indicating that the procedure is safe and effective in prevention of genetic defects as well as increasing the ART outcome. Besides its diagnostic value and expanding indications such as cancer predisposition, dynamic mutations and late onset disorders, a new feature, namely preimplantation human leuckocyte antigen (HLA) typing also demonstrates its novel therapeutic role in contemporary medicine. This article summarizes the recent status of PGD and discusses the current limitations and future perspectives associated with PGD techniques**.**

Key Words: *PGD, ART,FISH*

Introduction

It has been reported that nearly 50% of the cases with early pregnancy loss contain chromosomal abnormalities (Chandley 1984; Zenzes and Casper 1992; Jacobs ve Hassold 1995; Jobanputra *et al*., 2002). Although most of them are found to be eliminated before implantation, some anomalies such as trisomies of chromosomes 13, 18 and 21 can reach to blastocyst stage and even result in affected offspring (Sandalinas *et al*., 2001). Chromosomal aneuploidy has also been shown to increase under inappropriate stimulation protocols, suboptimal culture conditions, paternal factors and lack of certain growth factors (Munne *et al*., 1995; Janny and Menezo 1996; Kaye 1997; Moor *et al*., 1998; Calogero *et al*., 2003; Findikli *et al*., 2004).

Screening preimplantation embryos for certain chromosomal abnormalities is generally termed as PGD for aneupoloidy screening (PGD-AS). It is based on the principle that detection and elimination of chromosomally abnormal embryos before embryo transfer could increase the reproductive efficiency in certain cases where aneuploidy is proven or likely to exert a negative effect (Munne *et al*., 1995; Benadiva *et al*., 1996; Kuliev *et al*., 2002). So far, applications of PGD for aneuploidy screening to a large extent involved indications such as advanced maternal age, repeated implantation failures and recurrent abortion (Munne *et al*., 1999; Gianaroli *et al*., 2001; Kuliev *et al*., 2002; Munne 2002; Wilton 2002; Pehlivan *et al*., 2003; Rubio *et al*., 2003; Kahraman *et al*., 2004a). Due to their increased risk of producing aneuploid gamete cells, carriers of structural abnormalities such as inversions and translocations are also among other PGD candidates. Improved clinical outcome with decreased early abortions after selection of abnormal embryos with PGD have recently been reported by different groups on reciprocal and Robertsonian translocations (Conn *et al*., 1998; Scriven *et al*., 1998, 2000; Munne *et al*., 1998, 2000; Findikli *et al*., 2003). Furthermore, the positive effect of PGD application on clinical results was recently documented in severe male infertility, Klinefelter's syndrome and cases with abnormal gamete cell morphology, which are among other potential PGD indications (Gianaroli *et al*., 2001; Kahraman *et al*., 2000; 2003, 2004a, 2004b; Aran *et al*., 2004;). The data accumulated on approximately 5000 PGD cycles having above indications clearly shows that the prevalence of chromosomal abnormalities in oocytes as well as at cleavage stages can be as high as 50-70%. Elimination of such embryos prevents the birth of a trisomic child, decreases the abortion as well as high order pregnancy rates and has a positive impact on implantation, validating the beneficial approach of selecting euploid embryos for embryo transfer in PGD for certain indications (IWGPG 2001; Munne *et al*., 2003; Kuliev and Verlinsky 2004a).

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Preimplantation genetic diagnosis for single gene disorders

If one or both partners are carriers of a genetic

disease, in order to prevent the birth of an affected offspring, preimplantation embryos can be screened for a known genetic defect. Up to date, more than 300 healthy children have been born after approximately 1,500 PGD cycles for single gene disorders (ESHRE PGD Consortium Steering Committee 2000; Harper 2003). The technique involves the use of polymerase chain reaction (PCR) technology on a single cell and subsequent analysis by either conventional or advanced molecular genetics tools as DNA sequencing. Although, the first successful PGD application was based on sex selection for X-linked disorders, as the accuracy and the technical ease is improving, many autosomal dominant, autosomal recessive and X-linked genetic disorders, can now be diagnosed on preimplantation embryos by using one or two blastomeres obtained after embryo biopsy (Table I) (Handyside *et al*., 1990; Sermon 2002; Verlinsky and Kuliev 2002).

PGD for single gene disorders is further expanded to cancer predisposition, late onset disorders, or even serves as a therapeutic option for an affected sibling by preimplantation HLA typing (Verlinsky *et al*., 2001; Rechitsky *et al*., 2002, 2003). The latter is of importance, since it gives the unique opportunity for families in which an HLA compatible sibling can be born and its cord blood or bone marrow stem cells can be the ideal source for transplantation, leading to a successful restoration of the affected phenotype. Although the number of cases are currently limited to draw a general conclusion, reported results on 25 pregnancies obtained after 147 preimplantation HLA typing cycles are highly encouraging. However, certain clinical and patient specific factors can limit the successful pregnancy outcome. (Van de Velde *et al*., 2004; Fiorentino *et al*., 2004; Kuliev and Verlinsky, 2004b; Rechitsky *et al*., 2004; Kahraman *et al*., 2004c)

Table I. A subset of single gene disorders on which PGD can be applicable.

Autosomal Dominant Diseases
Myotonic dystrophy (MD)
Huntington Disease (HD)
Marfan syndrome
Osteogenesis imperfecta (OI)
Charcot-Marie-Tooth Disease (CMT)
Neurofibromatosis Type 1 (NF 1)
Autosomal Recessive Diseases
Cystic Fibrosis (CF) + CBAVD
Sickle Cell Anemia
β-Thalassaemia
21 β-Hydroxilase deficiency
Phenylketonurea (PKU)
Spinal Muscular Atrophy (SMA)
Gaucher Disease
Hurler Syndrome
X-Linked Diseases
Muscular dystrophy (DMD, BMD)
Hemophilia A, B
Fragile-X syndrome
Alport syndrome
Retinitis Pigmentosa
Ectodermal dysplasia
Hunter syndrome
Wiskott-Aldrich syndrome
G6PD deficiency
Adrenoleucodystrophy (ALD)

Besides its demonstrated diagnostic and therapeutic value, strict precautions should be taken, since several problems such as external contamination, allelic drop-out or preferential amplification effect the results and the reliability of the technique. Nowadays, designing sterile and dedicated area with special labware, apparatus and technical improvements such as the intruduction of nested and multiplex PCR systems seem to minimize these problems (Findlay *et al*., 1998; Lewis *et al*., 2001; Fiorentino *et al*., 2003).

Methodology and technical approaches

There are mainly two embryo development stages that sampling for PGD can be done: MII oocyte or prezygote stage and cleavage stage (Figure 1). First and second polar bodies of either an oocyte or fertilized zygote can be analyzed for a given chromosomal or DNA-sequence-based genetic defect. However, results obtained constitute only the maternal profile and do not give information regarding paternal contribution. On the other hand blastomere biopsy, reveals genetic information that is inherited from both parents. Advantages and disadvantages of these sampling stages on the analysis outcome are summarized in Table II.

Polar bodies are the by-products of the first and second meiotic divisions which appear after maturation of oocyte or fertilization. This type of analysis is usually preferred for the maternal indications which bring high aneuploidy risk in oocytes such as advanced maternal age and translocations in which female is the carrier. For other indications such as recurrent abortions, recurrent implantation failure and severe male infertility etc., evaluation of the blastomere is needed. In this case, biopsy is done by removing one or two blastomeres from a cleavage-stage embryo having 6-8 cells. Some centers use both polar body and blastoemere biopsy in order to increase the accuracy of the results (Kuliev *et al*., 2002). Also, biopsy can also be done at the blastocyst stage, involving the removal of multiple trophectoderm cells. Although the clinical data regarding the results are limited.

In all three stages, a partial opening on the zona pellucida should be created by either mechanical, chemical or laser-driven systems. A recent study compared the clinical outcome after different methods of zona opening and found insignificant differences of one technique to another (Joris *et al*., 2003). Therefore, subsequent aspiration of either polar bodies or a blastomere after zona opening is performed and obtained material is processed for either FISH (Figure 2) or single cell PCR. It has also been reported that, when compaction is observed during blastomere biopsy, short-term incubation of the embryo in Ca-Mg free media helps to facilitate the procedure (Kahraman *et al*., 2000). In order to study chromosomal abnormalities by FISH, biopsied samples are first fixed on a slide and subsequently analyzed after hybridization with probes

Table II: Biopsy stages.

Stages	Advantages	Disadvantages
▪ First polar body biopsy (M II oocyte stage)	▪ No known negative effect on embryo development. ▪ More time is available for analysis before embryo transfer	▪ Only one sample is available for analysis. ▪ Error rate is high. ▪ Only maternal anomalies can be detected
▪ First and second polar body biopsy (Prezygote stage)	▪ Provides more accuracy compared to first polar body biopsy. ▪ Both maternal and paternal anomalies can be analyzed.	▪ Limited time interval is available for biopsy.
▪ Blastomere biopsy (Cleavage stage)	▪ More accuracy can be obtained if two cells are used. ▪ Large clinical data is available.	▪ Probability of chromosomal mosaicism ▪ Selection of blastoemeres with nuclei are required
▪ Trophectoderm biopsy (Blastocyst stage)	▪ Multiple cells are available for analysis. ▪ Embryo selection can be done at a later stage. ▪ Higher implantation and lower multiple pregnancy rates	▪ Limited time is available for analysis. ▪ Number of embryos to be analyzed is decreased. ▪ Representative or only trophectoderm lineage. ▪ Clinical data is scarce.

Figure 1a. Polar body biopsy procedure.

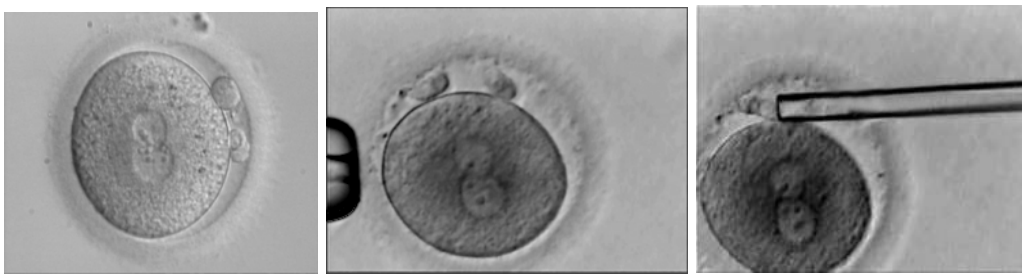


Figure 1b. Blastomere biopsy procedure.

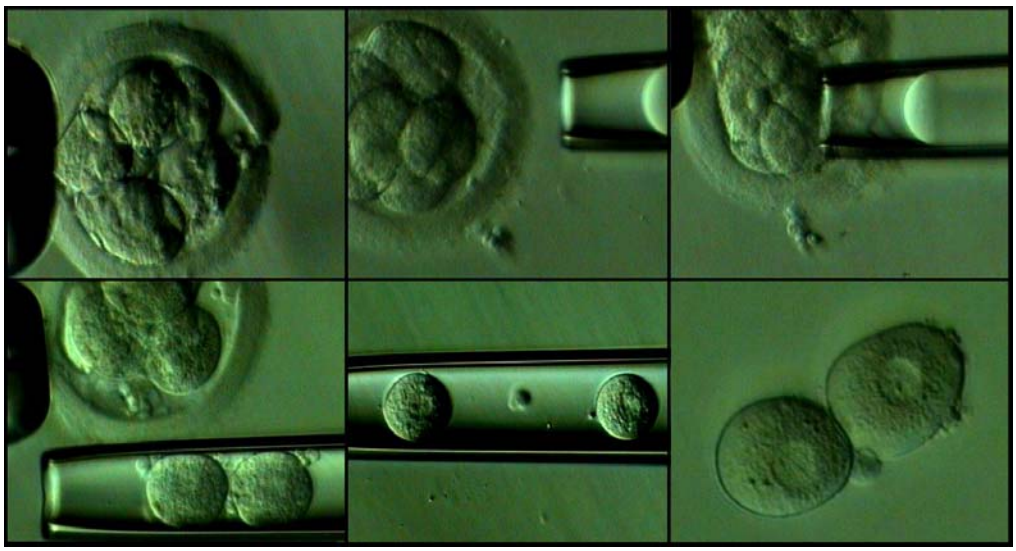


Figure 2. FISH images for corresponding aneuploidies:. a) Trisomy 13, monosomy 21; b) Trisomy 18, monosomy 21; c) Trisomy 18; d) Triploidy (3n)

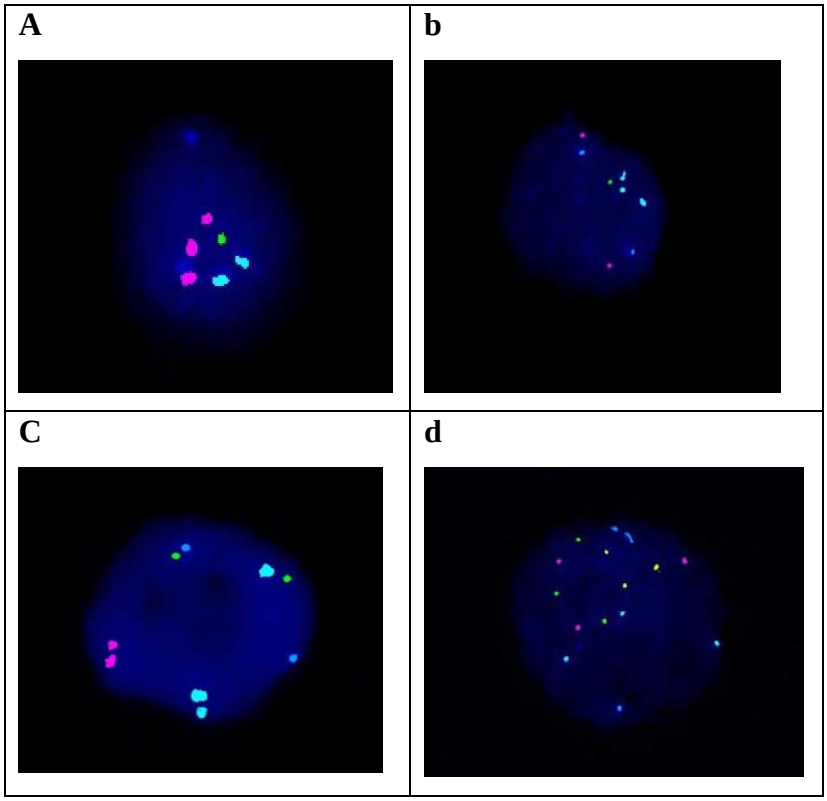
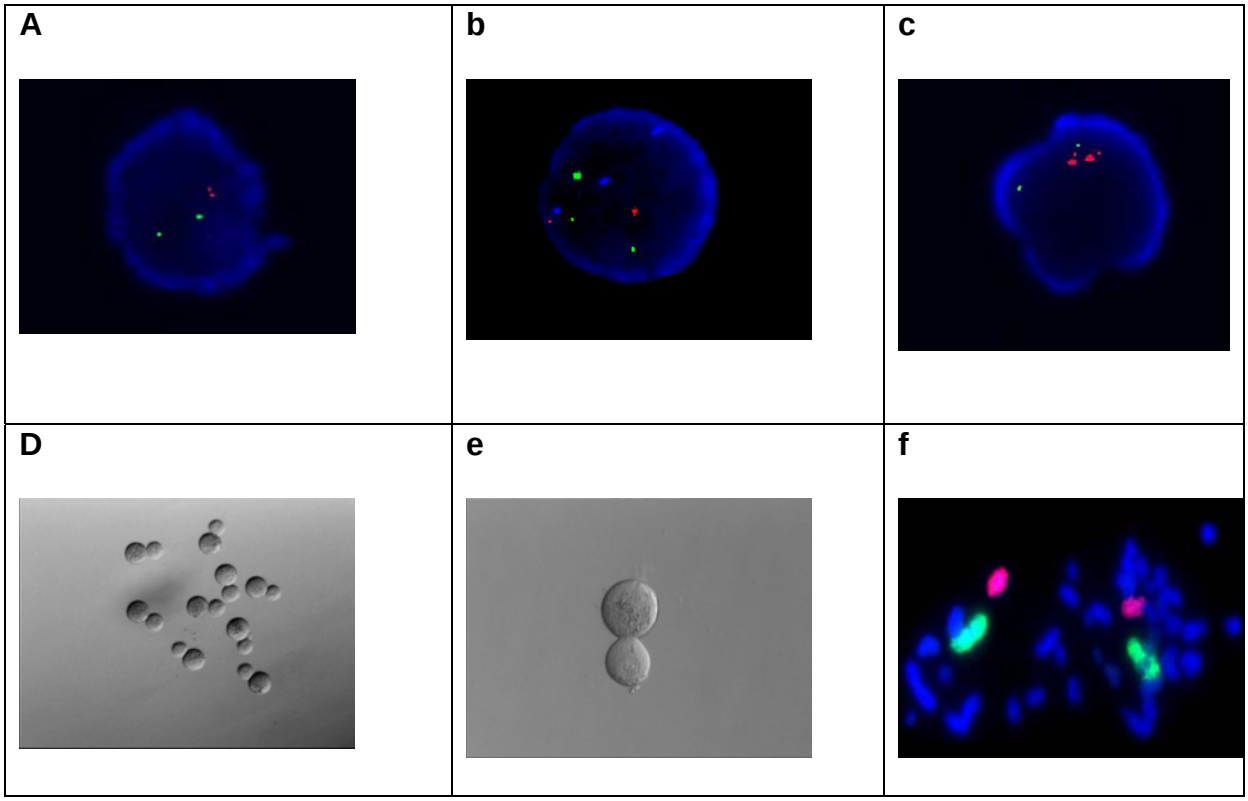


Figure 3. a) Robertsonian translocation 45 XY;rob t(13;14)(q10;q10) normal or balanced; b) Reciprocal translocation 46 XX;rcp t(11;22)(q25;q31) partial trisomy 11 (blue) and partial trisomy 22 (green); c)Reciprocal translocation 46 XX;rcp t(2;3)(q37;q27) normal or balanced; d) Blastomeres paired with mouse zygotes prior to electrofusion e) Fusion of two cells; f) FISH result on metaphase chromosomes after fusion.



specific for chromosomes to be analyzed. Several fixation methods are now available and their advantages and possible drawbacks such as the risk of misdiagnosis have recently been evaluated (Velila *et al*., 2002).

Current limitations and future prospectives

Although, the application of PGD becomes an invaluable tool for ART and clinical genetics, in order to increase its efficiency, several limitations should be overcomed. First, the fact that only a limited subset of chromosomes can be analyzed in conventional FISH techniques restricts the successful outcome in PGD-AS applications (Munne and Weier 1996; Munne *et al*., 1999). This limitation is mainly attributed as technical, since it involves chromosome analysis on interphase nucleus, other than metaphase spreads which could allow karyotyping hence making the analysis of all the chromosomes possible. Interphase FISH also fails to determine whether the analyzed arrangement is normal or balanced in the case of structural chromosomal abnormalities. However, it has recently been reported that the application of nucleus conversion technique, which involves the fusion of a biopsied sample with a bovine or a mouse zygote successfuly converted the interphase nucleus to a metaphase plate, giving reproducible and efficient results that can be analyzed for PGD (Evsikov and Verlinsky 1999; Willadsen *et al*., 1999). Representative images of this technique are shown in Figure 3. Application of this technique has recently been shown to be applied on 94 cycles, giving a 30.3% pregnancy rate (Verlinsky 2002).

Likewise, comparative genomic hybridization (CGH) has also been proposed as an alternative to interphase FISH. However, the time required (2-3 days) for the analysis requires cleavage stage embryos to be cryopreserved hence is not suitable for current clinical procedures. Although, successful pregnancies have been reported by CGH, cryopreservation after biopsy gives lower viability and poor ART outcome (Joris *et al*., 1999; Magli *et al*., 1999; Wilton *et al*., 2001; 2002). In the near future, improvements in the protocols, either shortening the time required for CGH or cryopreservation will create an alternative protocol for analyzing the whole set of chromosomes in a given embryo.

Another approach, which utilizes PCR and sequencing-based methods hence named as DNA fingerprinting has been developed and tested for the most common chromosomal abnormalities such as trisomy 21 (Katz *et al*., 2003). This technique initially included markers for 5 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. Although, the first impact would be the analysis of gene expression or mutation profiles on oocytes and embryos of different developmental stages which can provide potential targets for diagnosis. 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. Several microarray prototypes have already been designed for standard aneuploidy testing and for Robertsonian translocations; however, the technique requires further clinical confirmations and improvements (Kuliev and Verlinsky 2004a).

Although, the successful results are obtained in more than 90% of the blastomeres analyzed during conventional FISH analysis, the presence of mosaicism is of a major concern in PGD-AS cycles. 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; Munne *et al*., 2002; Sherman *et al*., 2003;). Obtained results can therefore carry a risk of representing false results, that is an embryo with majority of chromosomally normal blastomeres can be diagnosed as aneuploid and discarded from embryo transfer procedure.

Conclusion

In summary, cumulative analysis of more than 6000 PGD cycles performed to date indicates that application of PGD (i) prevents genetic disorders in couples at risk of having a child with a genetic disease, (ii) reduces the risk of high order pregnancies as well as repeated early abortions especially for translocation carrier couples and (iii) improves the ART outcome in poor prognosis patients such as women with increased maternal age. Expanding indications as well as novel approaches such as preimplantation HLA typing and the application of DNA microarray technologies also make PGD not only a diagnostic, but also a therapeutic tool for ART clinics. Although, there exist some limitations to be overcomed with technical protocols, results of the accumulated clinical data is encouraging and the validity as well as accuracy have already been proven. Therefore, PGD facilities have already become an integrated part of an increased number of ART clinics worldwide.

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