Increasing the probability of selecting chromosomally normal embryos by time-lapse morphokinetics analysis

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Objective: To study the differences in the cleavage time between chromosomally normal and abnormal embryos and to elaborate an algorithm to increase the probability of noninvasively selecting chromosomally normal embryos.

Design: Retrospective cohort study.

Setting: University-affiliated infertility center.

Patient(s): Preimplantation genetic screening patients (n = 125; n = 77 with ET), including cases of repeated implantation failure or recurrent miscarriage. A total of 504 embryos were analyzed.

Intervention(s): Embryo culture within a time-lapse system.

Main Outcome Measure(s): Kinetic variables included the time to 2 (t2), 3 (t3), 4 (t4), and 5 (t5) cells as well as the length of the second (cc2 = t3 − t2) and third (cc3 = t5 − t3) cell cycle, the synchrony in the division from 2 to 4 cells (s2 = t4 − t3), and the interval t5 − t2. Implantation and clinical pregnancy rates were also analyzed.

Result(s): A logistic regression analysis identified t5 − t2 (odds ratio [OR] = 2.853; 95% confidence interval [CI], 1.763–4.616), followed by cc3 (OR = 2.095; 95% CI, 1.356–3.238) as the most relevant variables related to normal chromosomal content. On the basis of these results, an algorithm for embryo selection is proposed to classify embryos from A to D. Each category exhibited significant differences in the percentage of normal embryos (A, 35.9%; B, 26.4%; C, 12.1%; D, 9.8%).

Conclusion(s): Chromosomally normal and abnormal embryos have different kinetic behavior. On the basis of these differences, the proposed algorithm serves as a tool to classify embryos and to increase the probability of noninvasively selecting normal embryos. (Fertil Steril® 2014;101:699–704. ©2014 by American Society for Reproductive Medicine.)

Key Words: Embryo kinetics, chromosome, arrayCGH, time lapse

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/basilen-embryos-time-lapse-morphokinetics/

Tremendous advances have occurred in the field of assisted reproduction technology (ART) over the past 30 years as the result of a combination of different factors in the IVF laboratory, including the introduction of groundbreaking techniques, such as intracytoplasmic sperm injection (ICSI) (1, 2), great improvements in culture media, and the introduction of preimplantation genetic diagnosis (PGD) (3). From a clinical point of view, physicians have learned to handle more pure and more powerful stimulation drugs that, along with increasing knowledge about the pathophysiology of ovarian hyperstimulation syndrome, have made the frequency of this syndrome almost anecdotic and the process of preparing a patient for IVF a controlled situation.

However, IVF success rates remain relatively low, with clinical pregnancy rates (PRs) of ~30% per transfer as reported by the Society for Assisted Reproductive Technology and by the European registers of the European Society of Human Reproduction and Embryology (https://www.sartcorsonline.com, 4). In addition, the number
of multiple births still remains a concern, and the pressing need to reduce the number of transferred embryos demands better selection methods. Thus, a big question remains: how can we move on to single ET (SET) programs while maintaining, or even improving, our clinical outcomes?

One approach could be based on the method used to evaluate and select embryos in the laboratory. Before the introduction of time-lapse technology, embryo grading and, most importantly, embryo selection were based only on static observations. These observations are inevitably linked to specific time points during the day and do not describe a dynamic process, such as embryo development, well enough. This subjective procedure has large flaws, such as inter- and intraobserver variation (5, 6), and controversial benefits for almost every scoring system.

The problem with applying a static scoring system to a dynamic process was solved in our laboratories by the introduction of time-lapse technology. Since that step, new kinetic markers associated with higher implantation potential have been proposed (7), the safety of these systems validated (8–11), and the effects of different intrinsic and extrinsic factors on the morphokinetic behavior of the human embryo analyzed (12–15). A morphokinetic era has evidently started in IVF.

Another approach could be based on the genetic screening of embryos. Several studies have demonstrated that chromosomal abnormalities are one of the most common causes of abnormal embryos in IVF (16–20), which translates into poor clinical outcomes. A recent publication highlighted the inherent imprecision of SET when conventional morphology is used alone, observing a 44.9% aneuploidy rate for blastocysts from patients with good prognosis (21).

Thus, embryo selection using morphokinetic markers combined with preimplantation genetic screening (PGS) could be the solution. Even though time-lapse technology is increasingly used in IVF laboratories, PGS is not always possible owing to legal or social reasons or simply because the clinic cannot perform the technique. The objective of the present study was to analyze the morphokinetic behavior of chromosomally normal and abnormal embryos to develop a new selection tool that increases the probability of noninvasively selecting chromosomally normal embryos. We retrospectively analyzed embryos using array-comparative genome hybridization (arrayCGH).

**Materials and Methods**

Embryo development was retrospectively analyzed using a time-lapse imaging system (Embryoscope, Unisense FertiliTech) in a total of 125 consecutive patients undergoing PGS (n = 504 embryos); of those, 77 received an ET between March 2011 and August 2012. All procedures and protocols were approved by the Institutional Review Board, which regulates and approves database analysis and clinical IVF procedures for research at IVI.

Embryo biopsy was done on day 3 and comprehensive chromosome screening performed through arrayCGH. This study included patients undergoing PGS for recurrent miscarriage (RM) and repeated implantation failure (RIF) only. RM was defined as two or more miscarriages before 20 weeks of pregnancy, and RIF was defined as the failure of a couple to conceive after 10 or more good-quality embryos transferred or after three IVF cycles (22).

**Ovarian Stimulation**

Ovarian stimulation was carried out as described elsewhere (23). Briefly, patients received a starting dose of recombinant FSH (Puregon, MSD; Gonal F, Merck-Serono) ranging from 150 to a maximum of 225 IU. GnRH antagonist (0.25 mg Ganirelix, Orgalutran) was administered daily starting on day 5 or 6 after FSH administration. Recombinant hCG (Ovitrelle, Merck-Serono) was administered as soon as two leading follicles reached a mean diameter ≥ 17 mm.

**Oocyte Retrieval, Embryo Culture, and Embryo Biopsy**

Oocyte retrieval was performed 36 hours after hCG under ultrasound guidance. After retrieval, the oocytes were kept in culture for 4 hours at 37.3°C and 5.8% CO2 until oocyte denudation. Oocyte denudation was performed by mechanically pipetting with 40 IU/mL of hyaluronidase (Vitrolife). ICSI was subsequently performed on all metaphase II oocytes. Fertilized oocytes were cultured individually in microdroplets of culture media (Vitrolife) until the day of blastomere biopsy. Biopsy was performed on day 3 for all embryos that were made up of 6 or more cells with less than 25% fragmentation as described elsewhere (24). Briefly, embryos were placed individually on a droplet containing CaCl2/MgCl2-free medium (G-PGD, Vitrolife), and the zona pellucida was perforated using laser technology (OCTAX). Patients with normal embryos were scheduled for blastocyst transfer on day 5 (approximately 120 hours) of development. Treatment cycles were selected at random for investigation by time-lapse image acquisition and subsequent retrospective analysis of the morphokinetic parameters of embryo development.

**ArrayCGH**

ArrayCGH was performed as described elsewhere (24). Briefly, a single cell from embryos was amplified using the Sureplex DNA amplification system (BlueGnome). Amplification quality was ensured by 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. Final results were obtained on day 5 using a laser scanner (710 Innoscan, Innopsys; and Powerscanner, TECAN). BlueFuse software was used to analyze the data (BlueGnome). The entire protocol for arrayCGH analysis was completed in 24 hours.

**Time Lapse Analysis and Recording of Kinetic Parameters**

The exact times for each embryo division and developmental parameters were calculated in hours postmicroinjection (hpi). Time-lapse images of each embryo were retrospectively analyzed using an EmbryoViewer software workstation (Unisense FertiliTech). Images were acquired every 15 minutes in five different focal planes over 120 hours of culture.
Development markers included the visibility of two pronuclei (2PN); pronuclear fading (PNF) when both pronuclei were no longer visible; the first cell division leading to two cells (t2); and subsequently the second (3 cells, t3), third (4 cells, t4), and fourth (5 cells, t5) cell divisions. We also calculated the intervals between two consecutive cleavages and defined the duration of the second cell cycle (cc2) as the time from division into a two-blastomere embryo to division into a three-blastomere embryo (cc2 = t2 – t2) and second synchrony (s2) as the time from division into a three-blastomere embryo to division into a four-blastomere embryo (s2 = t4 – t3). The duration of the third cell cycle was defined as cc3 (cc3 = t5 – t3), and the interval between 2 and 5 cells as the variable t5 – t2, which combines the concepts of cell cycle and synchrony.

Statistical Analysis

Analysis of variance (ANOVA) was used to test whether the mean times for embryonic events were significantly different between chromosomally normal and abnormal embryos. To describe the distribution of the probability of chromosomally normal embryos, times were converted from continuous variables into categorical variables by dividing them into groups based on their quartiles. Using this procedure, we avoided bias caused by differences in the total number of embryos in each category. Next, we calculated the percentage of chromosomally normal embryos for each time quartile to assess the distribution of normality in the different categories. Continuous data were analyzed using Student's t-tests when comparing two groups or ANOVA followed by Bonferroni’s and Scheffe’s post hoc analysis when multiple groups were considered. \( \chi^2 \)-tests were used to compare categorical data. For each time variable, the optimal range was defined as the combined range spanned by the two quartiles or as the range spanned by the one quartile with the highest proportion of normal embryo rates. A binary variable was defined by the value inside if the value of the time variable was inside, and vice versa for outside, the optimal range. The odds ratio (OR) of the effect of all binary variables generated for embryonic chromosomal normality was expressed in terms of the 95% confidence interval (95% CI) and significance. The effect of optimal ranges and other binary variables on chromosomal normality were quantified by logistic regression analysis. Receiver operating characteristic (ROC) curves were used to test the predictive value of all variables included in the model with respect to chromosomal normality. ROC curve analysis provides area under the curve (AUC) values between 0.5 and 1 and can be interpreted as a measurement of the global classification ability of the model.

RESULTS

The mean age of our female population was 36.1 years (95% CI, 35.3–36.9). The primary etiology of female factor infertility as an indication for PGS was RM in 40 women (51.9%) and RIF in 37 women (48.1%). Average E2 levels before hCG injection were 1,706 pg/mL (95% CI, 1494–1917). A total of 52 of the 114 transferred embryos were successfully implanted (gestational sac), resulting in a 46.48% (95% CI, 37.3–55.66) implantation rate. The biochemical PR per transfer was 63.3% (n = 49; 95% CI, 52.5–74.1), and the ongoing PR per transfer was 54.5% (n = 42; 95% CI, 43.4–65.6).

A total of 986 microinjected oocytes were cultured inside the time-lapse system: 717 were fertilized (72.5%; 95% CI, 69.7–75.3), and 504 (70.3%; 95% CI, 66.3–74.3) were analyzed by arrayCGH. Among the analyzed embryos, 143 (28.3%; 95% CI, 24.4–32.2) had normal chromosome content. Supplemental Table 1 shows the mean times for each cell division and for the intervals between consecutive divisions. Significant differences were observed between normal and abnormal embryos for t5, cc2, cc3, and t5 – t2. Figure 1 clearly shows a higher proportion of normal embryos within the optimal ranges defined for t5 – t2 and cc3. Table 1 describes the distribution in more detail: embryos falling within optimal ranges for t5 (47.2–58.2 hours), cc3 (11.7–18.2 hours), and t5 – t2 (>20.5 hours) exhibited a significantly greater proportion of normal embryos than those falling outside these ranges (34.7%, 33.4%, and 34.4% vs. 20.9%, 16.3%, and 10.4%, respectively).

Logistic regression analysis was used to select and organize the relevance of observed events (expressed as binary variables inside or outside the optimal range) to the selection of embryos with a higher probability of being chromosomally normal. The model identified t5 – t2 (OR = 2.853; 95% CI, 1.763–4.616) and cc3 (OR = 2.095; 95% CI, 1.356–3.238) as the most relevant variables related to normal chromosomal content. A ROC analysis to determine the predictive properties of this model with respect to chromosomal normality gave an AUC value of 0.634 (95% CI, 0.581–0.687).

Using the data provided by logistic regression, we created a hierarchical model using the corresponding decision tree procedure, which subdivided embryos into four categories (A–D) as shown in Figure 2. The classification procedure based on the binary variables t5 – t2 and cc3 was as follows. First, if the
value of $t_5 - t_2$ fell inside the optimal range, the embryo was
categorized as A or B; if the value of $t_5 - t_2$ fell outside the
optimal range, the embryo was categorized as C or D. If the value
of $cc_3$ fell inside the optimal range, the embryo was categorized
as A or C depending on $t_5 / C_0 t_2$; similarly, if the value of $cc_3$ fell
outside the optimal range, the embryo was categorized as B or D
depending on $t_5 / C_0 t_2$. Figure 3 describes a signifi-
cant decrease
in the percentage of normal embryos between the different cat-
egories (A, 35.9%; B, 26.4%; C, 12.10%; and D, 9.8%).

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td><strong>Percentage of normal and abnormal embryos in and out of defined optimal ranges.</strong></td>
</tr>
<tr>
<td></td>
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<tr>
<td>$t_5$</td>
</tr>
<tr>
<td>Out 47.2–58.2 hours</td>
</tr>
<tr>
<td>$P$ value</td>
</tr>
<tr>
<td>$cc_3$</td>
</tr>
<tr>
<td>Out 11.7–18.2 hours</td>
</tr>
<tr>
<td>$P$ value</td>
</tr>
<tr>
<td>$t_5 - t_2$</td>
</tr>
<tr>
<td>$&lt; 20.5$ hours</td>
</tr>
<tr>
<td>$P$ value</td>
</tr>
<tr>
<td>$&gt; 20.5$ hours</td>
</tr>
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<td>$P$ value</td>
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</table>

Note: $t_2$: time to 2 cell stage; $t_3$: time to 3 cell stage; $t_5$: time to 5 cell stage; $cc_3 = t_5 / C_0 t_3$; $t_5 / C_0 t_2$: interval between 2 and 5 cells.

**DISCUSSION**

This study combined time-lapse technology and PGS to
analyze and compare the morphokinetic behavior of chromo-
somally normal and abnormal embryos. Observations in a
large number of embryos ($n = 504$) resulted in an algorithm
that can increase the probability of selecting chromosomally
normal embryos in the absence of PGS.

The impact of chromosomal content on embryo
morphology and development has been described extensively
from a static point of view (20, 25–28). More recently, and as
a consequence of the increasing use of time-lapse technology
in IVF laboratories, the same approach has been carried out
in a more dynamic manner. Davies et al. (29) reported delayed
first and second cleavage and prolonged transitions between 2
and 4 cells among embryos with complex aneuploidies ($n = 62$
cleavage-stage embryos). The same study reported that irreg-
ular divisions (transition from 1 to 3 and 2 to 5 cells) and the
disappearance of asynchronous pronuclei predominate in em-
bryos with multiple aneuploidies. Chavez et al. (30) observed
that chromosomally normal embryos display strict and tightly
clustered cell cycle parameters up to the 4-cell stage, but only
30% of aneuploid embryos exhibit values within normal time
ranges ($n = 68$ cleavage-stage embryos). On the other hand,
Campbell et al. (31) reported no difference between euploid
and aneuploid embryos during early stages of development. However, in the periblastulation phase, aneuploid embryos had a significant delay in development compared with euploid embryos (n = 98 blastocysts). This study gave rise to an aneuploidy risk model based on the time from insemination to the start of blastulation (tSB) and the time from insemination to the formation of a full blastocyst (tB). More specifically, embryos with tB < 122.9 hpi and tSB < 96.2 hpi are classified as having low risk; embryos having tB < 122.9 hpi and tSB ≥ 96.2 hpi are classified as having medium risk; and embryos having tB ≥ 122.9 hpi are classified as having high risk. An elegant study by the same group applied the aneuploidy risk model to evaluate its effectiveness and potential clinical impact for unselected IVF patients without undergoing PGS after analyzing embryos with known implantation data (KID embryos). The study revealed significant differences in fetal heart rate (n = 88 KID embryos) and live-birth rate (n = 44 KID embryos) between risk classes (low, medium, high) (32). In agreement with the Davies et al. study but in opposition to Campbell et al.’s, our group found differences in the morphokinetic behavior of chromosomally normal and abnormal embryos during early stages of development, but with a much larger data set (n = 504 embryos), which is 5 times higher than the data sets from previous publications, giving our results statistical power and reducing the chances of random findings.

Interestingly, the variables included in our algorithm (t5 – t2 and cc3 = t5 – t3) can be detected as early as day 2 of development, avoiding the need to culture to the blastocyst stage to make a combined decision (morphology + kinetic markers associated with normal chromosomal content). Additionally, the algorithm here proposed is related to blastocyst formation and strengthens the relation of our findings to those reported by Campbell et al. but with a larger sample size. This aspect is a great advantage over Campbell et al.’s model because it can be applied not only to patients undergoing blastocyst transfer but also to those undergoing transfers on day 3 of development, a common situation in many IVF clinics. In addition, and considering again that the variables can be detected as early as day 2 (before embryo biopsy on day 3), our assumption would be that this algorithm could be applied regardless of the day of biopsy (day 3 vs. day 5), although we do not have data to support it. Another difference is that in Campbell et al.’s study, embryo biopsy was performed on day 5. In our study, we performed embryo biopsy of a single blastomere on day 3 with the existing probability of mosaicism that may bias the results of the study. However, in our program, confirmation rates on embryos biopsied on day 3, diagnosed as abnormal and rebiopsied on day 5, are similar to those biopsied directly on day 5, diagnosed as abnormal and rebiopsied on day 5, with results close to 97% (23).

High levels of in vitro–generated human embryos are chromosomally abnormal (33, 34), which constitutes one of the most common causes of IVF failure. This problem justifies the use of PGS and, if PGS is not possible, the use of time-lapse imaging to increase the probability of selecting chromosomally normal embryos. Moreover, embryo selection through time-lapse imaging is a noninvasive approach that can be applied not only to patients at risk of having chromosomally abnormal embryos but also to young patients, especially considering the high percentage of aneuploid oocytes and embryos reported in young donors (27) and good-prognosis patients (21). The observed discordance between ploidy status and morphology stresses the point that, without the extra information gained from PGS, we allow the transfer of morphologically normal, but still reproductively incompetent, embryos.

The selection of embryos through time-lapse technology should not be considered as a replacement for PGS. However, it does represent an excellent selection tool for good-prognosis patients who are not indicated for PGS or for patients who are indicated for PGS (history of implantation failure or early pregnancy loss) but who for any legal, social, or economic reasons do not wish or cannot have PGS performed. In these situations, a clear benefit is gained with morphokinetic screening and selection using the algorithm. In regards to cost, we must acknowledge that time-lapse technology currently involves expensive equipment and, in the short term, the cost differences between PGS and time-lapse are minimal. In the near future, we expect a reduced cost for PGS owing to the introduction of quantitative polymerase chain reaction, as well as less expensive time-lapse equipment owing to the existing competition between different brands.

Finally, careful assessment should be performed before generalizing the use of time-dependent variables and algorithms for selecting embryos in different clinical settings. Embryo development is a dynamic process that can be affected by several extrinsic and intrinsic factors (12–15). At this point, several algorithms are available for the clinical selection of embryos for transfer (7, 30, 35). Here we provide a new alternative that needs to be compared clinically in prospective studies in which all the alternatives are validated and their utility proven. This work is underway.

Acknowledgments: The authors thank María Cruz and the entire IVF and PGD team for their enthusiasm and support.

FIGURE 3

Percentage of chromosomally normal embryos according to hierarchical classification. N represents the total number of embryos in each category. *P < .001.

REFERENCES

<table>
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<tr>
<th>Variable</th>
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<th>n</th>
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<th>Upper limit</th>
<th>Minimum</th>
<th>Maximum</th>
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Note: 2PN = time of pronuclear appearance; PNF = time of pnf; t2 = time to 2 cells; t3 = time to 3 cells; t4 = time to 4 cells; t5 = time to 5 cells; cc2 = t3 / C0 t2; cc3 = t5 / C0 t3.