Application of three-dimensional fluorescence in situ hybridization to human preimplantation genetic diagnosis

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Objective: To report a simple and efficient fluorescence in situ hybridization (FISH) method for preimplantation genetic diagnosis (PGD).
Design: Technique and method.
Setting: A hospital in vitro fertilization (IVF) laboratory.
Patient(s): Women undergoing IVF or intracytoplasmic sperm injection (ICSI).
Intervention(s): None.
Main Outcome Measure(s): The intensity and clarity of signals, technical difficulty, the percentage of successfully treated blastomeres, and blastomere integrity after FISH.
Result(s): This paraformaldehyde fixation technique simplified the process of fixation of blastomeres for PGD without losing blastomeres during fixation. A total of 35 blastomeres derived from 10 arrested embryos or abnormally fertilized eggs (one pronucleus or three pronuclei) were used for three-dimensional (3D) FISH staining. Signals in all blastomeres were obtained successfully by this method. Approximately 0.1 μL of DNA probe was enough for the detection of the signals in each blastomere, less than the volume (1 μL) used in the conventional FISH.
Conclusion(s): The 3D-FISH technique for PGD is easy to learn, less damaging to blastomeres, and loses no blastomeres during fixation. It is efficient, feasible, and economic, which allows more patients to benefit from this technique. (Fertil Steril® 2009;92:1492–5. ©2009 by American Society for Reproductive Medicine.)

Key Words: Paraformaldehyde fixation, permeabilized method, 3D-FISH, PGD

Preimplantation genetic diagnosis (PGD) is becoming a widely accepted approach for the analysis of chromosomal integrity of embryos before transfer into uteri. Patients have to go through in vitro fertilization (IVF) procedures so that embryos are generated in the laboratory, and usually one to two cells are removed from the eight-cell embryo for PGD. Fluorescence in situ hybridization (FISH) is usually adopted to analyze the chromosomes in cells.

Since the time FISH was first applied to PGD (1), many improvements to the technique have been made. The blastomere fixation before FISH is a critical step in the analysis process. The most commonly used fixative for FISH in PGD is methanol/acetic acid. However, this method is difficult to master: if the technique is not done correctly, the biopsied blastomere may rupture and damage the embryo. Transfer and fixation of the blastomere is a critical step because only one or two blastomeres are analyzed and only a small number of embryos, which are all highly valuable for the patient, are available for biopsy (2). Furthermore, this fixation method requires removing all of the cytoplasmic materials to make the nuclear DNA accessible to the hybridization probes. A simpler and more reliable FISH technique is required for the further development of PGD.

Fluorescence in situ hybridization using DNA probes to preserve cells three-dimensionally, called 3D-FISH, allows 3D visualization of specific DNA or RNA targets within the interphase nucleus (3). The 3D-FISH technique uses paraformaldehyde as the fixative agent without cytoplasm removal. It has been used to study the spatial organization of the genome from individual chromosome territories and subchromosomal domains down to single gene loci and nascent RNA transcripts. Such manipulations allow the simultaneous visualization of numerous differently labeled nuclear or cytoplasmic targets. It can be applied to many kinds of cells cultured adherently or in suspension.

More cells are available from somatic sources, and the amount of cytoplasm in somatic cells is much less than that of blastomeres, which indicate that a different procedure for 3D-FISH of preimplantation embryos is needed. We evaluated a simplified fixation method in combination with

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permeabilization for FISH of blastomeres to determine its effectiveness in terms of the intensity and clarity of signals, technical difficulty, the percentage of successfully treated blastomeres, and blastomere integrity after FISH.

MATERIALS AND METHODS

Sample Collection

Abnormally fertilized human embryos were obtained from patients undergoing assisted fertilization at the Center of Reproductive Medicine of the Peking University Third Hospital. The samples were collected from patients undergoing intracytoplasmic sperm injection (ICSI), and developmentally arrested embryos from one-cell to eight-cell stages were collected on day 3. A total of 35 blastomeres from 10 embryos were used in the study.

Written informed consent was obtained from each infertile couple before using their donated embryos, and institutional review board approval was obtained.

3D-FISH Procedure

The probe used for hybridization was purchased from Vysis (Downers Grove, IL). The protocol for FISH on blastomeres was modified as previously described elsewhere (3). Briefly, after removing the zona pellucida in acidic G1 medium (pH 2.5; Vitrolife, Gothenburg, Sweden), embryos or blastomeres were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 30 minutes at room temperature to preserve the native 3D structure of the nuclei and then washed in PBS. During the procedure, air drying was carefully avoided. Embryos were permeabilized with 0.5% Triton X-100 in PBS for 30 minutes. After a bath in 20% glycerol in PBS for 20 minutes, the blastomeres were moved to the glass slides with some glycerol and freeze-thawed by briefly dipping the slides three times in liquid nitrogen. Blastomeres were put in 0.1 N HCl drop on a dish for 5 minutes and then preincubated in a hybridization buffer (50% formamide, 10% dextransulfate, 1×SSC).

Labeled probes were dissolved in a hybridization mixture. DNA-probes specific for chromosome X (CEP X, DXZ1, alpha satellite in spectrum-green) and embryonic DNA were denatured simultaneously on a hot block at 75°C for 5 minutes.

Hybridization was carried out in a humid atmosphere at 37°C overnight. The embryos or blastomeres were washed three times in 50% formamide/2×SSC solution, 10 minutes each time, then once in 2×SSC for 10 minutes and once in 2×SSC/0.1% NP-40 at 45°C for 5 minutes. Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI).

Standard procedures were employed for probe detection. Finally, the blastomeres were mounted on glass slides and examined with a confocal laser-scanning microscope (Zeiss LSM 510 META; Carl Zeiss Advanced Imaging Microscopy, Jena, Germany).

RESULTS

In the initial trial, most of the embryos or blastomeres stuck to the bottom of the dish and lysed when they were moved from 0.1 N HCl. To avoid specimen loss, 20% glycerol in PBS was added to the HCl drop, and whole sticky blastomeres were removed easily by mouth aspiration pipette. Tests of several other specimens achieved similar good results.

When applying the described technique to another 35 blastomeres, all of them were intact with good morphologic characteristics after manipulation, and 100% showed adequate FISH signals. We also attempted to perform PGD using a lesser volume of probes to maintain low costs and high specificity. Notably, only 0.1-μL of the DNA probe was sufficient for detection the signal in each blastomere, which is much less (10%) than the 1 μL used in conventional FISH. Furthermore, this technique does not require special training to achieve consistent results; rather, it merely requires moving the blastomere from one drop to another.

As shown in Figure 1, even though blastomere cytoplasmic materials were not removed, the DNA probe could still bind to the specimen's target sequence and produced clear signals after serial permeabilization steps.

DISCUSSION

Traditional cytogenetic staining techniques usually deal with hundreds or even thousands of cells on a single slide. Although most cells are usually not fixed well, it is not important for traditional analysis because sufficient information for diagnosis can be gained from only a few perfectly treated cells. During PGD, transfer and fixation of the blastomere is a key step and requires proficient operating skill because only one or two blastomeres in an embryo and only a small number of embryos are available for diagnosis.

The new technique has several major advantages over existing methods of FISH in PGD. First, 3D-FISH can provide information about the arrangement of chromosome territories, the organization of subchromosomal domains, and positions of individual genes and RNA transcripts. Nuclei in human cells have a radial organization. Chromosomes with the highest gene density are preferentially disposed toward the nuclear interior, and gene-poor chromosomes are located toward the nuclear periphery (4–6). Permeabilization treatments (incubation in Triton X-100, incubation in glycerol, freezing/ thawing, and brief incubation in HCl) do not greatly modify the fine structure of nuclei (7).

The second advantage is that none of the blastomeres were lost by this technique compared with the conventional FISH method. With the new technique, blastomeres are always added to different solution drops under a microscope. Therefore, the blastomere can always be visualized at every step. This essentially eliminates the risk of losing a specimen and avoids probes waste. This technique may also be helpful in the fixation of polar bodies, which are particularly prone to being lost because of their smaller size.