Multinucleation per se is not always sufficient as a marker of abnormality to decide against transferring human embryos

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Objective: To assess the developmental competence of human embryos with multinucleation (MN).
Design: Experimental study.
Setting: Research institute of private fertility center.
Patient(s): Forty-four couples donating 143 zygotes for confocal imaging study, and 78 couples included in the retrospective clinical study.
Intervention(s): Time-lapse imaging study using confocal and light microscopes.
Main Outcome Measure(s): Cytokinesis at first mitosis, MN, chromosomal behavior, euploidy, implantation, successful delivery of healthy baby.
Result(s): About 25% of the embryos showed abnormal cytokinesis (n = 34). All showed MN, and their development was greatly impaired. More than 75% of embryos that showed normal cytokinesis at first mitosis displayed MN (n = 81). However, the subsequent development of embryos with MN was similar to that of embryos without MN in vitro and in vivo. Most blastocysts were euploid. All chromosomes in several MNs took part in forming a bipolar spindle after the nuclear envelope breakdown followed by normal cleavage and development to the blastocyst stage. The implantation potential of embryos with MN was similar to that of embryos without MN, and healthy babies were born from the former group after transfer.
Conclusion(s): The presence of MN after the first mitosis does not adversely affect the subsequent development of embryos if they showed normal cytokinesis at this stage. The poor development of embryos with MN is mainly caused by abnormal first cytokinesis. (Fertil Steril® 2016;■:■–■. ©2016 by American Society for Reproductive Medicine.)

Key Words: Array CGH, chromosomal behavior, live birth, live cell imaging, multinucleation

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Multinucleated cells are frequently observed in human embryos in vitro. Multinucleation (MN) might decrease the rate of implantation of embryos after their transfer to the uterus, presumably from chromosomal aberrations and/or mosaicism (1–8). On the other hand, it has been shown that healthy babies can be born from embryos with MN (9, 10). Multinucleation might be a result as well as a cause of chromosomal aberrations such as chromosomal instability in Hela cells transfected with *Chlamydia trachomatis* (11). Although this kind of morphologic abnormality has been postulated to cause embryonic developmental failure in human embryos, its role in the pathogenesis of aneuploidy and implantation failure remains unknown.

Analysis using conventional snapshot images of fixed cells has contributed greatly to understanding their biology. However, when and how MN and chromosome abnormality might occur in embryogenesis is unknown. Because fixed cells are dead and cannot develop, it is not clear whether any particular morphologic defects in embryos are linked directly to their developmental failure. Time-lapse studies of changes in cellular morphology have revealed characteristic events in the

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development of human embryos in vitro from the first cleavage to the blastocyst stage (12, 13). However, it is difficult to observe chromosomal dynamics in each embryonic cell using conventional light microscopy. Therefore, time-lapse analysis of morphologic changes and of chromosome dynamics during preimplantation development in living embryos permits evaluation of the development of embryos with MN. A system enabling embryos to survive and develop normally after five-dimensional imaging (x, y, and z axes, time-lapse, and multicolor) allows retrospective linking between chromosomal dynamics and developmental capacity (14, 15). Here we used a live cell imaging technique to determine how the occurrence of MN might be involved in poor developmental competence and whether it would lead to aneuploidy at subsequent developmental stages.

MATERIALS AND METHODS

This study was approved by the local ethics institutional review board of IVF Namba Clinic and the Japan Society of Obstetrics and Gynecology (Registry No. 112). Vitrified-thawed pronuclear stage zygotes donated by couples who had completed their fertility treatment and provided informed consent were used for the in vitro imaging studies that did not involve embryo transfer. The clinical study was originally performed to assess the effect of continuous imaging inside an incubator. Embryo selection was based on the morphologic features on day 3 after checking normal fertilization, similar to conventional embryo selection without a time-lapse system. Therefore, the couples received full explanations regarding the treatment and gave their consent to being involved in imaging morphologic changes with the possible uterine transfer of embryos with MN. In this study, we retrospectively analyzed the relationship between abnormal cytokinesis at first mitosis or MN after normal cytokinesis and development after transfer.

Ovarian Stimulation and Insemination

Patients were treated with controlled ovarian stimulation according to their medical history as follows. For gonadotropin-releasing hormone (GnRH) agonist cycles, patients received oral contraceptive pills (1 mg of norethisterone and 0.05 mg of mestranol; Aska Pharmaceutical) on day 14 of the previous cycle, which was continued for 10 days, and GrRH agonist (600 μg/day, Suprecur nasal solution 0.15%; Mochida Pharmaceutical) on day 21 of the previous cycle until ovulation induction. On day 3 of the cycle, they received recombinant follicle-stimulating hormone (Gonal F; Merck Serono) ranging from 150 to 300 IU for 4 days followed by human menopausal gonadotropin (Ferring Pharmaceuticals) administration, ranging from 150 to 450 IU, until ovulation induction. For GnRH antagonist cycles, a GnRH antagonist (2.5 mg, ganirelix acetate; MSD) was administered daily after the leading follicles reached 13–14 mm in diameter. Ovulation induction was performed by human chorionic gonadotropin (hCG) administration when at least the leading follicles had reached 18 mm in diameter. Transvaginal follicle aspiration was performed 36 hours after the hCG injection. Insemination was done by a coculture of oocytes with 1.5×10^5 sperm/mL or intracytoplasmic sperm injection 40 hours after hCG injection.

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Definition of MN

We defined MN as having two or more nuclei, including micronuclei, in a blastomere. Binuclei was defined as having two nuclei with a diameter larger than 12 μm. When the diameter of one nucleus was smaller than 12 μm, it was categorized as micronuclei. Multinuclei was defined as having more than two nuclei.

Confocal Imaging Studies

Live cell imaging. Surplus embryos donated by 44 couples who had undergone fertility treatment were used in this study (Supplemental Fig. 1, available online). The mean age of donors was 34.8 $\pm$ 4.3 (standard deviation [SD]) years old when the oocytes were retrieved. All couples gave their informed consent. Normally fertilized zygotes were frozen at the pronuclear stage 16 to 18 hours after insemination by a slow-freezing method (16) or by vitrification (17). After thawing, the embryos were cultured in potassium simplex optimized medium containing amino acids (KSOM^{AA} medium), as described previously elsewhere (18).

The synthesis and microinjection of mRNA sequences were as described elsewhere (14). Briefly, they were synthesized using the RiboMAX Large Scale RNA Production Systems-T7 (Promega). The 5′ end of each mRNA sequence was capped using Ribo m7G Cap Analog (Promega). Synthesized RNAs were purified by phenol–chloroform treatment and gel filtration using a MicroSpin G-25 column (Amersham Biosciences).

In all, 143 frozen-thawed pronuclear stage zygotes were injected with a mixture of messenger RNAs (mRNAs) encoding enhanced green fluorescent protein (EGFP) coupled with α-tubulin (EGFP-α-tubulin) and monomeric red fluorescent protein-1 (mRFP1) fused with histone H2B (H2B-mRFP1) or a mixture of mRNAs encoding EGFP coupled with end binding protein 1 (19) (EB1, EB1-EGFP), and H2B-mRFP1.

The embryos were transferred to 5-μL drops of KSOM^{AA} medium covered with paraffin liquid (26137-85; Nacalai Tesque) on a glass-bottomed dish and imaged from the one-cell zygote to the blastocyst stage using a confocal laser microscope in an incubator (CV1000; Yokogawa Electronic) for 120 hours. Twenty-one images in the z-axis and two-color images (green, excitation 405 nm, emission BP525/50; and red, excitation 561 nm, emission BP617/73) were captured at 15-minute intervals. Developed blastocysts were categorized morphologically as reported elsewhere (13).

Microarray-based Comparative Genomic Hybridization

Twenty-six blastocysts were tested using microarray-based comparative genomic hybridization (CGH) to assess their ploidy. Briefly, whole individual blastocysts were placed in sterile 0.2-mL polymerase chain reaction tubes containing 2 μL of Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline

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