Frequency of embryo multinucleation detected by time-lapse system and its impact on pregnancy outcome

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Objective: To compare the detection rate of multinucleation with the time-lapse system and conventional control timing proposed by European Society of Human Reproduction and Embryology (ESHRE) consensus and evaluate its impact on pregnancy rates.
Design: Retrospective study.
Setting: A private IVF center.
Patient(s): A total of 686 embryos from 511 intracytoplasmic sperm injection (ICSI) cycles.
Intervention(s): None.
Main Outcome Measure(s): A time-lapse system was used to acquire embryo images until ET; the stored data were reviewed for the presence and persistence of multinucleation. The detection rate of multinucleation was compared with ESHRE/ALPHA consensus–proposed embryo evaluating times (23 ± 1, 26 ± 1, 44 ± 1 hours). Morphokinetic characteristics of multinucleated embryos and the effect of multinucleation on pregnancy rate were researched.
Result(s): Multinucleation was detected in 159 embryos of 145 ICSI cycles. Using ESHRE/ALPHA consensus embryo evaluating times, only 44 (27.6%) out of 159 multinucleated embryos could be identified. In cycles with multinucleated ETs compared with cycles with no multinucleated embryos, clinical pregnancy rates (respectively, 23.4 vs. 44) and implantation rates (respectively, 23.3 vs. 43.6) were significantly lower. Time to 2-cell, 4-cell, and 6-cell stage was significantly longer in multinucleated embryos. Patient age (odds ratio [OR], 0.95; confidence interval [CI], 0.92–0.98) and presence of multinucleation (OR, 0.37; CI, 0.24–0.56) were the only significant predictors of clinical pregnancy rate.
Conclusion(s): The time-lapse monitoring system seems to be a valuable tool to identify all cases with multinucleation. We conclude that the detection of multinucleation by time-lapse monitoring is associated with lower implantation and clinical pregnancy rates. (Fertil Steril® 2014;102:1029–33. ©2014 by American Society for Reproductive Medicine.)
Key Words: Embryo development, time-lapse, multinucleation, morphokinetic, pregnancy rate

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To prevent multiple pregnancies, it is important to reduce the number of embryos to be transferred, but at the same time pregnancy and implantation rates should not be decreased. Therefore, the choice of embryos with high implantation potential becomes more important. Although many new noninvasive techniques regarding the selection of embryos by metabolic status such as metabolomics and proteomics have been invented, they never got past the experimental stage and were never used for standard clinical applications (1–4).

Embryos are generally chosen for transfer based on their morphological properties. In day 2 and day 3 transfers, embryos are scored according to parameters such as early cleavage, number of blastomeres, and fragmentation rate. This score is used as a representation of the quality of developed embryos (5, 6). For day 5 and day 6 transfers, blastocyst scoring is performed based on Gardner's blastocyst scoring criteria (7).

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All embryo assessment criteria were reviewed, and standard embryo evaluation timings were given in a European Society of Human Reproduction and Embryology (ESHRE)/ALPHA consensus to evaluate the differences between labs and embryologists to standardize embryo evaluation (6). Shortly after ESHRE's publication, Meseguer et al. published their dynamic embryo selection model with completely different criteria based on exact cleavage timings (8). Usage of time lapse has greatly helped to assess dynamic embryo development. During time-lapse imaging, development of the embryos is monitored for 24 hours without the embryos being taken out of their stable environment. With this system, it is possible to observe the exact timing of blastomere divisions and select embryos with several dynamic markers during embryo assessment. Moreover, time lapse made morphological evaluation more flexible since it allows the replay of images. In a recent study assessing inter- and intraobserver variability of time-lapse annotations, a close agreement was found in terms of some dynamics parameters such as pronuclear breakdown and completion of blastocyst hatching, but measurements of the static morphological parameters such as multinucleation and evenness of blastomeres at the 2-cell stage presented fair to moderate agreement between embryologists (9).

In many embryo grading systems, the detection of multinucleation in blastomeres is an important criteria in embryo selection (10–12). Before the introduction of time-lapse techniques, multinucleation was evaluated only when it was detected during embryo check under a microscope in a limited time interval, and it was unknown whether it happened at any other time. However, multinucleation may occur at different times during the developmental period and can disappear in a very short time. Therefore, time lapse may be considered as the optimum technique to visualize multinucleation at any time point.

It has been suggested that multinucleated embryos are related to an increase in aneuploidy rate, lower blastocyst formation rate, lower implantation rate, and lower live birth rate (13, 14). Therefore, the purpose of the present study is to compare the multinucleation rate detected by the time-lapse system with conventional control timing proposed by the ESHRE consensus and evaluate its impact on pregnancy rate.

MATERIALS AND METHODS

Ovarian Stimulation

All patients were administered a GnRH antagonist (Cetrotide 0.25 mg, Merck Serono) protocol for ovulation induction. A single injection of 250 μg recombinant hCG (rhCG alpha; Ovitrelle, Merck Serono) was administered to induce final follicular maturation as soon as three follicles of $\geq$17 mm were observed. The luteal phase was supported by either micronized vaginal P (Progynex 200 mg, Farmaco Inc.), P in oil (Progynex ampul 50 mg, Farmaco Inc.), or vaginal gel (Crinone 8% gel, Merck Serono).

Oocyte Retrieval and Intracytoplasmic Sperm Injection (ICSI)

Transvaginal ultrasound–guided oocyte retrieval was performed 36 hours after hCG injection. All collected oocytes were kept in culture under the conditions of 37°C, 6% CO_2, 5% O_2 until denudation. For all steps related to embryo culture, two sequential culture media (Vitrolife, Sweden and Medicult) were used. The oocytes were denuded at around 3 hours after pick-up. The ICSI procedure was performed on all metaphase II (MII) oocytes in all cases since ICSI is the preferred method of fertilization by patients and clinics in our country to keep the fertilization rate as high as possible. Injected oocytes were placed into pre-equilibrated culture media in the standard incubator. The fertilization check was done at 16–18 hours after microinjection. The presence of two pronuclei was considered normal fertilization.

Embryo Culture

All fertilized oocytes were transferred into individual microwells of special culture slides (EmbryoSlide, Unisense FertiliTech) containing 35 μL of culture medium under 1.4 mL oil and were placed in a time-lapse monitoring system that uses an incubator with an integrated optical microscope to acquire images automatically (EmbryoScope, Unisense FertiliTech) until ET. Embryo development was recorded every 10 minutes on five different focal planes. All images and related data were recorded into EmbryoViewer (Unisense FertiliTech) and stored within the database.

ET

Before ET, embryologists annotated time-lapse images of the embryos and recorded morphokinetic variables. ET was performed at day 2, day 3, or day 5 after oocyte retrieval based on the number and quality of embryos. The choice of single versus double ET was done according to the legal regulations in Turkey. On the basis of these regulations, a single embryo was transferred to the patients who were under 35 years old and had one or two previous IVF attempts, and two embryos were transferred to the patients above 35 years old or who already had more than two previous IVF attempts. Embryos that were transferred were selected on the basis of their developmental quality based on morphokinetics and their final morphological appearance. The presence of multinucleation in these embryos was determined after obtaining clinical outcomes. Good-quality embryos that were not used for transfer were cryopreserved. Serum ß-hCG level was measured 12 days after ET. An ultrasound was performed 14 days after positive ß-hCG to ascertain gestational sac(s). Clinical pregnancy was evaluated within 7–14 days after the detection of the gestational sac by identification of fetal cardiac heartbeat. Implantation rate was calculated as the ratio between the number of gestational sacs and the number of transferred embryos.

Evaluation of Multinucleation

A total of 764 transferred embryos with known implantation data from 550 IVF-ICSI patients who were cultured in the time-lapse imaging system between June 2011 and December 2012 were retrospectively analyzed. All single ETs were included. Among two ETs, cases that resulted in two gestational sacs or no pregnancy were included to ascertain the

outcome resulting from the transferred embryos. Thirty-nine double ETs that presented only one gestational sac were excluded from the analysis as it was not possible to detect which embryo was implanted (multinucleated or not). All images of each transferred embryo were analyzed retrospectively with image analysis software (EmbryoViewer, Unisense Fertilitech) at the 2-cell stage for the presence and persistence of multinucleation. Radii and numbers of nuclei within a single blastomere at the 2-cell stage were also checked, and multinucleation was defined as the presence of more than one nucleus (either binucleated or micronucleated) within at least one of the blastomeres of 2-cell embryos (15). Evaluation of multinucleation was double checked by two embryologists using image analysis software. Based on time-lapse imaging, all embryos were rechecked according to embryo evaluation times proposed by ESHRE/ALPHA consensus (23 ± 1, 26 ± 1, 44 ± 1 hours) (Supplemental Fig. 1). A total of 145 patients were transferred a multinucleated embryo(s) (group 1) and 366 patients were not (group 2). Results of the transfer of embryos with and without multinucleation were compared.

The exact timings of embryo cleavage were examined with image analysis software. The timing of the cleavage of embryos into 2, 3, 4, 5, 6, 7, and 8 blastomeres was denoted as t2, t3, t4, t5, t6, t7, and t8, respectively. Morphokinetics were compared among embryos with and without multinucleation. Since this was a retrospective study, Institutional Review Board approval was not required.

Statistical Analysis

The statistical analysis of the data was performed using SPSS 13.0. The continuous variables were analyzed for normality distribution with the Kolmogrov-Smirnoff test. All continuous variables analyzed were normally distributed and were compared using Student's *t* test between the groups. The categorical data were compared between the two groups using the χ^2-test. Logistic regression analysis was used to predict the presence of clinical pregnancy using the forward Wald method. Patient age, body mass index (BMI), day 3 FSH level, number of MII oocytes retrieved, controlled hyperstimulation protocols used (agonist vs. antagonist), total FSH dose used for stimulation, indication for assisted reproduction (male factor, female factor, unexplained), t2 time, t3 time, t5 time, t8 time, and the medium used for embryo culture were included in the regression analysis. For all comparisons, probability $P<.05$ was considered statistically significant.

RESULTS

The mean age, BMI, basal FSH, previous ICSI cycles, and cause of infertility were similar between group 1 and group 2 (Table 1). The data on cycle characeristics and pregnancy rates are presented in Table 2. The rate of antagonist cycles, total gonadotropin dose used, and the mean number of MII oocytes aspirated were similar in both groups (Table 2). Single ET cycles were significantly higher in group 1 (n = 112, 77.2%) compared with in group 2 (n = 205, 66.1%; $P<.05$). Positive β-hCG result was lower in group 1 (n = 54, 37.2%) compared with in group 2 (n = 193, 52.7%; $P=.002$). The clinical pregnancy rate was lower in group 1 (n = 34, 23.4%) compared with in group 2 (n = 161, 44%; $P<.001$). The spontaneous abortion rate was similar between the two groups, whereas biochemical pregnancy was higher in group 1 (n = 20, 13.8%) compared with in group 2 (n = 32, 8.7%), although it did not reach statistical significance ($P=.08$). Implantation rate was lower in group 1 (37/159, 23.3%) compared with in group 2 (230/527, 43.6%; $P<.001$). In logistic regression analysis, patient age ($P=.001$, odds ratio [OR], 0.95; confidence interval [CI], 0.92–0.98) and the presence of multinucleation ($P<.001$; OR, 0.37; CI, 0.24–0.56) were the only significant predictors of clinical pregnancy rate.

Morphokinetic evaluation of the embryos is given in Table 3. Time to reach the 3-, 5-, 7-, and 8-cell stage was similar between multinucleated (n = 159) and nonmultinucleated (n = 527) embryos. Time to t2 was longer in multinucleated embryos (26.9 ± 3.0 hours) compared with in nonmultinucleated embryos (26.1 ± 2.7 hours; $P=.003$. Time to t4 was longer in multinucleated embryos (38.7 ± 4.0 hours) compared with in nonmultinucleated embryos (38.1 ± 3.5 hours; $P=.04$). Time to t6 was longer in

TABLE 1

Demographic data of the patients.

Variable	Group 1 (n = 145)	Group 2 (n = 366)	*P* value
Age (y)	30.5 ± 5.8	30.4 ± 5.3	.8
BMI (kg/m^2)	24.6 ± 3.7	25.2 ± 4.3	.1
Basal FSH (IU/L)	7.1 ± 2.2	7.0 ± 2.3	.6
Previous ICSI cycles	1.8 ± 1.4	1.6 ± 1.2	.1
Cause of infertility			.71
Unexplained	49 (33.7)	126 (34.4)	
Male factor	61 (42.1)	135 (36.4)	
PCOS	9 (6.2)	31 (8.5)	
Endometriosis	4 (2.8)	35 (9.6)	
Tubal factor	9 (6.2)	35 (9.6)	
Azoospermia	13 (9.0)	30 (8.2)	

Note: The data are presented as n (%) or mean ± SD from the mean.

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TABLE 2

Cycle characteristics and pregnancy outcome.

Variable	Group 1 (n = 145)	Group 2 (n = 366)	*P* value
Antagonist cycles	143 (98.6)	360 (98.4)	.83
Total gonadotropin dose (IU)	2,443 ± 963	2,509 ± 1,114	.50
MII oocytes aspirated	10.9 ± 6.1	11 ± 5.6	.85
Single ETs	112 (77.2)	205 (66.1)	<.05[a]
Double ETs	33 (22.7)	161 (43.9)	
Positive ß-hCG	54 (37.2)	193 (52.7)	.002[a]
Clinical pregnancy rate	34 (23.4)	161 (44.0)	<.001[a]
Spontaneous abortion rate	4 (2.8)	13 (3.6)	.65
Biochemical pregnancies	16 (11)	19 (5.1)	.01[a]
Implantation rate (N = 686)	37/159 (23.3)	230/527 (43.6)	<.001[a]

Note: The data are presented as n (%) or mean ± SD from the mean.

[a] Statistically significant, $P<.05$, χ^2-test.

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TABLE 3

Morphokinetic evaluation of embryos.

Embryo cleavage timing	Multinucleated embryos (n = 159)	No multinucleation detected (n = 527)	*P* value
t2 (n)	26.9 ± 3.0 (159)	26.1 ± 2.7 (527)	.003[a]
t3 (n)	37.5 ± 5.0 (159)	37.2 ± 3.7 (527)	.45
t4 (n)	38.7 ± 4.0 (158)	38.1 ± 3.5 (523)	.04[a]
t5 (n)	49.4 ± 6.2 (143)	49.4 ± 5.2 (455)	.96
t6 (n)	52.9 ± 5.6 (125)	51.7 ± 5.2 (416)	.02[a]
t7 (n)	54.8 ± 6.0 (117)	52.9 ± 5.3 (395)	.67
t8 (n)	56.5 ± 6.6 (109)	54.3 ± 6.0 (364)	.67

[a] Statistically significant $P < .05$, Student's *t* test.

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multinucleated embryos (52.9 ± 5.6 hours) compared with in nonmultinucleated embryos (51.7 ± 5.2 hours; $P = .02$).

Only 44 (27.6%) cases of multinucleated embryos could be detected within the embryo check time limits proposed by the ESHRE/ALPHA consensus. Among these 44 cases, most of them were detected between 25 and 27 hours (Table 4). The median onset of multinucleation was 30.6 hours (22.9–44.4 hours), and the median end of multinucleation was 36.2 hours (28–53.9 hours).

The multinucleated embryos (n = 159) were evaluated from different aspects according to being clinically pregnant (n = 37) or not (n = 122). The size of the nucleus was measured, and the difference between the largest and smallest nucleus was not significantly different between the pregnant cases (6.4 ± 4.6 μm) and nonpregnant cases (5.8 ± 4.2 μm; $P = .51$). The time of persistence of multinucleation was not significantly different between pregnant cases (6.4 ± 2.8 hours) and nonpregnant cases (5.7 ± 2.5 hours; $P = .17$). The percentage of blastomeres with multinucleation, the number of nucleoli, and the cell stage of multinucleation observed were not significantly different between pregnant and nonpregnant cases.

The predictors of clinical pregnancy were evaluated among the 686 embryos. Among these patients, age ($P = .001$; OR, 0.95; CI, 0.92–0.98) and presence of multinucleation ($P < .001$; OR, 0.37; CI, 0.24–0.56) were the only significant predictors of clinical pregnancy rate.

DISCUSSION

Observation of 2-cell stage multinucleation should be part of all routine embryo assessments to optimize embryo selection.

TABLE 4

Detection of multinucleation within embryo check times proposed by ESHRE/ALPHA consensus.

Embryo check times by ESHRE/ALPHA consensus	No. of embryos with multinucleated blastomeres (n/N = 44/159)
22, 23, 24 h (post-ICSI)	7/44
25, 26, 27 h (post-ICSI)	30/44
43, 44, 45 h (post-ICSI)	15/44

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However, this study showed that nuclear formation is a very dynamic process, and single observation could not accurately evaluate the nuclear status. The time-lapse monitoring system helps us assess multinucleation and provide new insights into the dynamic process of blastomere multinucleation.

Multinucleated blastomeres could be identified at any time between the first cleavage and blastocyst stage, although they were generally observed in 2-cell stage embryos. The visualization of nuclei may be more difficult when embryos have more cells and fragments (16).

Blastomere multinucleation has been associated with decreased embryo development and pregnancy outcome (11, 17, 18). It has been proposed that this nuclear abnormality has possible mechanisms of multinucleation including karyokinesis in the absence of cytokinesis, partial fragmentation of nuclei, or defective migration of chromosomes during mitotic anaphase (19).

Incidence of embryo multinucleation is increased with oocyte immaturity (20). It was found that there is a significantly higher incidence of embryo multinucleation and multinucleated cells per embryo at day 2 in in vitro–matured MIIs compared with in in vivo–matured MIIs (20–22). Blastomere multinucleation may also be stimulated by suboptimal culture conditions (19). Van Blerkom et al. showed that 2-cell stage multinucleation has been related to follicular underoxygenation (23). In addition, blastomere multinucleation was correlated with a high number oocytes retrieved, a high level of E_2 concentration on the day of hCG administration, and a very short duration of hormonal stimulation (14). In our study, the mean number of MII oocytes was similar between cases with or without multinucleation. In addition, asymmetric embryos and fragmented embryos have more multinucleation (14, 24). Several studies have proposed that maternal age does not correlate with multinucleation (14, 25, 26), which is also confirmed in our study.

The type and count of multinucleation were also detected in several studies. Meriano et al. showed that transfer of binucleated embryos resulted in higher pregnancy rates than transfer of micronuclei embryos (15). In our study, we did not see any differences in clinical pregnancy rate according to multinucleation count or multinucleation size. Klingman et al. showed that the incidence of chromosomal abnormalities was the same among embryos with two or three or more nuclei (13).

We found that both implantation rates and biochemical pregnancies were lower in cases with multinucleation. This can be explained by the possible relationship between multinucleation and aneuploidy (10, 15, 24). Very recently, Fauque et al. showed that birth rates significantly decreased with day 2 multinucleated ET, despite no significant impact on the implantation rate (27). More importantly, they observed no births in women above 35 years old when multinucleated embryos were transferred. This lower postimplantation development of multinucleated embryos can be explained by a higher rate of chromosomal aneuploidy (10, 13).

Owing to the importance of multinucleation, its detection becomes a critical issue. ESHRE/ALPHA consensus released the new criteria for embryo evaluation, which include day 2 and day 3 blastomere multinucleation. Furthermore, the consensus

released specific embryo control times for routine clinical practice. In our study, we concluded that 72.3% of transferred embryos with multicleated blastomeres cannot be detected by the embryo control timing proposed by the ESHRE/ALPHA consensus. Hnidia et al. found a 26% difference in the evaluation of blastomere multinucleation between traditional method and computer-controlled noninvasive multilevel analysis. Moreover, this showed us that even when using a specific timing, traditional methods can give wrong results compared with time-lapse monitoring. Hnidia et al. also proved that the error was even bigger with embryos with a fragmentation rate above 10 (28). When time factor is added to this study, the error will increase even more. In addition, using a time-lapse monitoring system showed that the nuclei in multinuclear blastomeres disappear at different times (15).

In the recent dynamic embryo selection model published by Meseguer et al., 2-cell stage multinucleation is not included in the exclusion criteria, while the 4-cell stage multinucleation is used (29). Our experiment shows that 2-cell multinucleation should also be included in exclusion criteria.

Since nuclear formation is a dynamic process, it may be wrong to evaluate the nuclear status relying on observations performed within a short time interval. Time-lapse monitoring gives us the chance to observe the embryo continuously and enables us to detect any changes that occur in or out of prespecified intervals. Therefore, it is important to consider the dynamic nature of embryo development and to include the time-lapse monitoring system in the embryo evaluation protocols that may affect clinical outcomes.

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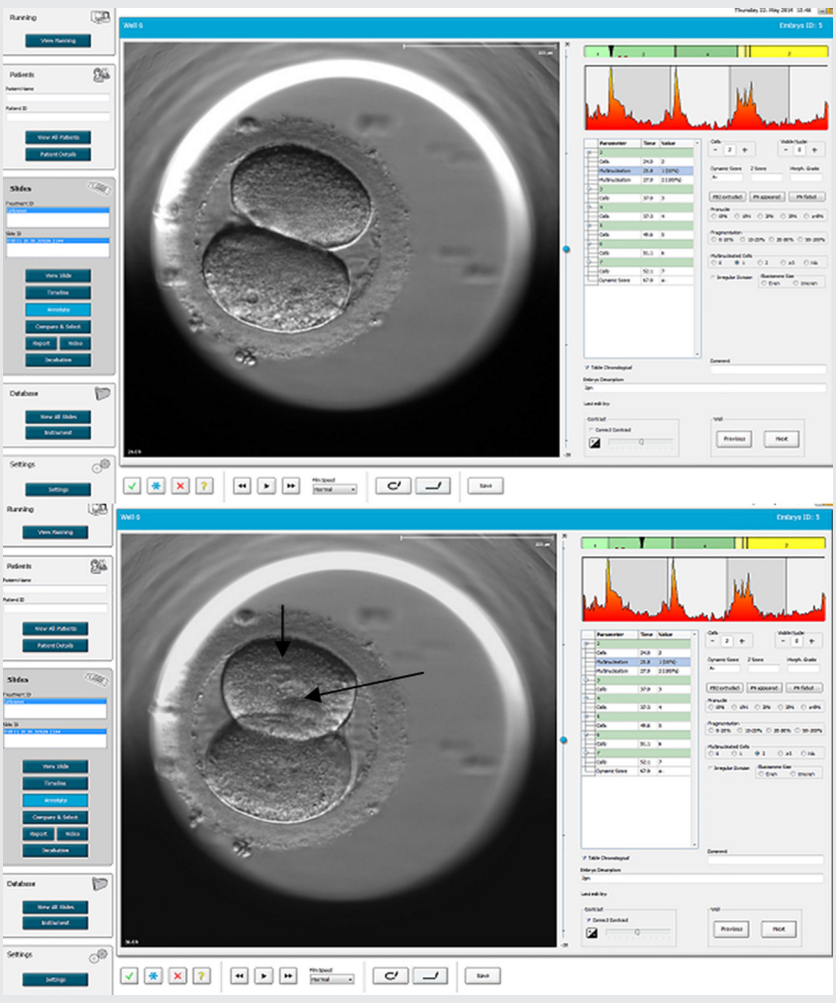
SUPPLEMENTAL FIGURE 1



The top panel represents a 2-cell stage embryo without multinucleation at 24 hours (within ESHRE/ALPHA consensus timings). The bottom panel shows the same embryo at 30.8 hours (outside EHSRE/ALPHA consensus timings) with multinucleation in one of the blastomeres.

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