Supernumerary Human Preembryos Provide Potential for Preimplantation Genetic Diagnosis

(FISH / IVF / preimplantation diagnosis)

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Abstract. Preimplantation genetic diagnosis is an alternative to the classical prenatal diagnosis for couples undergoing *in vitro* fertilization. It allows very early embryo selection – before the intrauterine embryo transfer. Prior to clinical application of preimplantation diagnosis in the Infertility Treatment Centre "Technobioassistance", Sofia Medical Faculty, we have developed preimplantation diagnosis models of human spermatozoa and untransferred 2–8-cell human embryos obtained *in vitro*. Directly fluorescein isothiocyanate-labelled probes specific for the centromeric regions of chromosomes 1, 5, 19 and X (Boehringer Mannheim) were used. Eighty-six point three percent of fixed blastomeres with normal size and shape had unfragmented nuclei with dispersed interphase chromatin or mitotic chromosomes and all of them demonstrated successful hybridization. In cases with more than 75% of embryo cells suitable for analysis we were able to estimate the presence of mosaicism among the blastomeres.

Preimplantation genetic diagnosis (PGD) is an alternative to the classical prenatal diagnosis for couples undergoing *in vitro* fertilization and embryo transfer (IVF-ET). It is aimed at detection of sex, chromosomal status and suspected gene mutations in cleaving preimplantation embryos at the 6–8-cell stage (Handyside et al., 1989; Handyside et al., 1992; Munne et al., 1993). PGD allows very early embryo selection – before the intrauterine transfer and implantation into the endometrium, circumventing the eventual necessity of abortion of affected foetuses.

The PGD procedure includes: microsurgical biopsy of single blastomeres through zona pellucida and their preparation for analysis; PCR amplification and related techniques to analyse unique DNA sequences or fluorescent *in situ* hybridization (FISH) for detection of ploidity of one or more chromosomal pairs; interpretation of the results. It is necessary to perform the genetic diagnosis, final selection of presumably healthy embryos and embryo transfer within 6–7 hours (Munne et al., 1993), avoiding the need of further culture or freezing. Briefly, there are two limiting factors for PGD – the time and the material for analysis – only one or two blastomeres per embryo.

The success of single-cell diagnosis depends not only on available technical equipment but also on manual skills of the researchers. The staff of our IVF center has good experience with micromanipulations of mammalian gametes and preembryos (Vatev el al., 1993; Vatev et al., 1997; Vatev et al., 1998). We are able to perform PCR amplification and related analysis of DNA of a single human cell (Delimitreva et al., 1998). At this stage of our work we are paying attention to preparing and performing FISH of single interphase blastomere nuclei and achieving adequate FISH success rate.

Material and Methods

The IVF, ICSI (intracytoplasmic sperm injection) and embryo transfer procedures were carried out according to the method used in the Human IVF-ET Unit "Technobioassistance" at the Department of Biology, Medical University, Sofia, Bulgaria (Vatev, 1988; Vatev et al., 1990; Vatev et al., 1993; Vatev et al., 1998). Untransferred preimplantation 2–8-cell-stage embryos and spermatozoa prepared for IVF were used to develop preimplantation diagnosis models. The investigation was approved by the Ethical Committee of the Preclinical University Centre – Medical University of Sofia, and the patients gave informed consent for the supernumerary embryos to be used for research purposes.

FISH was performed according to Munne et al. (Munne et al., 1993; Munne et al., 1994), including the following more important steps.

Zona pellucida was removed with acid Tyrode solution. After 15 min incubation at room temperature in hypotonic solution – 1% (w/v) Na citrate, individual blastomeres were fixed on clean slides. With sperm cells, nuclei were decondensed by 30 min exposure on 10 mM dithiothreitol followed by fixation. *In situ*

Received January 29, 2001. Accepted February 20, 2001.

The study has been supported by the Bulgarian Ministry of Education and Science, Grant No. L-431/94.

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Abbreviations: FISH – fluorescent *in situ* hybridization, FITC – fluorescein isothiocyanate, ICSI – intracytoplasmic sperm injection, IVF-ET – *in vitro* fertilization and embryo transfer, PGD – preimplantation genetic diagnosis.

hybridization was performed using directly FITC (fluorescein isothiocyanate)-labelled oligonucleotides specific for the centromeric regions of chromosomes 1, 5, 19 and X (Boehringer Mannheim, Austria). Interphase chromatin and probes were simultaneously denatured at 80°C for 10 min and then hybridized for 5 h in a humid chamber at 37°C. After immersion in formamide (42°C, 15 min) DNA was counterstained with DAPI (4,6-diamidino-2-phenylindole) or propidium iodide. The chromosome centromeres were observed by fluorescence microscopy (Zeiss Axioskop 20, Oberkochen, Germany) in the presence of anti-fade reagent DABCO (diazabicyclo[2.2.2]octane – Sigma, St. Louis, MO).

Results

The marker chromosome regions were clearly visible as coloured spots. Nearly half of the sperm nuclei showed one spot when the X probe was applied, while the rest was negative (Fig. 1). Chromosomes 1, 5 and 19 were observed simultaneously in more than 90% of sperm heads (Fig. 2).

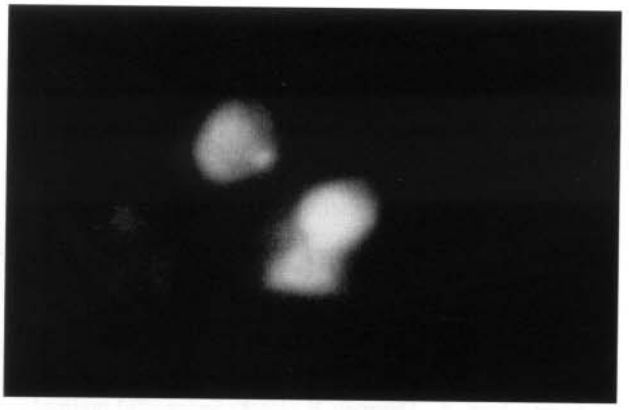

Fig. 1. Three human sperm nuclei after X-specific FISH – one of them demonstrates the X signal.

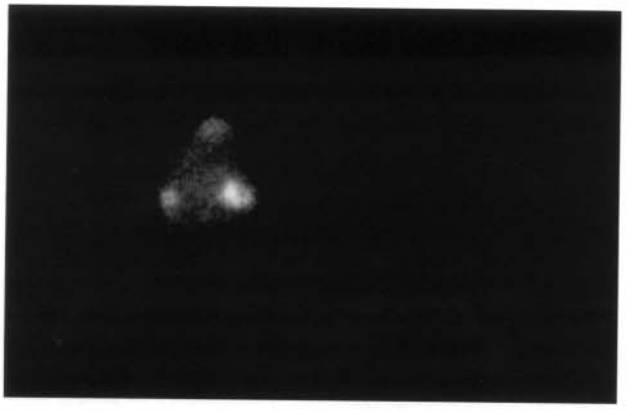

Fig. 2. Fluorescence micrograph illustrating hybridization FITC signals specific for centromeric regions of chromosomes 1, 5 and 19 in a human sperm head.

Fifty-seven of fixed 66 blastomeres with normal size and shape had unfragmented nuclei with dispersed interphase chromatin or mitotic chromosomes and all of them showed successful FISH reaction (Figs. 3, 4). These data are comparable to the figures reported in the literature (Munne and Weier, 1996; Staessen and Van Steirteghem, 1997; Vandervorst et al., 1998; Pellicer et al., 1999). More than 75% of blastomeres were suitable for analysis in 16 four to eight-cell embryos. We observed mosaicism among the blastomeres in four of them after applying the X-specific probe – simultaneous presence of nuclei with 1, 2 and 3 copies of the X chromosome in one embryo, and 0, 1 and 2 copies in three embryos.

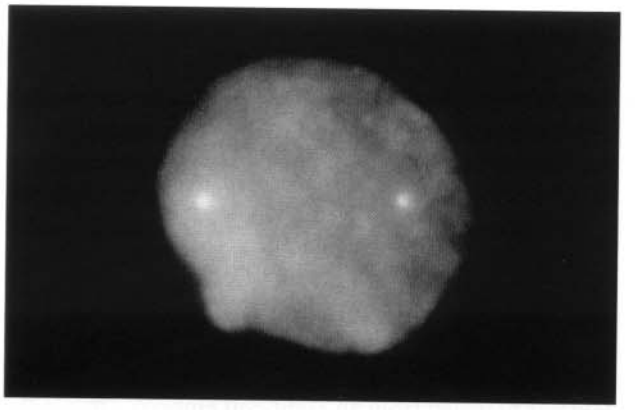

Fig. 3. Human blastomere nucleus with 2 X-specific signals.

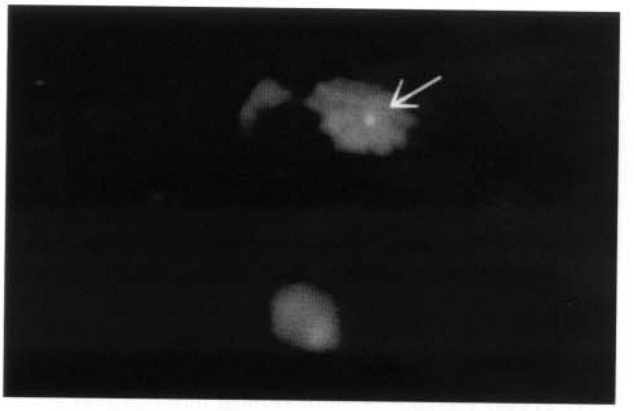

Fig. 4. Anaphase in a blastomere nucleus, demonstrating the X-specific signal (arrow).

Discussion

Researches who apply PGD clinically perform FISH analysis using blastomeres biopsed from embryos most suitable for intrauterine transfer (Benadiva et al., 1996; Vandervorst et al., 1998) – with adequate cleavage, number of blastomeres and minimum fragments (if possible). These authors report the total success rate of biopsy, fixation and FISH close to 85%. In our study we used donated untransferred embryos. Most of them (29 out of 36) displayed poor morphology – with more than