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RESEARCH ARTICLE

# Study of Morphokinetics in Day 3 Embryo with Implantation Potential and Effect of Sperm Cryopreservation on Embryogenesis

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## ABSTRACT

**Aim:** In recent past, many studies had come up with the combination of time-lapse (TL) imaging of embryo morphokinetics as a noninvasive means for improving embryo selection and *in vitro* fertilization (IVF) success. The primary objective of the study was to find out if there is significant variation in morphokinetics of embryos with different implantation potential and also to study the effect of sperm freezing on time points of embryogenesis events in embryos with implantation potential.

**Materials and methods:** Kinetic data and cycle outcomes were analyzed retrospectively in 142 patients who had undergone IVF/intracytoplasmic sperm injection (ICSI) cycles using semen with normal parameters and embryo transfer (ET) on day 3. For the surety of specificity of morphokinetics, only cases with single ET cycles were included in the study. Timing of specific events, from the point of ICSI, was determined using TL imaging. Kinetic markers like time to syngamy (t-pnf), t2, time to two cells (c), 3c (t3), 4c (t4), 5c (t5), 8c (t8), tMor, CC2, CC3, t5–t2, t5–t4, s1, s2, and s3 were calculated. The cleavage synchronicity from the 2–8 cell stage (CS2–8), from 4 to 8 cell stage (CS4–8), and from 2 to 4 cell stage (CS2–4) were calculated as defined elsewhere. Deoxyribonucleic acid replication time ratio (DR) was also included in the comparison. Analysis of variance test was used for comparison of the mean timing of cell division and cell cycle intervals.

**Results:** Morphokinetics t-pnf, t2, t8, CC2, S2, S3, CS2–8, CS4–8, and CS2–4 differed significantly between embryos with and without implantation potential, when embryos were developed using fresh semen, while t3, t4, t5, CC2, S2, t5–t2, CS2–4, and DR differed significantly between the embryos with and without implantation potential when frozen semen was used. No significant difference was found in mean value of any of the above-stated parameters when comparison was done between implanted embryos fertilized by either fresh or cryopreserved sperm.

**Conclusion:** Many morphokinetics parameters of embryogenesis vary significantly between embryos with different ability to implant; therefore, the criteria developed in our IVF lab can be useful for selection of suitable embryo even at day 3 of development with more chances of implantation.

**Clinical significance:** Study indicates necessity of development of individualized selection model based on morphokinetics for every IVF lab and also confirms freezing as an important tool for fertility preservation of males as it does not affect events of embryogenesis.

**Keywords:** Day 3 single embryo transfer, Embryogenesis, Morphokinetics, Time-lapse monitoring.

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## INTRODUCTION

The main goal of IVF procedure is delivering a single healthy newborn. It is estimated that over five million children have been conceived *in vitro*. Embryo selection for transfer becomes a crucial decision when few numbers of oocytes are retrieved and day 3 transfer become mandatory to ensure liveliness of embryo while transferred. However, the traditionally used method of morphologically

evaluation of embryo is not predictive enough to allow routine single embryo transfer (ET).

The TL imaging/monitoring provides a noninvasive tool for embryo culture and provides extra information on the cleavage pattern, morphologic changes, and embryo development dynamics which can help in identification of embryos with higher implantation potential.

Furthermore, studies in past concluded that transfer of cleavage-stage embryos gives higher cumulative clinical pregnancy rate than blastocyst transfer.[1] Many IVF laboratories prefer to transfer cleavage stage embryo(s) due to suspected negative effect of prolonged culture. Also, it remains the only choice when low numbers of embryos are available to avoid cancellation of transfer.

Many studies recommended that each laboratory needs to first characterize optimal growth patterns for embryos within their own *in vitro* culture system used for TL technology as contradictory conclusions were reached in relationship between culture media and embryo kinetics.[2,3]

Furthermore, cryopreservation of sperm became routine procedure in IVF setup as prophylactic measure for fertility preservation due to higher age, before chemotherapy or radiotherapy. The other reasons could be preservation of sperm when husband's absence is expected at a time of IVF cycle or just as backup sample. However, the impact of use of cryopreserved sperm on events of embryogenesis is still an area of extensive research.

The present retrospective study was undertaken to fulfill following objectives in our own IVF setting: (1) To evaluate the morphokinetic differences between embryos with and without implantation potential which were transferred at cleavage stage in single ET cycle, (2) to evaluate effect of sperm cryopreservation on embryogenesis events.

## MATERIALS AND METHODS

This was a retrospective study of prospectively acquired data of TL imaging of human embryos during *in vitro* growth. This study was conducted in a private IVF clinic from August 2013 to April 2016. Total 142 single-ET cycles on day 3 with self-egg and without surrogacy were included in this study. Only those embryos which were showing normal conventional day 3 morphology with less than 5% fragmentation were selected for inclusion in this study. *In vitro* fertilization/ICSI cycles which were of couples with normal endometrium and uterus along with normal semen parameters were included in this study.

### Conditions for Embryo Culture and Incubation

Each of the 12 individual wells of the EmbryoSlide® culture disk was filled with 25 µL of a single-step culture medium (Continuous Single Culture; Irvine Scientific, California, USA) and covered with an overlay of 1.5 mL paraffin oil (Irvine Scientific, California, USA). Following ICSI, injected oocytes positioned in the wells of the slide were placed in a TL incubator (EmbryoScope™; Unisense Fertilitech, Aarhus, Denmark) and incubated at 6% $CO_2$, 5% $O_2$, and 37°C for 5 days until ET. The culture medium was refreshed on the afternoon of day 3 by replacing the incubated slide with a new preequilibrated slide prepared as described earlier. Image stacks were acquired at seven focal planes every 10 minutes and data were continuously transferred to an external computer (EmbryoViewer® workstation; Unisense Fertilitech, Aarhus, Denmark).

### Embryo Transfer and Confirmation of Implantation

All ET procedures were performed using a stipulated standardized technique under ultrasound guidance. Viable implantation was confirmed at 7 weeks of pregnancy by the detection of fetal heartbeat under ultrasound.

### Equations defining Time Ratios

All data were recorded retrospectively from the EmbryoViewer® workstation and exported for further analysis into Microsoft Excel and then to Statistical Package for the Social Sciences (SPSS, version 17). Spreadsheet analysis was performed for the cleavage timings from t2 to t8, six cleavage cycle intervals (t3–t2; t4–t3; t5–t4; t5–t3; t8–t5; t8–t2) and for four ratios derived from morphokinetic parameters: CS2–8, CS4–8, CS2–4, deoxyribonucleic acid replication time ratio (DR) as described elsewhere.[4] The time of all mitotic events was expressed as hours post-ICSI. Annotations of all embryos included in the present study were performed by one embryologist, to minimize the interobserver variations.

### Statistical Analysis

The statistical analysis was done using F test in analysis of variance (ANOVA). All statistical analysis was performed using SPSS version 17.0 and $p < 0.05$ was considered statistically significant.

## RESULTS

A total of 142 transferred embryos with known implantation data (KID) results were retrospectively annotated for different biological events of embryogenesis. The cleavage timings from t2 to t8, six cleavage cycle intervals (t3–t2; t4–t3; t5–t4; t5–t3; t8–t5; t8–t2) and for four ratios derived from morphokinetic parameters: CS2–8, CS4–8, CS2–4, DR were calculated for all the transferred embryos. Out of 142 transferred embryos, 60 were fertilized using fresh semen

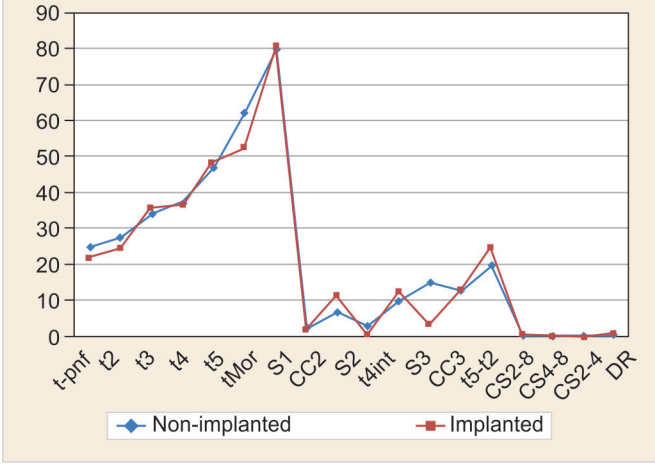
**Table 1:** Morphokinetic analysis of embryos with and without implantation potential developed by ICSI using fresh sperm

| *Morphokinetic parameters* | *Implantation* | *n* | *Mean* | *Standard deviation* | *95% confidence interval for mean* | | *ANOVA test sig. value* |
|---|---|---|---|---|---|---|---|
| | | | | | *Lower bound* | *Upper bound* | |
| t-pnf | Negative | 39 | 24.8923 | 2.57848 | 24.1745 | 25.6102 | 0.004 |
| | Positive | 21 | 22.1500 | 0.37417 | 21.8372 | 22.4628 | |
| t2 | Negative | 39 | 27.2462 | 2.49631 | 26.5512 | 27.9411 | 0.004 |
| | Positive | 21 | 24.5500 | 0.37417 | 24.2372 | 24.8628 | |
| t3 | Negative | 39 | 34.2462 | 4.52230 | 32.9871 | 35.5052 | 0.294 |
| | Positive | 21 | 35.9500 | 0.05345 | 35.9053 | 35.9947 | |
| t4 | Negative | 39 | 37.1538 | 3.38847 | 36.2105 | 38.0972 | 0.409 |
| | Positive | 21 | 36.1500 | 0.16036 | 36.0159 | 36.2841 | |
| t5 | Negative | 39 | 46.9769 | 6.62309 | 45.1330 | 48.8208 | 0.431 |
| | Positive | 21 | 48.8500 | 1.01559 | 48.0009 | 49.6991 | |
| t8 | Negative | 39 | 61.9923 | 7.55668 | 59.8885 | 64.0961 | 0.000 |
| | Positive | 21 | 52.0500 | 0.48107 | 51.6478 | 52.4522 | |
| S1 | Negative | 39 | 2.3538 | 0.28454 | 2.2746 | 2.4331 | 0.650 |
| | Positive | 21 | 2.4000 | 0.00000 | 2.4000 | 2.4000 | |
| CC2 | Negative | 39 | 7.0000 | 4.91049 | 5.6329 | 8.3671 | 0.015 |
| | Positive | 21 | 11.4000 | 0.32071 | 11.1319 | 11.6681 | |
| S2 | Negative | 39 | 2.9077 | 2.85340 | 2.1133 | 3.7021 | 0.010 |
| | Positive | 21 | 0.2000 | 0.10690 | 0.1106 | 0.2894 | |
| t4int | Negative | 39 | 9.8231 | 4.87252 | 8.4666 | 11.1796 | 0.103 |
| | Positive | 21 | 12.7000 | 0.85524 | 11.9850 | 13.4150 | |
| S3 | Negative | 39 | 15.0154 | 7.38008 | 12.9608 | 17.0700 | 0.000 |
| | Positive | 21 | 3.2000 | 1.49666 | 1.9488 | 4.4512 | |
| CC3 | Negative | 39 | 12.7308 | 4.32709 | 11.5261 | 13.9354 | 0.913 |
| | Positive | 21 | 12.9000 | 0.96214 | 12.0956 | 13.7044 | |
| t5–t2 | Negative | 39 | 19.7308 | 6.84694 | 17.8246 | 21.6370 | 0.066 |
| | Positive | 21 | 24.3000 | 0.64143 | 23.7638 | 24.8362 | |
| CS2–8 | Negative | 39 | 0.4860615 | 0.25169647 | 0.4159888 | 0.5561343 | 0.000 |
| | Positive | 21 | 0.8776353 | 0.04673119 | 0.8385670 | 0.9167035 | |
| CS4–8 | Negative | 39 | 0.5944409 | 0.24229190 | 0.5269864 | 0.6618954 | 0.000 |
| | Positive | 21 | 0.1982175 | 0.08613339 | 0.1262082 | 0.2702268 | |
| CS2–4 | Negative | 39 | 0.3663206 | 0.33593748 | 0.2727950 | 0.4598462 | 0.005 |
| | Positive | 21 | 0.0173952 | 0.00953653 | 0.0094224 | 0.0253679 | |
| DR | Negative | 39 | 0.8513071 | 1.34661266 | 0.4764077 | 1.2262065 | 0.937 |
| | Positive | 21 | 0.8896739 | 0.09121742 | 0.8134142 | 0.9659336 | |

and 82 were fertilized using frozen semen. Pregnancy rate using fresh sperm was 35% (21/60) and 39% (32/82) using cryopreserved sperm (p-value not significant).

Table 1 and Graph 1 depict the morphokinetics of embryos with and without implantation potential fertilized by ICSI using sperm from fresh semen. Statistically significant difference was found in t-pnf, t2, t8, S3, CS2–8, CS4–8, CS2–4, and DR between the embryos with and without implantation potential.

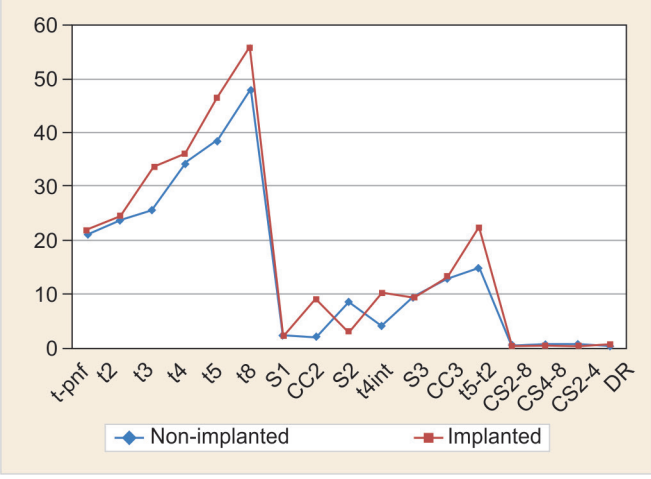
Table 2 and Graph 2 depict the morphokinetics of embryos with and without implantation potential fertilized by ICSI using cryopreserved sperm. Statistically significant difference was found in t3, t4, t5, CC2, S2, t5–t2, CS2–4, and DR between the embryos with and without implantation potential.




**Graph 1:** Morphokinetic differences between implanted and non-implanted embryos fertilized with fresh sperm

**Table 2:** Morphokinetic analysis of embryos with and without implantation potential developed by ICSI using frozen sperm

| *Morphokinetic parameters* | *Implantation* | *n* | *Mean* | *Standard deviation* | *95% confidence interval for mean* | | *ANOVA test sig. value* |
|---|---|---|---|---|---|---|---|
| | | | | | *Lower bound* | *Upper bound* | |
| t-pnf | Negative | 50 | 21.4000 | 0.00000 | 21.4000 | 21.4000 | 0.217 |
| | Positive | 32 | 22.0750 | 1.03376 | 21.5241 | 22.6259 | |
| t2 | Negative | 50 | 23.6000 | 0.00000 | 23.6000 | 23.6000 | 0.137 |
| | Positive | 32 | 24.3250 | 0.91324 | 23.8384 | 24.8116 | |
| t3 | Negative | 50 | 25.6000 | 0.00000 | 25.6000 | 25.6000 | 0.005 |
| | Positive | 32 | 33.5000 | 4.85359 | 30.9137 | 36.0863 | |
| t4 | Negative | 50 | 34.3000 | 0.00000 | 34.3000 | 34.3000 | 0.001 |
| | Positive | 32 | 36.3000 | 0.86101 | 35.8412 | 36.7588 | |
| t5 | Negative | 50 | 38.5000 | 0.00000 | 38.5000 | 38.5000 | 0.033 |
| | Positive | 32 | 46.6250 | 6.87890 | 42.9595 | 50.2905 | |
| t8 | Negative | 50 | 48.1000 | 0.00000 | 48.1000 | 48.1000 | 0.085 |
| | Positive | 32 | 56.0500 | 8.55928 | 51.4891 | 60.6109 | |
| S1 | Negative | 50 | 2.2000 | 0.00000 | 2.2000 | 2.2000 | .0776 |
| | Positive | 32 | 2.2500 | 0.33862 | 2.0696 | 2.4304 | |
| CC2 | Negative | 50 | 2.0000 | 0.00000 | 2.0000 | 2.0000 | 0.010 |
| | Positive | 32 | 9.1750 | 4.89483 | 6.5667 | 11.7833 | |
| S2 | Negative | 50 | 8.7000 | 0.00000 | 8.7000 | 8.7000 | 0.014 |
| | Positive | 32 | 2.8000 | 4.23698 | 0.5423 | 5.0577 | |
| t4int | Negative | 50 | 4.2000 | 0.00000 | 4.2000 | 4.2000 | 0.067 |
| | Positive | 32 | 10.3250 | 6.16111 | 7.0420 | 13.6080 | |
| S3 | Negative | 50 | 9.6000 | 0.00000 | 9.6000 | 9.6000 | 0.968 |
| | Positive | 32 | 9.4250 | 8.49671 | 4.8974 | 13.9526 | |
| CC3 | Negative | 50 | 12.9000 | 0.00000 | 12.9000 | 12.9000 | 0.857 |
| | Positive | 32 | 13.1250 | 2.40707 | 11.8424 | 14.4076 | |
| t5–t2 | Negative | 50 | 14.9000 | 0.00000 | 14.9000 | 14.9000 | 0.047 |
| | Positive | 32 | 22.3000 | 6.78862 | 18.6826 | 25.9174 | |
| CS2–8 | Negative | 50 | 0.2530612 | 0.00000000 | 0.2530612 | 0.2530612 | 0.077 |
| | Positive | 32 | 0.6077630 | 0.37097272 | 0.4100856 | 0.8054404 | |
| CS4–8 | Negative | 50 | 0.6956522 | 0.00000000 | 0.6956522 | 0.6956522 | 0.234 |
| | Positive | 32 | 0.4541628 | 0.38399722 | 0.2495451 | 0.6587804 | |
| CS2–4 | Negative | 50 | 0.8130841 | 0.00000000 | 0.8130841 | 0.8130841 | 0.012 |
| | Positive | 32 | 0.2535250 | 0.39065738 | 0.0453583 | 0.4616916 | |
| DR | Negative | 50 | 0.1550388 | 0.00000000 | 0.1550388 | 0.1550388 | 0.011 |
| | Positive | 32 | 0.6691235 | 0.35747739 | 0.4786373 | 0.8596098 | |




**Graph 2:** Morphokinetic differences between implanted and non-implanted embryos fertilized with cryopreserved sperm

Table 3 and Graph 3 demonstrate morphokinetic differences between implanted embryos fertilized by ICSI using fresh and cryopreserved sperm. We did not find statistically significant difference in any morphokinetics between implanted embryos irrespective of fertilization with fresh/frozen sperm.

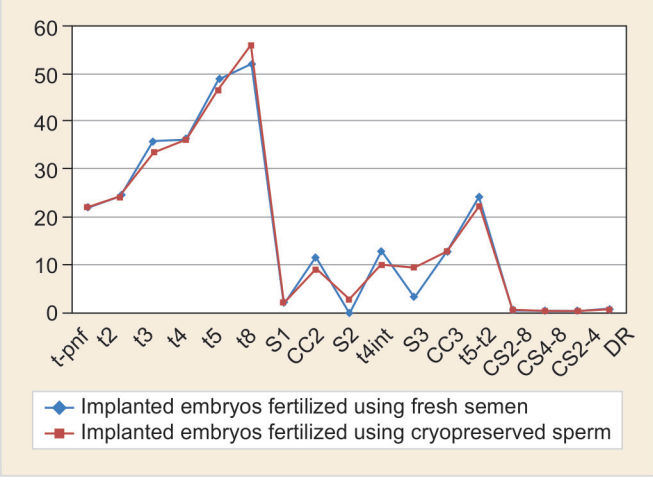
The results reveal that CC2, S2, and CS2–4 are the parameters which differ between the embryos with and without implantation potential irrespective of fertilization by fresh or cryopreserved sperm.

## DISCUSSION

Although relatively expensive, TL technology enables the collection of significantly increased volumes of data regarding embryo development without interrupting the

**Table 3:** Morphokinetic analysis of embryos developed from ICSI using fresh and frozen sperm

| *Morphokinetic parameters* | *Type* | *n* | *Mean* | *Standard deviation* | *95% confidence interval for mean* | | *ANOVA test sig. value* |
|---|---|---|---|---|---|---|---|
| | | | | | *Lower bound* | *Upper bound* | |
| tPNF | Fresh | 21 | 22.1500 | 0.37417 | 21.8372 | 22.4628 | 0.846 |
| | Frozen | 32 | 22.0750 | 1.03376 | 21.5241 | 22.6259 | |
| t2 | Fresh | 21 | 24.5500 | 0.37417 | 24.2372 | 24.8628 | 0.514 |
| | Frozen | 32 | 24.3250 | 0.91324 | 23.8384 | 24.8116 | |
| t3 | Fresh | 21 | 35.9500 | 0.05345 | 35.9053 | 35.9947 | 0.172 |
| | Frozen | 32 | 33.5000 | 4.85359 | 30.9137 | 36.0863 | |
| t4 | Fresh | 21 | 36.1500 | 0.16036 | 36.0159 | 36.2841 | 0.634 |
| | Frozen | 32 | 36.3000 | 0.86101 | 35.8412 | 36.7588 | |
| t5 | Fresh | 21 | 48.8500 | 1.01559 | 48.0009 | 49.6991 | 0.378 |
| | Frozen | 32 | 46.6250 | 6.87890 | 42.9595 | 50.2905 | |
| t8 | Fresh | 21 | 52.0500 | 0.48107 | 51.6478 | 52.4522 | 0.205 |
| | Frozen | 32 | 56.0500 | 8.55928 | 51.4891 | 60.6109 | |
| S1 | Fresh | 21 | 2.4000 | 0.00000 | 2.4000 | 2.4000 | 0.228 |
| | Frozen | 32 | 2.2500 | 0.33862 | 2.0696 | 2.4304 | |
| CC2 | Fresh | 21 | 11.4000 | 0.32071 | 11.1319 | 11.6681 | 0.217 |
| | Frozen | 32 | 9.1750 | 4.89483 | 6.5667 | 11.7833 | |
| S2 | Fresh | 21 | 0.2000 | 0.10690 | 0.1106 | 0.2894 | 0.100 |
| | Frozen | 32 | 2.8000 | 4.23698 | 0.5423 | 5.0577 | |
| t4int | Fresh | 21 | 12.7000 | 0.85524 | 11.9850 | 13.4150 | 0.295 |
| | Frozen | 32 | 10.3250 | 6.16111 | 7.0420 | 13.6080 | |
| S3 | Fresh | 21 | 3.2000 | 1.49666 | 1.9488 | 4.4512 | 0.054 |
| | Frozen | 32 | 9.4250 | 8.49671 | 4.8974 | 13.9526 | |
| CC3 | Fresh | 21 | 12.9000 | 0.96214 | 12.0956 | 13.7044 | 0.803 |
| | Frozen | 32 | 13.1250 | 2.40707 | 11.8424 | 14.4076 | |
| t5–t2 | Fresh | 21 | 24.3000 | 0.64143 | 23.7638 | 24.8362 | 0.420 |
| | Frozen | 32 | 22.3000 | 6.78862 | 18.6826 | 25.9174 | |
| CS2–8 | Fresh | 21 | 0.8776353 | 0.04673119 | 0.8385670 | 0.9167035 | 0.055. |
| | Frozen | 32 | 0.6077630 | 0.37097272 | 0.4100856 | 0.8054404 | |
| CS4–8 | Fresh | 21 | 0.1982175 | 0.08613339 | 0.1262082 | 0.2702268 | 0.079 |
| | Frozen | 32 | 0.4541628 | 0.38399722 | 0.2495451 | 0.6587804 | |
| CS2–4 | Fresh | 21 | 0.0173952 | 0.00953653 | 0.0094224 | 0.0253679 | 0.105 |
| | Frozen | 32 | 0.2535250 | 0.39065738 | 0.0453583 | 0.4616916 | |
| DR | Fresh | 21 | 0.8896739 | 0.09121742 | 0.8134142 | 0.9659336 | 0.103 |
| | Frozen | 32 | 0.6691235 | 0.35747739 | 0.4786373 | 0.8596098 | |




**Graph 3:** Morphokinetic analysis of implanted embryos developed from ICSI using fresh and frozen semen

culture conditions. Associations were reported to exist between embryo morphokinetic parameters and their subsequent implantation potential.[5,6]

Gardner et al[7] propose using a hierarchical predictive model to identify embryos with the highest developmental potential. This model is based on morphological screening, presence or absence of exclusion criteria, timing of cell division to the five-cell phase, synchrony of divisions from the two- to four-cell phases, and duration of the second cell cycle. However, more recent evidence has shown potential issues in the transferability of Gardner et al's[7] algorithm between different clinics,[8-11] possibly due to different embryo growth rates in diverse settings (i.e., oxygen concentration, culture media, or patient population) in different laboratories. Therefore, it

is highly recommended that each IVF laboratory develop its own cut-off values for quantitative parameters based on KID, although methodology maybe adopted from published studies.

The present study is in concordance with results of other studies stating that the morphokinetics vary significantly between embryos with and without implantation potential.[12,13]

In this study, statistically significant difference was found between t-pnf, t2, t8, S2, S3, CS2–8, CS4–8, CS2–4, and DR of embryos with and without implantation potential when fertilized with fresh semen. Also, when embryos which were fertilized with frozen semen were taken into account, statistically significant difference was found in t3, t4, t5, CC2, S2, t5–t2, CS2–4, and DR. The results are not in complete agreement with the study in which morphokinetics of t-pnf, t2, t3, t5, t8, S1, and t5–t2 were found to be different between statistically implanted and nonimplanted embryos,[14] which suggest that human embryo morphokinetics may vary between laboratories. Therefore, TL algorithms emphasizing quantitative timing parameters may have reduced interlaboratory transferability; qualitative measures are independent of cell division timings, with potentially improved interlaboratory reproducibility, which is in concordance with results by Liu et al.[11]

However, these results are in agreement with the study by Lemmen et al[15] who state that the timing and coordination of events during early embryo development (from zygote to cleavage stage) are connected with embryo quality and implantation rate.

It shall be noted that S2, CS2–4, and DR differ significantly in all implanted embryos irrespective of type of semen used for fertilization, which suggest that relative kinetic expressions defining cleavage synchronicity are better predictors of implantation than absolute time points.[4]

No difference was found in morphokinetics of implanted embryos irrespective of fertilization by fresh or frozen semen, which indicates that embryogenesis events do not get affected by origin of sperm used for ICSI. These results were found to be in concordance with the study by Eastick et al,[16] who found that there are no differences in the morphokinetic parameters of early embryo development when either fresh or frozen ejaculate sperm is used for ICSI insemination.

Though the study shows promising results to propose embryo selection model for single ET cycle, the small sample size of the present study requires large-scale prospective randomized controlled trials with validation of results by analysis of outcomes.

## CONCLUSION

In conclusion, time for specific events, of embryogenesis to occur, varies from other data available, which signify the necessity of each lab to establish their own criteria based on morphokinetics for selection of embryo with greater possibility of implantation. This observational study has shown that there are no differences in the morphokinetic parameters of early embryo development when either fresh or frozen ejaculate sperms were used for ICSI insemination, which confirms the usefulness of freezing for fertility preservation without any adverse impact on events of embryogenesis. Further studies could include embryo monitoring and annotation up to the blastocyst stage with higher sample size.

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