ORIGINAL ARTICLE: ASSISTED REPRODUCTION

Is there a relationship between time-lapse parameters and embryo sex?

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Objective: To study if it is possible to identify embryo sex from embryo cleavage timings.
Design: Retrospective and observational study.
Setting: University-affiliated private fertility center.
Patient(s): Women undergoing preimplantion genetic diagnosis.
Intervention[s]: All biopsied embryos were cultured in an Embryoscope incubator with time-lapse technology.
Main Outcome Measure(s): Cleavage timing from insemination to day 3 and all kinetic parameters that have been described in previous studies by our group.
Result(s): The study included 421 embryos from our Compressive Chromosome Screening program, conducted from January 2012 to December 2012. Embryos were grouped according to their sex: male (176 embryos) and female (161 embryos). Chromosomal abnormal rate was similar for the two groups (male 62.5%, female 58.4%). When morphokinetic parameters were separated in different quartiles and grouped, we found statistical differences between male or female embryos. By logistic regression analysis we found that two specific kinetic variables were relevant: second synchrony (>2 hours) and timing of morula formation (80.8–90.9 hours). With the use of these parameters, we propose an algorithm with four different categories reflecting the range from 71% to 42% in the likelihood of an embryo being female.
Conclusion(s): Embryo development was affected by embryo sex, and the sex ratio could be affected by the embryo selection method for transfer based on kinetic parameters. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
Key Words: Growth, preimplantation, sex, time-lapse, embryo development

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The development of assisted reproductive technology (ART) has enabled the best embryos to be selected for transfer to increase the likelihood of successful implantation. Embryo evaluation includes morphology parameters such as cell number, cell size, fragmentation rate, multinucleation, etc. (1–4). These parameters have been chosen to select the embryo with the highest implantation potential based on morphologic criteria. However, little is known about whether these parameters affect the embryo sex differently. Are we disturbing the sex ratio by using these criteria?

There is still controversy in the literature regarding the kinetics of embryos. Authors have reported that sex could have an effect on embryo kinetics in nonhuman animals, but the studies have shown both faster and slower development of male embryos than female embryos (5–7). Other studies did not find such a correlation (8–11). Several human embryology studies have also tried to find a correlation between embryo sex and embryo development. Some of these observed a faster development in male embryos (12–17), and others failed to find any difference in cleavage times between male and female embryos (18–21).

All of the earlier studies are based on a static morphologic assessment. Since the invention of time-lapse technology, we have the opportunity to study the whole cleavage process and not just isolated points along it (22–25). With this technology we can observe the morphokinetic parameters of the embryo and identify any sex-specific differences. Moreover, if we study the morphokinetics of the embryo from patients who have

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undergone compressive chromosome screening (CCS), we can obtain sex information for all of the biopsied embryos of the patients and not only those that have been transferred.

The aim of the present study was to assess the relationship between embryo sex and embryo morphokinetics with the use of time-lapse technology in a retrospective cohort study. As far as we know, this is the first study of its kind to link embryo sex for all developing embryos with their morphokinetic parameters.

MATERIALS AND METHODS

Study Population

This study was conducted at the Instituto Valenciano de Infertilidad (IVI) in Madrid, Spain. The procedures and protocols were approved by the Institutional Review Board, which regulates and approves database analysis and clinical IVF procedures for research at IVI. A total of 421 embryos from 93 patients were included in the study, which ran from January 2012 to December 2012. All patients were undergoing CCS because of recurrent pregnancy loss (RPL), defined as two or more miscarriages before 20 weeks of pregnancy, or repeated implantation failure (RIF), defined as the failure of a couple to conceive after the transfer of ten or more good-quality embryos or after three IVF cycles (26). Because the complex aneuploidy rate increases in patients older than 40 years, women >39 years old were excluded. Additionally, advanced maternal age is our main group of patients in the preimplantation genetic diagnosis program, but we do not have time-lapse data from patients with advanced maternal age, because we do not provide them this technology. Embryo development was retrospectively analyzed with the use of a time-lapse imaging system (Embryoscope; Unisense Fertilitech).

Ovarian Stimulation and Oocyte Retrieval

For ovarian stimulation and oocyte retrieval, the patients were treated as described by Garcia-Velasco et al. (27). Briefly, the women received a starting dose of recombinant FSH (Puregon, Organon; or Gonal F, Merck Serono) ranging from 150 IU to 225 IU (maximum) and 0.25 mg GnRH antagonist ganirelix daily (Orgalutran; Organon) starting on day 5 or 6 after FSH administration. The patient's cycle was monitored according to the individual policy of the clinic. Recombinant hCG (Ovitrelle; Serono) was administered as soon as two leading follicles reached a mean diameter $\geq$17 mm, and oocyte retrieval was performed 36 hours later. Intracytoplasmic sperm injection (ICSI) was performed subsequently on all metaphase II oocytes; a single morphologically normal-appearing spermatozoon was selected for ICSI.

Embryo Culture and Time-lapse Monitoring

All oocytes were cultured individually in microdroplets of a culture medium (Life Global) until the day of blastomere biopsy. Fertilization was confirmed by the presence of two pronuclei and extrusion of the second polar body. In addition to the morphokinetic parameters, cell number, fragmentation pattern (defined as the embryonic volume occupied by the enucleated cytoplasm and expressed as a percentage), symmetry, and multinucleation were recorded. All embryos were cultured under an atmosphere of 6.3% of CO_2 and 20% O_2.

Images were recorded every 15 minutes in five different focal planes for >120 hours of culture. The exact times for each embryo division and developmental parameters were calculated in hours after microinjection. Time-lapse images of each embryo were retrospectively analyzed with the use of an Embryoviewer software workstation (Unisense Fertilitech). The morphokinetic markers used in the study were those previously identified as good markers by Meseguer et al. (23): appearance of two pronuclei (2PN), pronuclear fading (PNF) when both pronuclei have become invisible, division to two cells (t2), and subsequently to three cells (t3), four cells (t4), five cells (t5), six cells (t6), seven cells (t7), and eight cells (t8), and morula formation (tM). Durations of events such as the second cell cycle (cc2 = t3 − t2), third cell cycle (cc3 = t5 − t3), second synchrony (s2 = t4 − t3), and interval between two and five cells (t5 − t2) were also calculated. According to Meseguer et al. (23), three more dynamic morphology criteria are used in the study of embryo morphokinetics and could be related to embryo sex and were therefore also included: 1) uneven blastomere size at the 2-cell stage (determined when cell nuclei appear); 2) abrupt division from one to three or more cells; and 3) multinucleation at the 4-cell stage.

Embryo Biopsy and Chromosomal Analysis

Embryo biopsy was performed on day 3 in embryos with more than five cells and <25% fragmentation. The zona pellucida was perforated with the use of laser technology (Octax) (28). In all cases, one cell was removed and all blastomeres were placed in a tube to perform the chromosomal analysis. All embryos were biopsied at the same time after insemination. The biopsied embryos were cultured until they reached the blastocyst stage (day 5). Embryo transfer was performed on day 5 when a normal embryo was available. The maximum number of embryos transferred was two.

Array comparative genome hybridization (CGH) was performed for chromosomal analysis. The protocol used for processing biopsied cells was as previously described by Mir et al. (29). Briefly, a single cell from each embryo was lysed and its DNA amplified with the use of the Sureplex DNA amplification system (Bluegnome). Amplification quality was ensured with the use of gel electrophoresis (Lonza). Cy3 and Cy5 fluorophores were used to label the sample and control DNA, respectively. Labeling mixes were combined and hybridized on a 24sure array (Bluegnome) for 6–12 hours. The obtained fluorescence was measured and correlated with copy numbers of the chromosomes. Final results were obtained on day 5 with the use of a laser scanner (710 Innoscan, Innopsys; or Powerscanner, Tecan). Bluefuse software (Bluegnome) was used to analyze the data. The entire protocol for array-CGH analysis was completed in 24 hours.

Statistical Analysis

The times, in hours after ICSI, of embryo events in male and female embryos usually, but not always, followed normal