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PRECONCEPTION AND PREIMPLANTATION DIAGNOSIS FOR CYSTIC FIBROSIS

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SUMMARY

Preimplantation diagnosis provides couples at high genetic risk the possibility of avoiding genetic disease without the need for prenatal diagnosis and selective abortion of the affected pregnancy. Following extensive background work on the reliability of genetic diagnosis in a single cell, we offered on a research basis preimplantation diagnosis to five couples at risk for offspring with the delta-F508 mutation (the major mutation causing cystic fibrosis). There was no detrimental effect from polar body removal on either fertilization or preimplantation development. Genetic analysis, undertaken in 22 polar bodies and 15 corresponding blastomeres, identified 21 embryos of which ten were transferred.

KEY WORDS Preimplantation diagnosis Polar body removal Cystic fibrosis Embryo biopsy

INTRODUCTION

Preconception and preimplantation genetic analysis has been initiated for couples at high risk of conceiving children with genetic diseases as an alternative to prenatal diagnosis and termination of affected fetuses. The first attempts at genetic analysis of preimplantation mammalian embryos (Gardner and Edwards, 1968; Leonard *et al.*, 1987; Monk *et al.*, 1988; Monk and Handyside, 1988; Summers *et al.*, 1988; Handyside *et al.*, 1989, 1990) demonstrated that material could be removed from mammalian pre-embryos (including human pre-embryos) and successfully analysed without destroying the viability of the pre-embryo.

Babies without detectable birth defects have been born following blastomere biopsy for gender determination (Handyside *et al.*, 1990), demonstrating that embryo biopsy can be performed safely in humans.

Preconception and preimplantation genetic analysis for human gametes and pre-embryos is now feasible due to the refinement of techniques for embryo handling, *in vitro* fertilization (IVF), and the advent of the polymerase chain reaction (PCR).

The only approach for preconception diagnosis at present is genetic analysis of the first polar body (Verlinsky *et al.*, 1990), although both spermatozoa and the second polar body could be used in principle. Preconception diagnosis by polar body removal (PBR) has clear advantages compared with preimplantation genetic analysis by blastomere biopsy. First of all, the first polar body has no known function in embryonic development. Second, PBR does not involve any damage to the developing embryo as no embryonic material is removed. Finally, the polar body is removed within 24 h following oocyte retrieval and genetic analysis is completed

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by the second day, with no risk of missing the implantation window as embryo development *in vitro* occurs at a slower rate than *in vivo* (Bavister, 1988).

However, PBR will not make it possible to establish genetic diagnosis of the embryo in a number of situations: (1) paternal alleles cannot be analyzed; (2) the test might not be efficient for telomeric loci as crossing-over will occur with high frequency leading to the heterozygous state of the polar body; and (3) gender determination is not possible. In these situations, blastomere biopsy becomes an important adjunct to PBR.

The fact that by PBR the genotype of the oocyte is inferred rather than directly determined has also been suggested as an additional weakness. However, our experience so far demonstrates an accurate prediction of the genotype of the corresponding oocyte (Verlinsky *et al.*, 1990; Strom *et al.*, 1990).

This paper summarizes our experience with PBR and blastomere biopsy for preconception and preimplantation genetic diagnosis of the delta-F508 mutation.

MATERIALS AND METHODS

The following couples volunteered to participate in our research programme: four couples who have had a child affected with cystic fibrosis (CF), and one couple participating in the IVF programme found to be heterozygous for the delta-F508 mutation by carrier detection. All research protocols have been approved by the Institutional Review Board and Ethics Committee of Illinois Masonic Medical Center.

Follicular stimulation was performed using a standard clomiphene citrate/human menopausal gonadotropin/protocol. Oocytes were retrieved through vaginal ultrasound-guided aspiration. Culture of oocytes took place in Ham's F-10 medium (Gibco) with 10 per cent heat-inactivated maternal serum in plastic four-well multidishes (Nunc) under light paraffin oil in an atmosphere of 5 per cent CO_2, 5 per cent O_2, and 90 per cent N_2. After incubation for 3 h, oocytes were treated with hyaluronidase (200 IU/ml) in IVF medium to remove the cumulus cells. For micromanipulation, oocytes that had extruded their first polar bodies were selected and transferred one at a time into individual drops of IVF medium supplemented with 0·1 M sucrose, under sterile paraffin oil.

PBR was performed using Narishige micromanipulators under an inverted Nikon Diaphot microscope fitted with Hoffman Modulation Contrast optics. All equipment was located in a vertical laminar flow hood (Holten LaminAir A/S). Holding and bevelled pipettes were made as described elsewhere (Hogan *et al.*, 1986). Once in the needle, the polar body was expelled into a tube with HPLC grade water, and the tip of the needle was broken off in the water to ensure that all material was transferred (Verlinsky *et al.*, 1990; Strom *et al.*, 1990).

After PBR, the sucrose was washed from the oocytes using IVF medium. After an additional 2 h of culture, oocytes were inseminated with $1{\cdot}5 \times 10^5$ motile sperm prepared using the percoll gradient technique. Approximately 15 h after insemination, oocytes were examined for the presence of pronuclei and after an additional 24 h in culture, for cleavage. At this time, diploid embryos whose first polar body was found to be homozygous for the abnormal gene were transferred back to the

Table 1. Fertilization and development of human oocytes without and following polar body removal

Cycles	Oocytes	Fractured/ atretic	Fertilization Monospermic	Dispermic	Total	Cleavage
Following PBR						
10	121	29	54/92 58·7%	7/92 7·6%	61/92 66·3%	54/54* 100%
Without PBR						
40	266	30	151/236 64·2%	12/236 5·1%	163/236 69·3%	147/151* 97·4%

*Triploid embryos were not included.

patient with the hope of achieving a pregnancy. The remaining embryos were biopsied and those that developed to blastocyst stage were frozen.

The embryo biopsy was performed at the 4- to 8-cell stage. The microtools used for embryo biopsy were the same as those for PBR, except for the use of a larger-diameter bevelled pipette (approximately 18–25 mcm). As during the aspiration of a blastomere its membrane usually breaks, blastomeres with a clearly visible nucleus were chosen, paying special attention to aspirate nuclei into the pipette. Once aspirated, the blastomere nucleus was expelled into the microcentrifuge tube as during PBR.

For PCR and DNA analysis, each polar body or blastomere was lysed by freezing at −70°C for 20 min followed by treatment at 98°C for 20 min. PCR was performed as described previously (Strom *et al.*, 1990). All buffers, oligonucleotide primers, and reagents, including Taq polymerase, were first digested with *Mse*I, in order to eliminate contaminating DNA sequences, and this was followed by incubation at 95°C for 10 min to inactivate the restriction enzyme.

The strategy of the study was as follows. When PBR revealed that the polar body was homozygous for the defective allele, the corresponding pre-embryos were transferred. The remaining pre-embryos (those whose polar bodies were normal, those that were heterozygous, and those whose analysis failed) were biopsied at the 4- to 8-cell stage, and unaffected embryos were transferred in the same cycle or frozen for subsequent transfer.

RESULTS AND DISCUSSION

When considering the safety of preimplantation diagnosis it is important to examine any possible detrimental effect of the micromanipulations and biopsy on fertilization and embryo development. Since pregnancy rates in IVF are already low, if micromanipulation further reduced the likelihood of pregnancies, preimplantation diagnosis could be impractical.

Table 1 shows our data on the effect of PBR on *in vitro* fertilization and development from ten patient cycles in comparison with intact control oocytes retrieved from an additional 40 IVF patients who were randomly chosen. PBR was performed

Table 2. Preimplantation genetic analysis of delta-F508 mutation

	Normal	Heterozygous	Affected	Failed PCR	Total
Oocyte (after PBR)	5	5	8	4	22
Blastomere (after PBR)	5	7	1	2	15*
Oocyte	1	0	0	0	1*

*The same oocytes following PBR.

on a total of 92 non-atretic oocytes: in addition to oocytes retrieved from women who were carriers for CF, those obtained from carriers for alpha-1-antitrypsin deficiency or haemophilia A were also included for the viability study. Eighteen of these oocytes were found to be immature on the day of aspiration, so that PBR and insemination were performed 24 h later. When we attempted to remove the polar body from some of the oocytes, it was found that the polar body was still attached to the ooplasm. In these cases, after an additional 3 h of incubation the polar body was detached and successfully removed.

As can be seen from Table 1, there was no decrease in the fertilization rate for oocytes following PBR as compared with control oocytes (66·3 and 69·3 per cent, respectively), the percentage of embryos entering cleavage being similar in oocytes subjected to PBR compared with control oocytes (100 vs. 97·4 per cent). There was no increase in the percentage of polyspermic embryos in the PBR oocytes with respect to control oocytes (7·6 vs. 5·1 per cent).

Therefore, following PBR, oocytes demonstrated no significant differences in fertilization rate, polyspermia, or development to the cleavage stage, indicating that PBR had no deleterious effect on fertilization or pre-embryonic development.

This is in agreement with the assumption that the first polar body does not play a role in embryo development. In fact, it begins to degenerate shortly following its extrusion (Rodman, 1971) and it was observed that normal babies have been born following the destruction of the first polar body during partial zona dissection (PZD) as a treatment for male factor infertility (J. Cohen, personal communication). Although we do not go along with the suggestion that the first polar body can be fertilized and develop along with its corresponding oocyte as a triploid 'twin' (Bieber *et al.*, 1981), removal of the polar body eliminates such a possibility.

It has been found that PZD before insemination increases the risk of polyspermia (Gordon *et al.*, 1988; Malter and Cohen, 1989). Although the zona pellucida had been punctured during PBR, there was no significant increase in the rate of polyspermy. In fact, the slit in the zona remaining after the withdrawal of the microneedle can hardly be considered as a 'dissection' made according to the PZD protocol. However, we cannot dismiss the possibility that the puncture in the zona pellucida during PBR may cause an increase in polyspermy, though our data failed to demonstrate such a possibility.

Table 2 shows the results of genetic analysis of a total of 22 polar bodies, 15 blastomeres, and 1 oocyte for delta-F508 mutation of CF. Successful diagnosis

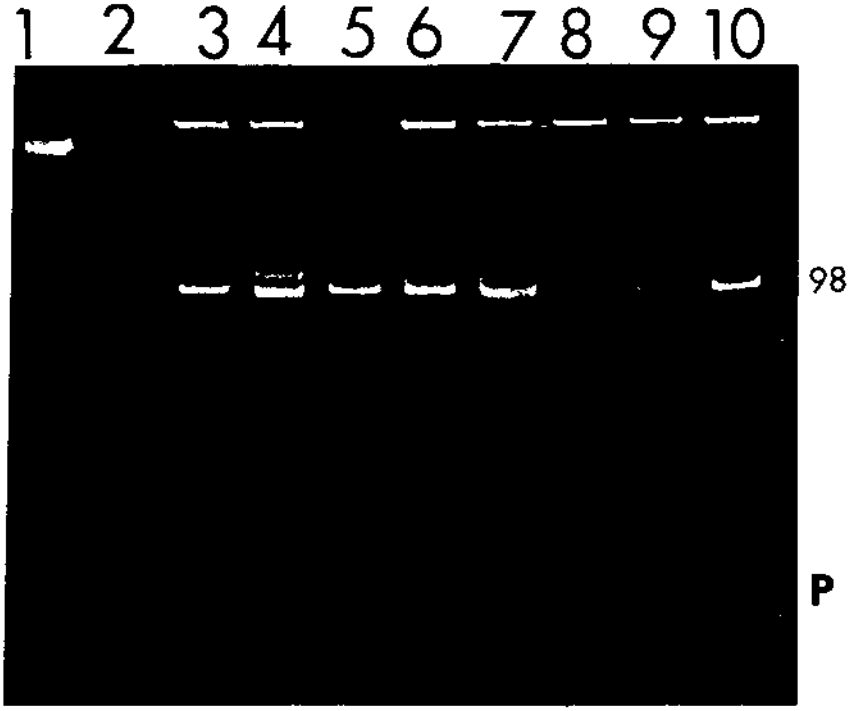


Figure 1. PCR analysis of DNA from the first polar body following PBR. Lane 1: Size standards (PBR322 DNA digested with *Hae*III). Lane 2: Negative control (buffer only). Lane 3: Homozygous normal DNA. Lane 4: Heterozygous DNA (normal/F508). Lane 5: Polar body DNA (homozygous normal). Lane 6: Polar body DNA mixed with normal DNA. Lane 7: Polar body DNA mixed with delta-F508 DNA. Lane 8: Polar body DNA (homozygous delta-F508). Lane 9: Polar body DNA mixed with normal DNA. Lane 10: Polar body DNA mixed with delta-F508 DNA

was achieved in 18 polar bodies: eight polar bodies (44 per cent) appeared to be homozygous normal, five (28 per cent) heterozygous, indicating that an odd number of crossover events had occurred, and five (28 per cent) homozygous for delta-F508 (Figure 1).

The follow-up of each diagnosis by PBR and the subsequent genetic analysis of corresponding pre-embryos are shown in Table 3. Of five oocytes whose polar bodies were homozygous delta-F508, four fertilized and were found to be acceptable for transfer, while one did not fertilize and was determined to have a normal allele, thus confirming the polar body analysis.

Of eight oocytes whose polar bodies were homozygous normal, seven fertilized of which two appeared to be triploid (one did not amplify and the other was not studied), whereas five resulted in pre-embryos which were analysed by blastomere biopsy with the following results: one was determined to be homozygous (Strom *et al.*, 1990) and four heterozygous delta-F508.

Of five oocytes whose polar bodies were heterozygous, four resulted in pre-embryos whose genotype was determined by blastomere biopsy to be homozygous normal, and one heterozygous.

Of four oocytes whose polar bodies did not amplify, one did not fertilize, one appeared to have a single pronucleus after insemination, and two were diploid.

Table 3.

Oocyte No.	First PB genotype	Pronuclei	Blast genotype	Oocyte genotype	Transfer*
1	N/N	2	1st No amp 2nd CF/CF	—	No
2	No amp	2	CF/N	—	No*
3	CF/N	2	N/N	—	Yes
4	No amp	2	N/N	—	No*
5	CF/CF	2	—	—	Yes
6	N/N	0	—	—	No
7	CF/CF	0	—	N/N	No
8	CF/CF	2	—	—	Yes
9	CF/CF	2	—	—	Yes
10	No amp	1	CF/N	—	No*
11	N/N	2	CF/N	—	Yes
12	CF/N	2	N/N	—	No†
13	N/N	2	CF/N	—	Yes
14	N/N	2	CF/N	—	No*
15	N/N	3	No amp	—	No
16	N/N	2	CF/N	—	Yes
17	N/N	3	—	—	No
18	CF/CF	2	—	—	Yes
19	CF/N	2	CF/N	—	No*
20	No amp	0	—	—	No
21	CF/N	2	N/N	—	Yes
22	CF/N	2	N/N	—	Yes

*Unaffected embryos that were not transferred were due to poor *in vitro* development or other technical reasons.

†Frozen for subsequent transfer.

No. amp = No PCR amplification.

The latter three were determined by blastomere biopsy to be heterozygous (2) and homozygous normal (1).

Therefore, as a result of genetic analysis undertaken in 22 oocytes, 21 embryos were identified either by PBR or by blastomere biopsy, of which only ten unaffected embryos were transferred. The other unaffected embryos either showed poor *in vitro* development, or were frozen for subsequent transfer, or were not transferred for technical reasons (Table 3).

As our data show, there seems to be no need for further information about embryos whose first polar bodies are found to be homozygous for the abnormal recessive gene: these embryos represent between 25 and 50 per cent of all embryos (depending on the distance between the gene being analysed and the centromere). For a recessive or a dominant gene carried by the mother, fertilization of such oocytes will result in an unaffected fetus. If the frequency of crossing-over is high, analysis of the second polar body from the heterozygous oocytes could determine the status of another fraction of embryos. This means that the analysis of the polar bodies could theoretically identify all oocytes that contain the unaffected gene. However, in recessive diseases, 50 per cent of oocytes containing the abnormal gene

will be fertilized with sperm carrying the normal gene and will be unaffected. Therefore, polar body analysis will theoretically be able to identify only two-thirds of all normal embryos in recessive diseases, the remaining unaffected embryos to be identified by embryo biopsy and genotype analysis during preimplantation development.

Therefore, for application in clinical practice both preconception and preimplantation genetic analysis should be available to enable genotyping and confirmation of preimplantation diagnosis for couples at risk for autosomal recessive, dominant, and X-linked disorders.

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