Pre-implantation Genetic Diagnosis: A Preliminary Report of 2 Years of Experience

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Background: Pre-implantation genetic diagnosis (PGD) is defined as making a diagnosis or screening embryos or gametes before implantation. It has the advantage of avoiding repeated spontaneous abortions or therapeutic termination of pregnancy resulting from abnormal embryos. Here, we present our preliminary report of 2 years of experience.

Methods: From March 2001 through October 2002, couples seeking assistance for in vitro fertilization (IVF) were referred for PGD due to chromosomal problems or for aneuploidy screening (PGD-AS). One or two blastomeres were aspirated on day 3 and analyzed using the fluorescent in situ hybridization (FISH) technique. Probes to chromosomes X, Y, and 18 were used for aneuploidy screening and individual specific probes were chosen for chromosomal translocations. Unaffected embryos were transferred on day 5.

Results: There were 25 cycles for aneuploid screening (group 1) and four cycles for chromosomal translocation (group 2). In group 1, 73 embryos were biopsied with a successful biopsy/fixation rate of 72.6% and a diagnosis rate of 96.2%. Fifteen unaffected embryos were transferred in 11 cycles, achieving two sets of twins and four singleton pregnancies (implantation rate: 53.3%). In group 2, 27 embryos were biopsied with a successful biopsy/fixation rate of 66.7% and a diagnosis rate of 88.9%. Seven non-affected embryos were transferred in three cycles, resulting in one set of twins (implantation rate: 33.3%). All antenatal amniocentesis confirmed the diagnosis. Post-natal physical examination showed no evidence of major abnormalities.

Conclusions: PGD is an alternative method for having healthy children in selected couples with chromosomal abnormalities. In addition, PGD-AS may increase the implantation rate in infertile couples seeking IVF assistance.
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Key words: pre-implantation genetic diagnosis (PGD), fluorescent in situ hybridization (FISH).

The prenatal diagnosis of inherited genetic diseases conventionally involves chorionic villus sampling during the first trimester of pregnancy and amniocentesis during the second trimester. Couples

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with a chance of having affected children have difficulties in making decisions of whether to continue or terminate affected pregnancies. Some couples repeatedly terminate pregnancies in attempts to have a healthy child.

Pre-implantation genetic diagnosis (PGD) is defined as making a diagnosis or the screening of embryos or gametes before implantation. It has the advantage of transferring only unaffected embryos into the uterus, and thus avoiding repeated spontaneous abortions or therapeutic termination of a pregnancy resulting from implantation of abnormal embryos. The procedure consists of in vitro fertilization (IVF) and genetic diagnosis. Basically, there are two kinds of techniques used to make diagnosis: polymerase chain reaction (PCR) and fluorescent in situ hybridization (FISH).[1,2] The former is applied successfully for the diagnosis of a single gene defect and the latter is used successfully for X-linked diseases, chromosomal abnormalities and aneuploidy screening. PGD-aneuploidy screening (PGD-AS) is performed in patients with advanced maternal age (>35 years), repeated miscarriages, and repeated IVF failures.[3]

The first live birth from successful PGD was reported in 1990.[4] Later, more and more medical centers have adopted PGD and the number of PGD cycles has been increased. Since 1997, the European Society of Human Reproduction and Embryology (ESHRE) PGD Consortium has undertaken a long-term study of the efficacy, safety, and clinical outcomes of patients undergoing PGD. There have been at least 2000 PGD cycles reported, resulting in more than 200 babies born.[3] In our reproductive center at Chang Gung Memorial Hospital, the PGD technique was introduced in March 2001. To date, seven healthy babies have been born from 29 treatment cycles. Here, we present a preliminary clinical report of the use of PGD during a 2-year period.

METHODS

Patients

From March 2001 through October 2002, 20 couples were referred for PGD or PGD-AS. Among them, 18 infertile couples underwent PGD-AS, and 2 female carriers with chromosomal translocation underwent PGD. In the latter, both couples presented with habitual abortion. Analysis of the aborted tissue and karyotype of both couples revealed the problem of chromosomal translocation. One female carrier had Robertsonian translocation, 45, XX, der (13; 14)(q10; q10) and the other one had reciprocal translocation 46, XX, t(14; 22)(q24; q13). After genetic counseling, they decided to undergo this procedure. The other 18 infertile couples that had factors of advanced maternal age, repeated miscarriages and repeated IVF failures underwent PGD-AS. All couples gave informed written consent and understood the current success rates of the procedures.

Ovarian stimulation and oocytes retrieval

The main stimulation protocol used was the long protocol. A gonadotropin-releasing hormone (usually leuprolide acetate, Lupron; TAP Pharmaceuticals, Chicago, Ill, USA) was given on day 21 of menstrual cycle. When menses came, the patients received examinations using vaginal ultrasound and serum estradiol. Once the ultrasounds showed ovarian quiescence with no ovarian cysts and estradiol levels below 30 pg/ml, administration of gonadotrophins was initiated with recombinant follicle-stimulating hormone at 150 IU (Gonal F; Serono laboratories, Randolph, Mass USA) per day. The daily dose was adjusted by individual response. Oocyte retrieval using transvaginal ultrasound was carried out 34-36 hours after administration of human chorionic gonadotrophin (HCG, Profasi; Serono, Italy) when two or more follicles reached the mean diameter of 18 mm.

Oocyte collection, insemination and embryo culture

Oocytes were collected in 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)-buffered human tubal fluid medium (HTF). After serial washing, each oocyte recovered was maintained at 37˚C in a separate drop of IVF-50 culture medium (Universal IVF, Medi-Cult) equilibrated with 5% CO_2 in air. Conventional IVF or intracytoplasmic sperm injection was carried out using motile spermatozoa prepared using the swim-up procedure. The zygotes were checked for the presence of pronucli (PN) and polar bodies at 18-21 hours after the insemination or microinjection. Zygotes with two PN were cultured at 37˚C in a separate drop of IVF-50 culture medium (Universal IVF, Medi-Cult) equilibrated with 5% CO_2 in air until day 3.

Embryos transfer and follow up

After the diagnoses of biopsied blastomeres were made (see following procedure), the unaffected embryos or normal diploid embryos were transferred back immediately (usually on day 4 post-oocyte retrieval). Pregnancies were checked using results of urinary tests or serum β-human chorionic gonadotropin levels at 2 weeks after embryo transfer. Clinical pregnancy was defined as a distinct intrauterine gestational sac seen on transvaginal or abdominal ultrasound. Antenatal amniocentesis during the secondary trimester of pregnancy was recommended to confirm our PGD results. Post-natal karyotyping and physical examinations were performed.

Embryo biopsy and blastomere fixation

Embryos with 7 or more cells (grade I or II) on day 3 were selected for biopsy. Zona drilling was carried out using a 1.48 µm (infrared) diode laser (Fertilase®; MTM, Medical Technologies, Montreux SA, Switzerland) connected to an inverted microscope (Diaphot 300; Nikon, Tokyo, Japan). Four pulses of 10 ms duration were used for zona opening. An opening of approximately 25-30 µm was made to allow placement of the embryo biopsy pipette (Cook IVF, Queensland, Australia). Only one blastomere from each embryo was aspirated with a clear nucleus seen. On some occasions when the blastomere fixation failed, a second blastomere was aspirated.

The aspirated blastomere was transferred and washed in phosphate buffered saline at room temperature. It was then placed on a drop around 10 µl of fixatives (0.1% Tween 20, 0.01 M HCl in distilled water) on top of a poly-1-lysine-coated slide. The fixative was spread by continuous and gentle blowing until the cytoplasm dissolved. The final position of the nucleus was marked using a diamond pen. After dehydration in serial ethanol (70%, 90%, and 100%), the slides were dried and ready for FISH.

Whole embryo spreading and blastomere preparation

For the non-transferred embryos, they were not considered as normal embryos and they were spread to investigate the chromosome status under the patients' consent. Each embryo was washed in PBS at room temperature and then placed on a drop of approximately 10 µl of fixative (0.1% Tween 20, 0.01M HCl in distilled water) on top of a poly-1-lysine-coated slide. The fixative was spread by continuous and gentle blowing until the cytoplasm dissolved. The final position of the nucleus was marked using a diamond pen. After dehydration in serial ethanol (70%, 90%, and 100%), the slides were dried and ready for FISH.

FISH

The FISH method that are set up in our laboratory follow the procedures described by Harper and Delhanty.[5-7] The efficiency of the FISH procedure was tested in each experiment on interphase nuclei of male human leukocytes.[6-8]

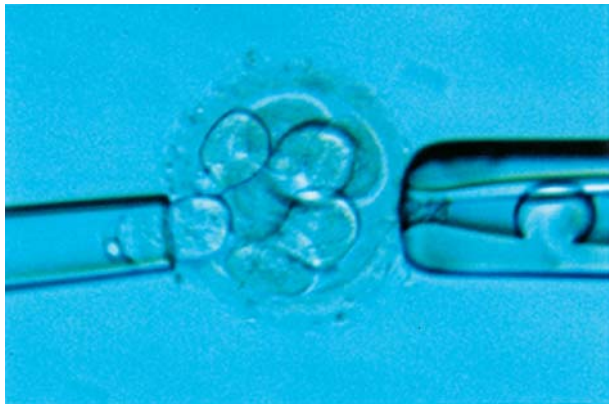

Fig. 1 A blastomere was aspirated gently through a hole on the zona which was made with the assistance of a laser.

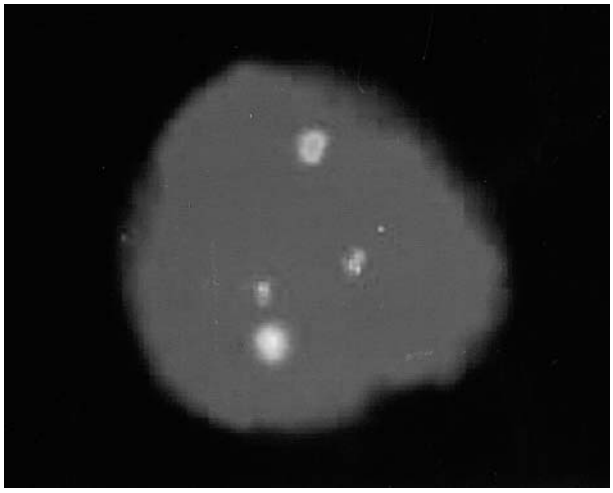

Fig. 2 A blastomere showed one green signal for chromosome X, one orange signal for chromosome Y and two white signals for chromosome 18.

Probes used in aneuploidy screening were pBam X5, alphoid probe (insert size 2.0 kb), specific for the centromeric region of the X chromosome, cY98, specific for the 3.77 kb region on the long arm of the Y chromosome and L1.84, alphoid probe, specific for the centromeric region of human chromosome 18. These probes were kind gifts from Prof. Joyce Harper and Prof. Joy Delhanty at the University College London, UK. For translocation couples, commercial probes from Vysis were used (LSI 13, TelVysion 14q and LSI DiGeorge/VCSF/LSI ARSA).

The details of the FISH protocols for probes to 18, X, and Y are the same as described before.[5,6] The procedure of the FISH for commercial probes followed the product sheet. FISH signals were counted using the criteria described by Hopman et al.[9] The chromosomal abnormalities detected using the FISH studies included aneuploidy, haploidy and polyploidy. Embryos comprising more than 2 cell lines were classified as mosaic embryos.[7,10] Since chromosomes 18, X, and Y were used in this study, analytic criteria and classification were adopted from those of Munné et al.[10]

RESULTS

According to the indications, 29 IVF cycles from 20 patients were included in the study and were divided into two groups. Eighteen patients with 25 cycles who underwent PGD-AS were included in group 1. The remaining two patients with four cycles were carriers of chromosomal translocation and were included in group 2.

In group 1, the mean age of those patients was 36.2 years (range, 32 to 41 years). Twenty-five cycles were started and 6 cycles were cancelled due to poor response. Totally, 250 mature MII oocytes were retrieved (Table 1) and inseminated. ICSI were performed in eight cycles. Of them, 153 oocytes were fertilized normally (normal fertilization rate, 64.3%). There were no normal fertilized embryos in one of the 19 cycles. In three cycles, there were no good embryos suitable for biopsy. Of the remaining 15 cycles, only 73 embryos met the criteria for biopsy. Fifty-three biopsied embryos were fixed successfully (success rate, 72.6%) and 51 were diagnosed using FISH (diagnosis rate, 96.2%). Fifteen embryos were transferred in 11 cycles leading to 6 clinical pregnancies including 2 sets of twins and 4 singleton pregnancies. It resulted in the blighted ovum and 5 viable fetuses. The pregnancy rate per transfer was 54.5% and the implantation rate was 53.3%. Antenatal amniocentesis of every pregnancy was confirmatory. Five healthy infants were achieved and post-natal physical examination showed no evidence

Table 1. Clinical Details of Cycles Proceeding to Embryo Biopsy between the Group with Aneuploidy Screening and the Group with Chromosomal Abnormalities

	Group 1 (n = 18) *	Group 2 (n = 2)	Total (n = 20)
Cycles	25	4	29
Cycles to oocyte retrieval	19	4	23
Number of oocytes aspirated	250	67	317
Number of inseminated oocytes	238	67	305
Number of oocytes fertilized normally	153	37	180
Number of biopsied embryos	73	27	100
Number of successful biopsies	53	18	71
Number of diagnosed	51	16	67
Number of embryos transferred back	15	7	22
Cycles to embryo transfer	11†	3‡	14
Number of clinical pregnancy	6	1	7
Number of gestational sac	8	2	10
Number of positive fetal heart beat	5	2	7
Births of babies	5	2	7

* Number of patients expressed in parentheses

† No normal fertilization in 1 cycle, poor embryo development without biopsy in 3 cycles, and no embryos suitable for transfer in 4 cycles

‡ No embryos suitable for transfer in 1 cycle

of congenital anomalies. The results are shown in detail in Table 2 and Table 3.

In group 2, 67 oocytes were retrieved from four cycles, including three cycles for Robertsonian translocation and one cycle for reciprocal translocation. Thirty-seven oocytes were fertilized normally (fertilization rate: 55.2%) and 27 embryos were suitable for biopsy. Eighteen embryos were biopsied and fixed successfully (success rate, 66.7%) and 16 were diagnosed using FISH (diagnosis rate, 88.9%). Seven non-affected embryos (normal or balanced translocation karyotype) were transferred in three cycles, resulting in one twin pregnancy. Amniocentesis of the twins showed 45, XX, der(13; 14)(q10; q10) and 46, XX, 9ph. Post-natal karyotyping confirmed the results and physical examination showed no evidence of major abnormalities.

For those non-transferred embryos, the chromosome status of whole embryos were analyzed and classified in Table 4.

DISCUSSION

Translocations occur at a frequency of approximately 0.1% in the general population.[11] In addition, they were found in 0.6% of infertile couples and 9.2% of fertile couples having more than three consecutive first-trimester abortions (recurrent spontaneous abortion).[12] Our 2 patients with chromosomal translocations both presented with repeated habitual abortions. Because the patients were attempting to have unaffected infants, they may encounter a lot of physiological and psychological traumas from recurrent spontaneous abortions or termination of pregnancies. Such patients may benefit from in vitro fertilization followed by PGD.[2,13,14] After using PGD, a significant reduction in spontaneous abortions was reported from 95% to 13%.[15] In our study, the pregnancy rate of Robertsonian translocation was 33.3% (one out of three cycles), in contrast with that reported from ESHRE (29% of per transfer cycle).[3] Only one cycle of reciprocal translocation has not

Table 2. List of Pregnancy Outcomes in Detail

	Patient	Outcome				
		No of fetuses	Gestational age Delivery method	Birth weight (gm)	Prenatal amniocentesis	Postnatal examination
Group 1	A	Singleton	GA 39 weeks, C/S	3440	Confirmed	Normal
	B	Twin (one blighted ovum)	GA 39 weeks, SD	3320	Confirmed	Normal
	C	Twin	GA 36 weeks, C/S	2860, 2870	Confirmed	Normal
	D	Singleton	GA 35 weeks, SD	2180	Confirmed	Normal
	E	Singleton (blighted ovum)	D & C†	-	-	-
	F	Singleton (blighted ovum)	D & C†	-	-	-
Group 2	G*	Twin	GA 35 weeks, C/S	2100, 2140	Confirmed‡	Normal

Abbreviations: GA: gestational age; SD: spontaneous delivery; C/S: cesarean section; D&C: dilatation & curettage

* A carrier of Robertsonian translocation, 45, XX, der(13; 14)(q10; q10)

† Tissue from D&C confirmed the diagnosis

‡ Karyotype revealed 45, XX, der(13; 14)(q10; q10) and 46, XX, 9ph

Table 3. Clinical Outcomes in Detail

	Clinical outcome			
	Implantation rate	Pregnancy rate (per oocyte retrieval)	Pregnancy rate (per embryo transfer)	Take baby home rate
Group 1	8/15 = 53.3%	6/19 = 31.6%	6/11 = 54.5%	5/11 = 45.5%
Group 2	2/ 7 = 28.6%	1/ 4 = 25%	1/ 3 = 33.3%	1/ 3 = 33.3%
Total	10/22 = 45.5%	7/23 = 30.4%	7/14 = 50%	6/14 = 42.9%

Table 4. Summary of Chromosomal Status of Non-transferred Embryos in Each Group

	Group 1	Group 2	Total
Diploid			
Aneuploid	8	2	10
Monosomy	3	2	5
Monosomy and mosaic	2		2
Trisomy18 and mosaic	3		3
Mosaics	17	5	22
2N/N	3	1	4
2N/N/4N	6	2	8
2N/aneuploid/variable ploidy	8	2	10
Haploid mosaics	2	2	4
Mosaic N/2N		1	1
Mosaic haploid/aneuploid	2	1	3
Polyploid:	9		9
All cells the same ploidy	2		2
5N	1		1
6N	1		1
Mosaic polyploids	7		7
Two ploidies (xN/yN)	3		3
Complex polyploid mosaic	4		4
Total mosaics (any ploidy)	29	7	36
Total abnormal	36	9	45
Total abnormal other than mosaics	7	2	9

achieved pregnancy yet.

The techniques developed for PGD have also been applied to aneuploidy screening (PGD-AS) in couples seeking reproductive assistance.[16] When a woman is 36 years or older, the risk of aneuploidy is of about 1/250. As the woman's age increases, two major impacts occur. First, there is a higher risk of aneuploidy which inevitably induces more pregnancy wastage. Trisomy is the most common class of chromosome abnormality occurring in 25% of spontaneous abortion.[17] In addition, the most frequently surviving aneuploidy types are trisomy 21, 18, 13 and monosomy X and thus leads to affected children. Second, decreasing ovarian reserve accompanies aging.[17] In our study, the cancellation rate related to poor response in those women for PGD-AS was as high as 24% (6 cycles out of 25 cycles). Only 57.9% of oocyte retrieval cycle (11 out of 19 cycles) achieved embryo transfer cycles. This result may be explained by maternal aging associated to the lower number of oocytes retrieved per cycle, poor embryo quality (not suitable for biopsied), and lower number of oocytes transferred per cycle. However, the clinical outcomes showed a 53.3% (8 out of 15) implantation rate, 31.6% pregnancy rate per oocyte retrieval, and 54.5% pregnancy rate per transfer cycle. It was claimed that the selection of chromosomally normal embryos to transfer into the uterus increased the chance of implantation in women of advanced reproductive age.[18,19] Our preliminary results are similar to their results. However, our number of cases was too small to produce a significant difference statistically in this stage. More patients must be included in the future studies. In addition, more kinds of probes should be included to screen out aneuploid embryos.

The number of referrals for aneuploidy screening has increased to 14.2%.[3] PGD-AS has been performed for women of high reproductive age (> 35 years), recurrent IVF failure (> 3 attempts with no pregnancy), and recurrent miscarriages with normal karyotypes in the couples.[2,3] Commercial multi-color FISH probes are available for aneuploidy now but they are expensive. It was reported that 34% of the embryos destined to be aborted due to abnormal karyotypes might be detected with FISH using combinations of 13, 18, 21, X and Y probes for PGD prior to embryo transfer.[18] Moreover, up to 65% of abnormal karyotypes could be detected if additional chromosome 16 and 22 probes were added.[20] In this report, we just used combinations of 18, X, and Y probes for aneuploidy screening. This may be associated with the higher rate of pregnancy loss during the first trimester (3 out of 8, as 37.5%). In the future, combinations of more probes than 18, X, and Y will surely improve the results. There are several reasons to use only 3 probes in this series. First, the study was based on the economic considerations. The commercial multi-color FISH probes for aneuploidy are quite expensive. Second, the probes for chromosomes 18, X, and Y used in this study were homemade and were directly labeled. To mix them with commercial probes of chromosomes 13 and 21 (locus specific) needs further refinement of the procedure. Third, in this preliminary series of PGD, our reliable probes to chromosomes 18, X, and Y were used. Commercial probes for chromosomes X, Y, 18, 13 and 21 will be used in the next step. The inclusion of the probe for chromosome 21 will fulfill the important screening of trisomy 21.

There have been several reports of misdiagnosis in PGD sporadically.[4,21] The rate of misdiagnosis in

PGD is 1.8% (0.9% in the FISH group, 3.4% in the PCR group).[3] So it was well accepted to perform prenatal diagnosis in a resulting pregnancy. All patients in our small series received amniocentesis and the results confirmed the diagnosis of that using PGD. The tissue from the D & C in the 2 patients with blighted ovum also confirmed the diagnosis. The number of cases in this preliminary study was small and it is hardly to say the accuracy rate. However, our experience as well as the result of other large series in the world provides a positive view on the use of PGD with the FISH technique.

In conclusion, PGD is an alternative method to achieve healthy children and prevent repeated spontaneous abortion in couples carrying high risk of producing offspring with genetic disorders. In addition, PGD-AS may increase the implantation rate and avoid births of trisomy in couples with advanced maternal age.

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著床前胚胎遺傳診斷：兩年的經驗報告

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背 景： 著床前遺傳物質診斷是對於著床前配子或胚胎作診斷或篩檢，它的好處是可以避免不正常的胚胎造成的重複性流產或必須終止妊娠，我們報告2年來的初步經驗。

方 法： 從2001年3月到2002年10月，尋求試管嬰兒治療的夫婦因爲染色體問題，接受著床前胚胎遺傳物質診斷或是接受染色體非整倍數的篩檢。第3天的胚胎取出一或二個細胞以螢光原位雜交來作分析診斷。染色體非整倍數的篩檢是使用染色體X, Y及18的標記。染色體轉位的病人則需個別的標記來診斷。診斷正常的胚胎於第5天植入。

結 果： 一共有25個治療週期是作染色體非整倍數的篩檢（第一組），4個治療週期是爲了染色體轉位（第二組）。第一組中，73個胚胎接受切片，固定成功率爲72.6%，診斷率爲96.2%，11個週期植入15個胚胎得到兩次雙胞胎及四次單胞胎（著床率53.3%）。第二組中，27個胚胎接受切片，固定成功率爲66.7%，診斷率爲88.9%，7個不受影響的胚胎分3個週期植入得到一對雙胞胎（著床率33.3%）。所有懷孕均有羊水檢查來確認診斷，產下的嬰兒身體檢查並無重大畸形。

結 論： 對於有染色體問題的夫婦而言，獲得健康胎兒的另一種選擇是著床前遺傳物質診斷。此外，染色體非整倍數的篩檢，可以提高著床率，在某些情況下值得使用。

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關鍵字：著床前遺傳物質診斷，螢光原位雜交。

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