Preimplantation Genetic Diagnosis for the Better Management of Couples During Assisted Reproduction

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ABSTRACT Intracytoplasmic Sperm Injection (ICSI) offers the real prospect of genetic parenthood for men with profound oligozoospermia and azoospermia. However, it may result in transgenerational transmission of genetic defect, which substantially increases the recurrence risk of infertility in the offspring of couples treated with ICSI. Recent developments in the field of genetics and assisted reproduction have led to emergence of preimplantation genetic diagnosis (PGD). PGD helps in the negative selection of the aneuploid and abnormal embryos. For PGD, the embryo is biopsied and 1-2 blastomeres are removed from the 6-10 cell embryo. For establishing the diagnosis, the genetic analysis is carried out using FISH (for structural and numerical chromosomal anomalies) or PCR (for single gene disorders). Only the normal embryos are transferred back to the uterus, thus ensuring a normal pregnancy. Spare or arrested embryos obtained following ICSI were used for the study. FISH was performed on 5 embryos using CEP X spectrum green probes. PCR was done for cystic fibrosis common mutation ∆F508 and for β-thalassaemia IVS1→5 and 619 bp deletion using blood as positive control. Mosaicism was noted in two of the five embryos using FISH. The blastomeres were found to be normal for cystic fibrosis and β-thalassaemia. PGD has an important role in helping patients to avoid the risk of transmission of genetic defect or abnormalities and also helps in avoiding repeated medical termination of pregnancy.

INTRODUCTION

Intracytoplasmic Sperm Injection (ICSI) has become a routine practice in management of infertility especially in cases of severe male factor infertility (Palmero et al. 1992). ICSI offers the real prospect of genetic parenthood for men with profound oligozoospermia and even with azoospermia (Kurinczuk et al. 1997). During ICSI all the natural sperm selection processes are bypassed, that may be weeding out the ab-

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normal sperm and hindering it from fertilization of the egg. Because of the visual sperm selection based on morphology, there is a potential risk of using sperms carrying genetic abnormality or sperms with structural defect (Kurinczuk et al. 1997). Hence, the major cause of concern with ICSI is the possibility of transgenerational transmission of genetic defects (Meshede et al. 2000). The common genetic abnormalities associated with male infertility include cystic fibrosis gene mutation (Patrizio et al. 1993), Y chromosome microdeletions (Bardoni et al. 1991; Foresta et al. 1997), and chromosomal abnormalities (structural and numerical) (Baschat et al. 1996; Testart et al. 1996). Defects and abnormalities may also be transmitted through the female arising most commonly due to non-disjunction at maternal meiosis I and II.

Though ICSI cannot avoid transmission of a genetic abnormality to the offspring, the identification of these defects in the embryo prior to implantation helps in overcoming the fear of having an abnormal baby. This is now possible with the advent of Preimplantation Genetic Diagnosis (PGD). PGD is a new and exciting advance in prenatal diagnosis bringing the hope of healthy babies to couples at risk of passing heritable diseases to their offspring. PGD, as the name suggests, diagnoses the genetic disease or chromosomal abnormality at the preimplantation stage i.e. before implantation (Egozcue et al. 2000). PGD thus helps in negative selection of the affected embryos before the pregnancy has been established and thus avoids the risk of recurrent abortion and/or repeated termination of pregnancy (Grifo et al. 1997).

PGD involves isolation of genetic material from the oocyte or the embryo (biopsy), genetic analysis of the cells for establishing the diagnosis (FISH or PCR) and transfer of unaffected embryos. Biopsy can be done at the oocyte stage (polar body biopsy) or at the embryo stage (blastomeres from the cleavage stage embryo or

trophectoderm cells from the blastocyst). The diagnosis is established using PCR or FISH. PCR is the process through which a single copy of DNA can be amplified millions of times, thereby making it possible to analyze the genetic make-up of single cells. However, the most common genetic abnormalities in humans are related to an abnormal number of chromosomes and are not detectable by PCR. For the study of chromosomal abnormalities in human embryos, FISH is the method of choice. FISH is based on the use of labeled DNA probes that bind specifically to the DNA of fixed cells. By using different fluorescently labeled probes, multiple chromosomes in a single cell can be simultaneously analyzed in much less time than it takes for classic karyotype analysis. Secondly, FISH allows chromosome enumeration to be performed on interphase cell nuclei, thus circumventing the need for culturing cells or preparing metaphase spreads as is necessary with karyotype analysis. FISH is currently the only method available to detect numerical chromosomal abnormalities as well as translocations in single cells from embryos (Munne et al. 1998). It is also used to selectively implant female embryos in cases of X linked genetic disorders like Hemophilia and DMD.

Present Status of PGD in the World

Multicolor FISH is being used to detect chromosomal aneuploidy on single cells for chromosomes 13, 18, 21, X and Y. These five sets of chromosomes are responsible for almost 95% of cytogenetic defects in live births.

PGD for cystic fibrosis is offered at many centers for human reproduction in USA and UK (Handyside et al. 1992). At this time, the detection of the DAZ gene deletion has not been utilized as a clinical test with PGD. Specific tests for Cystic fibrosis, Duchenne Muscular Dystrophy, Beta-Thalassemia, Tay-Sachs disease, Hemophilia, Fragile-X syndrome, Sickle cell disease, Retinitis Pigmentosa, Marfan syndrome, Huntington's disease, Fanconi's anemia have been developed and successfully applied in PGD using PCR.

The first case of PGD was reported in 1994 for a sex-linked disorder (Handyside et al. 1994). Over a period of the last 7 years, data is avail-

able on 886 patients who have undergone 1318 PGD cycles and data on 163 pregnancies and 162 babies are available from the ESHERE PGD consortium steering in December 2000 and the results have been shown to be very promising (ESHERE PGD Consortium, 2000).

Studies have been initiated at our Center at Jaslok Hospital, Mumbai, to set up PGD for major chromosomal aneuploidies. We have successfully standardized FISH procedure on single cell preparations collected from arrested embryos. Using specific probes from Vysis (UK), for the X chromosome, we studied the FISH signals in five samples (more than two cells were analyzed of each sample). Mosaicism was noted in two samples. Further evaluation using MultiVysion set of probes (Vysis, UK) is under progress. We have also standardized PCR for β-thalassemia and cystic fibrosis on single cells.

MATERIAL AND METHOD

ICSI was performed using an inverted microscope (Labovert SS, Lietz) at 250 X magnification and Lietz micromanipulation system with Narishige oil syringes (IM-6). Metaphase II oocytes were placed in the medium (GAMETE Medium, Scandinavia) and the sperms were placed in the central droplet in PVP (Medicult, Denmark). The droplets were covered with paraffin oil. The oocyte was stabilized with the holding pipette with the polar body at the 6 or 12 o'clock position. The injection micropipette containing the single sperm was then pushed through the zona pellucida and oolemma into the cytoplasm of the oocyte at the 3 o'clock position. The injection pipette was withdrawn gently and the oocyte released from the holding pipette. The fertilized oocytes were cultured in G1.2 medium (Scandinavia) at 37°C and 5% CO_2 till day 3 when the embryo reached the 6-10 cell stage.

Biopsy was performed on the spare or the arrested embryos (embryos failing to grow beyond 2-4 cell stage) under oil using an inverted microscope and micromanipulator system (Research Instruments, U.K). The embryo was secured with the holding pipette at 9 o'clock by gentle suction in such a way that the cell to be biopsied was at 3 o'clock. The zona was drilled using Laser (Fertilase®, MTG Medical

Technologies G.M.B.H. Germany). A 1.48 mm diode non-contact laser beam was used which brought about photolysis of the zona pellucida to form an opening. The aspiration pipette was placed through the hole in the zona and one or more cells were aspirated by gentle suction. In cases where spare embryos were used, post biopsy the embryos were cultured in labeled drops of G1.2 medium under pre-equilibrated mineral oil at 37°C and 5% carbon dioxide to evaluate the effect of biopsy on further embryo development.

FISH Protocol for PGD

Single cells biopsied from the embryo were placed on prepared glass slides and fixed (Munne et al. 1998). They were incubated in 2 X SSC at 37ºC for 30 min followed by dehydration in chilled alcohol grades for 1 minute each. After wiping the excess alcohol, 3 µl of Vysis probe cocktail containing CEP X spectrum green probe, was placed on the cell, covered with small glass coverslip and sealed with rubber cement. The target DNA in the cell and the probe were co-denatured at 75ºC for 5 min. Hybridization was done overnight at 37ºC in a moist chamber. The slides were then washed with 0.4 X SSC at 70ºC (for 2 minute), 2X SSC at room temperature for 1 minute, counter stained in Propidium Iodide for 35 sec, rinsed in PBS, mounted in antifade solution, sealed with nail paint and kept at 4°C for 30 min. The signals were observed under Zeiss Axioskop-2 with an FITC filter.

PCR Protocol

PCR was performed for cystic fibrosis common mutation ΔF508 on single blastomere DNA using blood DNA as positive control from a carrier patient. The blastomere was taken in the PCR tube and incubated in a boiling water bath for 10 min to lyse the cell and the extract was tested. DNA from whole blood (positive control) was extracted using Quiagen Kit. PCR was performed with a separate tube for normal and mutated primer for each patient. The denaturation was done at 95°C for 1min, followed by annealing at 56°C for 1min for 70 cycles and final extension at 72°C. The PCR products were analyzed using agarose gel electrophoresis.

For β-thalassemia IVS 1→5 and 619 bp deletion mutations were tested using blood from known carrier as positive control. The protocol used was similar to that used for cystic fibrosis except that the denaturation was done at 93°C for 1min, followed by annealing at 66°C for 1min for 100 cycles and final extension at 66°C.

RESULTS

FISH: Five embryos (two cells per embryo) were analyzed and mosaicism was noted in two embryos (Table 1). In embryo 1, one blastomere showed 2 signals and the other blastomere showed 3 signals for the X chromosome. In embryo 4, one blastomere showed 1 signal and the other showed 2 signals for the X chromosome. These 2 embryos showed X chromosome mosaicism. Two blastomeres each from embryos 2 and 3 showed the presence of only one signal. Embryo 5 showed two signals in both the blastomeres.

Table 1: Details of the FISH signals in two blastomeres obtained from the embryos

Embryo No.	Number of signals in Blastomere "a"	Number of signals in Blastomere "b"
1	2	3
2	1	1
3	1	1
4	1	2
5	2	2

PCR: Figure 1 shows the gel run for cystic fibrosis. Well 1 in the gel contained DNA from the blastomere and normal primer. It showed presence of the band of size 78bp confirming normal gene. Well 2 contained DNA from the blastomere and mutated primer. It did not show any of the bands and confirms absence of the mutation. Well 3 contained DNA from the blood (Carrier patient) and normal primer. The band of 78bp showed presence of the normal gene. Well 4 was empty. Well 5 contained DNA from the blood (Carrier patient) and mutated primer. It showed presence of a band of size 75bp, which confirmed the single stranded deletion of the gene. Hence from wells 3 and 5 the carrier status of the mutation was observed. Wells 6 and 7 contained normal and mutated primers respectively with distilled water as test sample. Absence of bands in both shows purity of PCR. Well
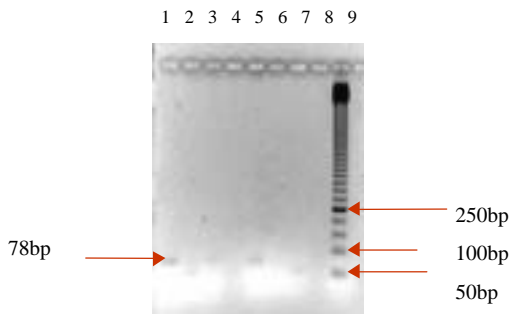
Fig. 1. PCR for Cystic Fibrosis Mutation D F 508
1. Normal: Blastomere DNA
2.Mutated: blastomere DNA
3. Normal: Blood DNA
4. Blank
5. Mutated: Blood DNA
6. Normal: Distilled Water
7. Mutated: Distilled Water
8. Blank
9. Standard Molecular Weight Marker

8 was left blank and well 9 contains standard molecular weight marker with 50bp difference in bands. Thus the blastomere under investigation is normal.

Figure 2 shows the gel run for β thalassemia IVS 1→5 and 619 bp deletion mutations. Well 1 in the gel contained DNA from the blastomere and normal primer for mutation IVS 1→5. It showed the presence of the band of size 285bp confirming normal gene. Well 2 contained DNA from the blastomere and mutated primer for mutation IVS 1→5. It did not show any band and confirmed absence of the mutation. Well 3

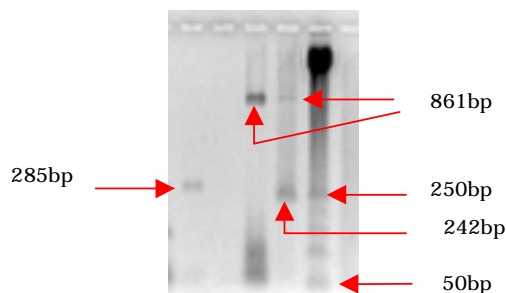**Fig. 2. PCR for β-Thalassemia Mutations**
1. IVS 1→5 Normal: Blastomere
2. Mutated: blastomere
3. 619bp Deletion: Blastomere
4. 619bp Deletion - DNA from lymphocytes

contained DNA from another blastomere and primer for mutation of 619bp deletion. The band of 861bp showed presence of the normal gene. Well 4 contained DNA from the blood (carrier patient) and primer for mutation of 619bp deletion. It showed presence of two bands, one of size 861bp, which confirmed the presence of the normal gene and the second of 242bp, which confirmed the deletion of 619bp. Hence from well 4 the carrier status of the mutation was observed. Well 5 contains standard molecular weight marker with 50bp difference in bands.

CONCLUSION

The desire for most couples harboring the genetic abnormalities associated with infertility, to have a child, overrides their concern of passing the defect or abnormality of the same traits to their progeny. Interestingly, even after genetic counseling, the decision to proceed with ICSI for the overwhelming majority of couples remains unchanged. They want to carry on with the treatment only with the hope that there will be more advanced assisted reproductive technologies in future to circumvent infertility. It is the responsibility of the physician to counsel the couple about the risks of potential infertility in their children, as well as more life-threatening situations such as cystic fibrosis disease and the unknown consequences of autosomal and Y-chromosome defects. Here the patients should be informed about PGD so as to avoid the birth of abnormal babies. PGD also helps to detect the defects or abnormalities at the preimplantation stage and hence it helps in avoiding medical termination of pregnancy, which is not acceptable in many communities on social grounds.

REFERENCES

Bardoni B, Zuffardi O, Guioli S, Ballabio A, Simi P, Cavalli P 1991. A deletion map of the human Yq11 region: implications for the evolution of the Y chromosome and tentative mapping of a locus involved in spermatogenesis. *Genomics,* **11(2)**: 443-51.
Baschat AA, Kupker W, al Hasani S, Diedrich K, Schwinger E 1996. Results of cytogenetic analysis in men with severe subfertility prior to intracytoplasmic sperm injection. *Hum Reprod,* **11(2)**: 330-3.
Egozcue J, Snatalo J, Gimenez C, Perez N, Vidal F 2000. Preimplantation Genetic Diagnosis, *Mol. Cell. Endocrinol,* **166(1)**: 21-5.

ESHERE PGD Consortium Steering 2000. ESHERE Preimplantation Genetic Diagnosis (PGD) Consortium data collection II, *Hum Reprod,* **15(12)**: 2673-83.

Foresta C, Ferlin A, Garolla A, Rossato M, Barbaux S, De Bortoli A 1997. Y-chromosome deletions in idiopathic severe testiculopathies. *J Clin Endrocrinol Metab,* **82(4)**: 1075-80.

Grifo JA, Tang YX, Kery L 1997. Update in preimplantation genetic diagnosis. Age, genetics, and infertility. *Ann N Y Acad Sci,* **828**: 162-5.

Handyside AH, Lesko JG, Tarin JJ, Winston RM, Hughes MR 1992. Birth of a normal girl after in vitro fertilization and preimplantation diagnostic testing for cystic fibrosis. *N Engl J Med,* **327(13)**: 905-9.

Kurinczuk JJ, Bower C 1997. Birth defects in infants conceived by intracytoplasmic sperm injecton: an alternative interpretation. *BMJ,* **315(7118)**: 1260-5; discussion 1265-6.

Meschede D, Lemcke B, Behre HM, De Geyter C, Nieschlag E, Horst J 2000. Clustering of male infertility in the families of couples treated with intracytoplasmic sperm injection. *Hum Reprod,* **15(7)**: 1604-8.

Munne S, Fung J, Cassel MJ, Marquez C, Weier HU 1998. Preimplantation genetic analysis of translocation: case specific probes for interphase cell analysis. *Hum Genet,* **102(6)**: 663-74.

Palmero S, Benahmed M, Morera AM, Trucchi P, Fugassa E 1992. Indentification of nuclear tri-iodothyronine receptors in Sertoli cells from immature piglet testes. *J Mol Endocrinol,* **9(1)**: 55-9.

Patrizio P, Ord T, Silber SJ, Asch RH 1993. Cystic fibrosis mutations impair the fertilization rate of epididymal sperm from men with congenital absence of the vas deferens. *Hum Reprod,* **8(8)**: 1259-63.

Testart J, Gautier E, Brami C, Rolet F, Sedbon E, Thebault A 1996. Intracytoplasmic sperm injection in infertile patients with structural chromosome abnormalities. *Hum Reprod,* **11(12)**: 2609-12.

Tiepolo L, Zuffardi O 1976. Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y-chromosomes long arm. *Hum Genet,* **34(2)**: 119-24.

Tuerlings JH, Mol B, Kremer JA, Looman M, Meuleman EJ, Buys CH, Merkus HM et al. 1998. Mutation frequency of cystic fibrosis transmembrane regulator is not increased in oligozoospermic male candidates for ICSI. *Fertil Steril,* **69(5)**: 899-903.

Van Opstal D, Los FJ, Ramlakhan S, Van Hemel JO, Van Den Ouweland AM, Brandenburg H, et al. 1997. Determination of the parent of origin in nine cses of prenatally detected chromosome aberrations found after intracytoplasmic sperm injection. *Hum Reprod,* **12(4)**: 682-6.

Veld PA, Weber RF, Los FJ, den Hollander N, Dhont M, Pieters MH, et al. 1997. Two cases of Robertsonian translocations in oligozoospermic males and their consequences for pregnancies induced by intracytoplasmic sperm injection. *Hum Reprod,* **12(8)**: 1642-4.