PREIMPLANTATION DIAGNOSIS

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INTRODUCTION

With the report of the birth of the first in-vitro fertilization (IVF) infant, 'Louise Joy Brown' in 1978, a lot of progress have been achieved in the reproductive endocrinology field (1). It involves the steps of hyperstimulation of the ovaries to achieve many oocytes in good quality, aspiration of these mature oocytes and bringing them together with the previously collected and prepared sperm to get fertilization. Afterwards the early conception product, namely the embryo is transferred back to the uterus. Now the IVF is considered to be safe and so far nearly 100.000 infants born through this procedure have been reported. IVF has opened the door to several reproductive advances such as oocyte donation, embryo freezing and assisted fertilization procedures like the recently introduced and very successful procedure of intracytoplasmic sperm injection (ICSI) for couples with severe male factor infertility and lastly the preimplantation diagnosis.

Genetic disease is a significant cause of illness and mortality in infants. The overall incidence at birth including those resulting from chrosomal abnormalities and single gene defects, is about 1% to 3%. Although most single gene defects are rare, close to 5000 have now been identified. Efforts are being made to develop methods for somatic gene therapy, that is the replacement of normal gene function in the somatic cells of affected individuals. A promising example of this is the possibility of using aerosol sprays to deliver vectors with the normal gene to the lungs of patients affected by cystic fibrosis (CF) (2). Other diseases in which somatic gene therapy may be possible are those in which the defect can be corrected by alteration of blood cells temporarily removed for transfectin with the normal gene. These include adenine deaminase deficiency which causes severe combined immunodeficiency syndrome and is normally lethal within weeks at birth.

Current methods of prenatal diagnosis involve sampling cells of fetal origin, for example amniocentesis in the second trimester or chorion villus sampling (CVS) in the first trimester of pregnancy and use of cytogenetic biochemical or DNA methods to detect the genetic defect. If the pregnancy is affected, however couples face the difficult decision of whether to terminate the pregnancy. Preimplantation diagnosis makes it possible to transfer only unaffected embryos to the uterus, avoids the possibility of a termination following diagnosis at later stages of pregnancy.

Preimplantation diagnosis refers to a technique whereby the genetic diagnosis of an oocyte or an early cleavage-stage embryo is carried out before implantation. IVF or ICSI are currently used to provide embryos for preimplantation diagnosis (3-6). So the patients who will undergo preimplantation diagnosis should also undergo a full IVF cycle with or without ICSI and the embryos will be genetically screened through micromanipulative techniques and the ones proved to be genetically normal can be transferred back to the uterus.

INDICATIONS AND DIFFERENT APPROACHES FOR PREIMPLANTATION DIAGNOSIS

In fact, the indications for preimplantation diagnosis are like those as for conventional amniocentesis and CVS. Couples who may benefit are at risk of;

- chromosomal disorders especially translocations
- monogenic X-linked, autosomal recessive or autosomal dominant disorders
- mitochondrial diseases

For the purpose of preimplantation diagnosis, the first polar body from an oocyte, blastomere(s) from an

early-cleavage-stage embryo or trophectoderm cells from blastocysts can be obtained by micromanipulation.

Preconception diagnosis (polar body biopsy): Preconception diagnosis refers to the genetic analysis of gametes either sperm or oocyte. In fact the genetic analysis of a single sperm is possible (7) but it is not feasible for the purpose of preimplantation diagnosis, because sperm cells are destroyed during the analysis. So till more efficient technique to analyse the sperm cell without any damage is achieved, currently the preconception diagnosis is defined as the sampling and genetic analysis of the first polar body of an oocyte from which the genetic information regarding the genetic status of the oocyte is achieved.

Polar body biopsy involves the removal of the nonfunctioning haploid set of chromosomes of the first meiotic division. After removal of the surrounding cumulus and corona cells, the polar body becomes clearly visible and can be removed by micromanipulation. This approach has some advantages; 1) the first polar body is normally extruded by the oocyte after meiosis I, and is not thought to play a critical role in the further development after fertilization of the oocyte, 2) the risk associated with the removal of the polar body using micromanipulation may be less than those resulting from the removal of blastomeres from early-cleavage-stage embryos unless the first polar body is fragmented and sticks to the membrane of the oocyte, 3) it allows diagnosis before conception, which may be good for the couples ethically opposed to abortion.

Normal development of the embryo to the blastocyst stage after removal of the first polar body was reported (8). Subsequently, two human pregnancies were reported after fertilization of uneffected oocytes (9). If a mutant allele for an autosomal recessive disorder is detected in the polar body, it may be concluded that the primary oocyte contains a normal allele. Contrary, if the polar body contains the normal allele, the primary oocyte has the mutant allele and will not be inseminated. It has some disadvantages like, in cases where the male partner is at risk of transmitting an autosomal dominant disease this approach cannot be offered. Also this method cannot be used for the purpose of gender determination simply because the sex of the embryo is determined by the sperm that fertilizes the oocyte. Cross-over is another problem in the analysis of the first polar body; the primary oocyte and the first polar body may contain copies of the two alleles if cross-over occurs between homologous chromosomes; the genotype of the secondary oocyte cannot be predicted without further testing of the second polar body after fertilization. Polar body diagnosis has had limited application and success in clinical practice.

Genetic analysis of blastomeres: Currently, almost all successful clinical preimplantation diagnoses have involved in removal of one or two blastomeres through micromanipulation from 6 to 10 cell stage embryos. This approach is mostly preferred since under normal culture conditions in IVF, more than 60% of fertilized oocytes will develop to this stage, 6 to 10 cell stage at day three after insemination. It has been shown that when one or two cells are removed at 8 cell stage embryos, the embryos will develop normally to the blastocyst stage with no alteration in cell number (10).

Obviously there are fewer cells to analyse compared to blastocyst biopsy. Technically there are three different procedures for removal of the blastomeres: 1) a two-step procedure combining zona drilling and blastomere aspiration, 2) a three-step procedure combining partial zona disection, squeezing of the embryo and aspiration of the extruded blastomeres and 3) direct puncture of the embryo and aspiration of the blastomeres. All three procedures require micromanipulation. The most widely used method is the combination of drilling a hole in the zona pellucida with acid tyrode and aspiration of the blastomeres (Fig I).

Blastocyst biopsy (trophectoderm biopsy): In average, a in vitro cultured human blastocyst contains about 58 to 126 cells between 5 and 7 days after insemination (10). This technique is carried out by creating a slit or an opening with a microneedle in the zona pellucida opposite the inner cell mass; the trophectoderm cells (10-30) will herniate through the opening and are then separated with a microneedle (11). These cells will then be used for DNA or chromosome analysis.

This approach has some advantages. First, the embryo reaches a maximum number of cells before implantation and for the analysis there will be more cells available for analysis than the use of polar body biopsy or blastomere(s) of the early-cleavage embryos. Besides, this technique is a safe procedure, since the trophectoderm cells are extraembryonic and will contribute only to placental tissue after implantation. So on the other side developing fetus is not under risk while at this stage the embryonic gene expression is well established. Unfortunately only about 35% of the embryos reach the blastocyst stage after IVF which will decrease the change of pregnancy. In literature, the pregnancy rate following IVF and transfer of blastocyst-stage embryos was reported to be low (12). So it could not find place as a diagnostic technique in preimplantation diagnosis.

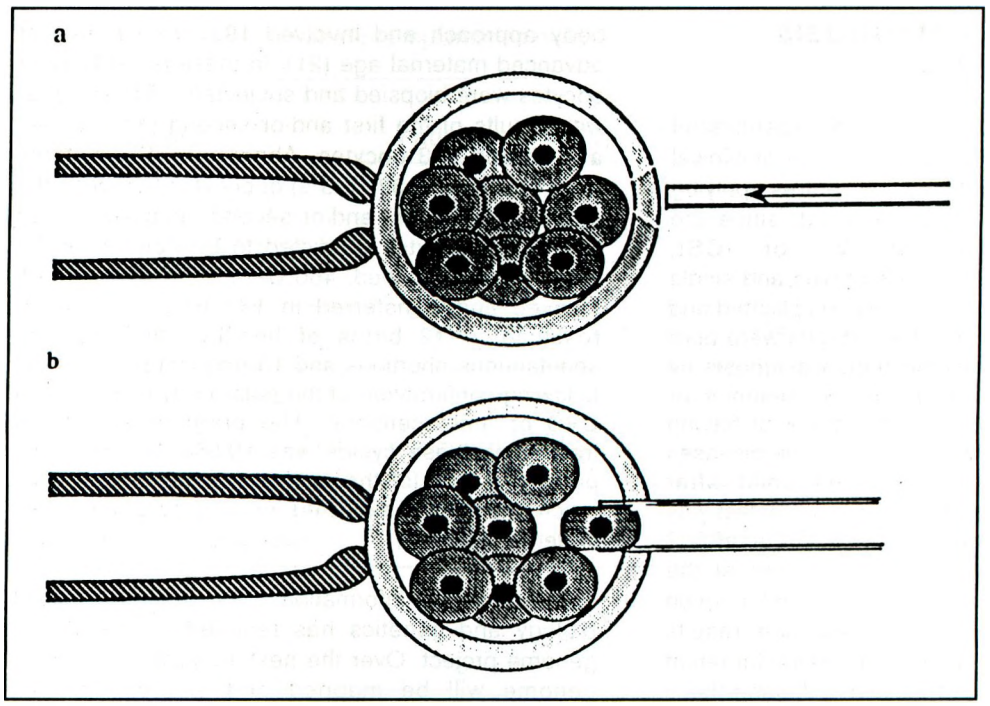

Fig. 1: Zona drilling and aspiration of blastomere(s): the 8-cell embryo is held by a holding pipette. A hole is made in the zona pellucida by squirting acidic tyrode solution (a). A blastomere is aspirated into a biopsy pipette (b).

DIAGNOSTIC TECHNIQUES USED IN PREIMPLANTATION DIAGNOSIS

Classically in other prenatal diagnosis techniques, the analysis is carried out on a few mg of chorionic tissue or on a few millions of amniocytes. In preimplantation diagnosis, there are only one or two blastomeres available to carry out this diagnostic analysis. So extremely sensitive methods should be used. Two techniques are currently used for preimplantation diagnosis of genetic diseases in clinical practice: polymerase chain reaction (PCR), which is used to identify single gene defects and flourescent in situ hybridization (FISH), which is used to detect chromosome abnormalities. Both PCR and FISH can be used for gender determination of preimplantation embryos.

In situ hybridization (ISH) with sex chromosome specific probes is an effective alternative to karyotype analysis and chromosome banding for sexing embryos, and it can be used for the detection of X and Y chromosomes and chromosome abnormalities, such as aneuploidy. With the development of non-isotopic FISH methods, it has advantages of high specificity and sensitivity and the fact that the procedure can now be completed in as short as two to four hours (13). The FISH technique applied to cleavage-stage-embryos seems to have become the preferred method for gender determination since the simultaneous detection of X and Y-chromosome specific probes is likely to be the most reliable technique for identifying the sex of an embryo and aberrations in the number of sex chromosomes can be detected as well. It can also be used to detect abnormalities in other chromosomes such as trisomy of chromosome 21,18, and 13 (14).

Another very powerful technique is the PCR. Genetic disorders may be caused by several changes in DNA. These changes may involve the modification i.e. point mutation, the deletion or insertion of one or a few base pairs. Through PCR, that small portion of DNA will be copied as millions. And it becomes easy to detect an error in the amplified portion of this DNA since millions of copies of DNA will be present. Theoretically, PCR can be used for any genetic disease if the mutant gene is known. Its application on single cells in preimplantation diagnosis requires a special attention. The time required for the process of generating multiple copies of the target DNA sequences is short and can be completed within a day. DNA amplification using PCR is an alternative to FISH for gender determination in preimplantation diagnosis. DNA amplification of only Y-specific sequences is however not reliable enough for preimplantation diagnosis because of amplification failure (15). Now such diagnoses are based on PCR assays looking simultaneously for X- and Y-signals (16,17). So now PCR assay is believed to be suitable for preimplantation diagnosis for couples at risk of X-linked genetic disease.

PREIMPLANTATION DIAGNOSIS IN CLINICAL PRACTICE

Preimplantation diagnosis is still an experimental procedure with only about 6 years' history of clinical applications. There are still very few centres applying preimplantation diagnosis in the world, since the combined technologies of IVF or ICSI, micromanipulation of oocytes or embryos and single cell PCR and FISH analysis are very complicated and hard to perform as a whole. The first girls were born in 1990 after IVF and preimplantation diagnosis by gender determination based on the presence or absence of a Y signal in couples at risk of having children with a variety of X-linked recessive diseases affecting only boys (18) The first child after preimplantation diagnosis of a single gene defect was born to a couple at risk for the CF DeltaF508 mutation in 1992 (4). Recently, 8 centres at the Fourth Meeting of the International Working Group on Preimplantation Genetics presented their results (Table I) (9). To that date, 164 preimplantation diagnosis cycles have been achieved. Among these 42 pregnancies, 3 misdiagnoses have been carried out and 42 pregnancies have been observed: one after gender determination probably due to failed amplification and the two were in compound heterozygous embryos for unexplained reasons may be due to contamination or failure of amplification of one allele.

Lately in 1996, Soussis et al presented the obstetric outcome of their first 16 clinical pregnancies after the premiplantation diagnosis of inherited diseases (19). From 1989 to 1993, in their clinics thirty-three women underwent a total of 58 cycles and resulted 42 embryo transfers following preimplantation diagnosis. They achieved 16 pregnancies (12 singletons and 4 twins). They reported that three pregnancies were lost in the first trimester and of the remaining pregnancies, two had no prenatal diagnosis, six cases of X-linked disease had the sex confirmed by ultrasound and CVS was performed in the remaining five. All the singleton pregnancies had an uneventful antenatal course and the birthweights and Apgar scores of the babies were normal. The twin pregnacies presented obstetric complications but they comment these were not unusual.

For the ongoing debate about the routine preimplantation diagnosis of common aneuploidies in infertile couples of advanced maternal age, the experience reviewed by the Fifth Annual Meeting of International Working Group on Preimplantation Genetics has demonstrated the feasibility of the approaches of FISH analysis of either blastomeres or polar bodies removed from the oocytes (20). The majority of the cases have been performed by polar body approach and involved 193 IVF patients of advanced maternal age (21). In these patients 1293 oocytes were biopsied and subjected FISH analysis, with results of the first and/or second polar bodies available in 993 oocytes. Abnormal FISH patterns were observed in 328 (33%) of oocytes based on the analysis of the first and/or second polar bodies. Of 665 (67%) oocytes predicted to be normal for the chromosomes studied, 460 were normally fertilized, cleaved and transferred in 187 treatment cycles resulting in 12 births of healthy children, nine spontaneous abortions and 18 ongoing pregnancies following confirmation of the polar body diagnosis by CVS or amniocentesis. The pregnancy rate per transfer in these cycles was 19.9% well within he pregnancy rate for the routine IVF cycles and even much higher than expected for couples of advanced maternal age.

The explosion of information in the field of molecular biology and genetics has resulted in the human genome project. Over the next 10 years the human genome will be mapped and sequenced. The understanding of genetic disease will be revolutionized as particular genes will be localized and sequenced. The defects in the genetic code will be traced by these techniques and the abnormal protein present in each genetic disorder will be recognized.

Clinical details in preimplantation diagnosis for single-gene-defect disorders by PCR are summarised in Table II. The single-gene-defect where the preimplantation diagnosis was first applied is the cystic fibrosis. Later, most preimplantation diagnoses were done for this very common disease (Table II). It is inherited as an autosomal recessive disease affecting between 1/2000 to 1/2500 children (22). Parents who are carriers have a 1 in 4 risk of having an affected child. The disease is caused by mutations in the CF transmembrane conductance regulator gene, producing a deficient protein leading to inadequate ion transport (23). The major clinical manifestations of CF are chronic pulmonary disease and pancreatic enzyme insufficiency. In males with CF, there is also an abnormality of epididymis and vas deferens. Here, these tubes end in blind channels instead of connecting through to the urethra. Approximately 97% of CF males have this problem from birth and, as a result, most of them are sterile, although they have normal spermatogenesis. The gene of cystic fibrosis is identified in 1989 (24) and now it is known that there are more than 500 mutations responsible for the clinics of CF. Especially in male factor infertilities with congenitally absent bilateral vas deferens, the patients should be screened and through the PCR that mutation on that specific gene should be searched whether it is

Table I. Results obtained after preimplantation diagnosis at 8 different centres worldwide (9).

Indications	Patients	Cycles	Transfers	Pregnancies	Babies
X-linked disorders	54	83	70	23	21
Monogenic disorders	38	56	49	14	9
Chromosomal abn.	24	25	15	5	0
Total	**116**	**164**	**134**	**42**	**30**

Table II. Results of preimplantation diagnosis for single gene defect disorders by PCR (9).

Monogenic disorder	Patients	Cycles	Transfers	Pregnancies	Babies
Cystic fibrosis	27	33	31	9	6
Tay-Sachs disease	3	5	3	1	1
Lesch-Nyhan disease	2	4	3	1	1
Duchenne musc dystrophy	1	1	1	1	1
Hemophilia	2	6	6	1	0
Alpha-1-antitrypsin def.	1	5	3	0	0
Retinitis pigmentosa	1	1	1	0	0
Fragile-X syndrome	1	1	1	1	0
Total	**38**	**56**	**49**	**14**	**9**

passed onto the embryo or not. Preimplantation diagnosis is also very effective in other single-gene-defects like Tay-Sachs disease, Lesch-Nyhan disease, Duchenne Muscular Dystrophy, hemophilia, etc (Table II).

The use of assisted reproductive technologies such as conventional IVF or ICSI and the development of sensitive molecular methods at the single cell level allow preimplantation diagnosis of an inherited disease in early human embryos before implantation into the uterus. Preimplantation diagnosis may be considered a very early form of prenatal diagnosis in which disease free embryos are selected prior to establishing a pregnancy, so termination of pregnancy at later stages is then avoided.

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