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Original Article

Effect of morphokinetics and morphological dynamics of cleavage stage on embryo developmental potential: A time-lapse study

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ABSTRACT

Objective: Using a non-invasive method to select the most competent embryo is essential in in vitro fertilization (IVF). Since the beginning of clinical application of time-lapse technology, several studies have proposed models using the time-lapse imaging system for predicting the IVF outcome. This study used both morphokinetic and morphological dynamic parameters to select embryos with the highest developmental potential.

Materials and Methods: A total of 23 intracytoplasmic sperm injection treatment cycles with 138 fertilized oocytes were included in this study. All embryos were cultured to the blastocyst stage, and embryo development was recorded every 10 min by using a time-lapse imaging system. Morphokinetic parameters and eight major abnormal division behaviors were studied to determine their effects on blastocyst formation. The most influential variables were used in hierarchical classification for blastocyst formation prediction.

Results: Several parameters were significantly related to the developmental potential. Embryos with the timing of pronuclear fading (tPNF) of >26.4 h post insemination (hpi), the timing of division to two cells (t2) of >29.1 hpi, and the timing of division to four cells (t4) of >41.3 hpi showed the lowest blastocyst formation rate. The abnormal division behaviors of fragmentation >50%, direct cleavage, reverse cleavage, and delayed division or developmental arrest were found to be detrimental to blastocyst formation. On the basis of these results, we propose a hierarchical model classification, in which embryos are classified into groups A-D according to their developmental potential. The blastocyst formation rates of groups A, B, C, and D were 80.0%, 77.8%, 53.7%, and 22.2% ($p < 0.001$). The good blastocyst rates of groups A, B, C, and D were 60.0%, 44.4%, 14.6%, and 11.1% ($p = 0.007$).

Conclusion: We propose a hierarchical classification system for blastocyst formation prediction, which provides information for embryo selection by using a time-lapse imaging system.

Introduction

Selecting the most competent embryo to achieve a singleton pregnancy is the ultimate goal of in vitro fertilization (IVF). Extended embryo culture with blastocyst transfer is considered a useful method in IVF for selecting embryos with a high implantation potential [1] and for reducing multiple pregnancies [2,3]. However, prolonged in vitro culture has been reported to potentially increase the risks of epigenetic disorders and preterm deliveries [4–6]. In patients with insufficient embryos, prolonged culture may be associated with the risk of no embryos remaining for transfer. A solution for the aforementioned conflicts is the identification and transfer of embryos with a high developmental potential in the cleavage stage.

Over the past three decades, embryologists have evaluated embryos' quality through conventional morphology assessment [7–10] at distinct time points. Since the first time-lapse microscopy system was approved for clinical use in June, 2009, more details of the cytokinetic process of embryo development have been revealed. Using the time-lapse microscopy system, morphokinetic and morphological dynamic parameters have been applied

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predicting blastocyst formation [11,12], implantation potential [13–15], and aneuploidy [16,17].

Compared with embryos that cleave late, those that cleave early are more developmentally competent [18,19]. Wong et al. (2010) first reported the feasibility of recording the cleavage timing by using a time-lapse imaging system for predicting the embryo developmental potential [11]. Thereafter, numerous studies have used morphokinetic parameters to predict the IVF outcome [13,16,17]. However, these results have several limitations and should be evaluated with caution. First, the morphokinetic parameters were reported to be affected by several factors, such as stimulation protocols [20,21], patient population [22], culture condition [23,24], and sperm DNA fragmentation [25]. Second, some time-lapse equipment, such as the Embryoscope (Vitrolife), can only set the same starting time point for all cultured embryos, which made the sperm entry time in each individual embryo imprecise. Because of the aforementioned limitations, the results from each laboratory varied. Third, the clinical effectiveness and cost effectiveness of improving overall outcomes by using morphokinetic parameters for predicting IVF outcome remain controversial [26–28].

To reduce the variation and improve the transferability of the time-lapse algorithm, some researchers have used division patterns, rather than division timings, as the time-lapse parameters [12]. Yang et al. (2015) demonstrated that some specific cleavage patterns adversely affect the embryo developmental potential [12]. Moreover, some studies have found that direct cleavage (DC) and reverse cleavage (RC) exert deleterious effects on the implantation potential [15,29]. Recently, instead of the insemination timing, the timing of pronuclear fading (tPNF) was proposed as a reference starting time point to eliminate the error associated with variation in sperm entry time and settings of different time-lapse equipment [30].

This study proposes a concise and feasible time-lapse model for predicting the embryo developmental potential by using both morphokinetic and morphological dynamic parameters of the early cleavage stage. Moreover, the relationship between each studied parameter and the developmental potential was analyzed.

Materials and methods

Patient selection and patient management

This retrospective cohort study was performed at Changhua Christian Hospital from September 2014 to April 2016. A total of 23 intracytoplasmic sperm injection (ICSI) treatment cycles with 138 fertilized oocytes were included in this study. The mean age (±SD) of women (including oocyte donors) in the treatment cycles was 34.2 ± 4.2 years. Embryos with cleavage-stage biopsy or assisted hatching conducted at the cleavage stage were excluded. This retrospective study was approved by the Institutional Review Board of Changhua Christian Hospital. All patients provided informed consent for data collection.

Ovarian stimulation was performed using a standardized gonadotropin-releasing hormone (GnRH) antagonist (0.25 mg of ganirelix, Merck Sharp and Dohme, or 0.5 mg of cetrorelix, Merck Serono) protocol or GnRH agonist (leuprorelin, Takeda) protocol. Different dosages of recombinant follicle-stimulating hormone (follitropin-α, Merck Serono, or follitropin-β, Merck Sharp and Dohme) and human menopausal gonadotropins (Ferring) were administered according to the patients' body weight, ovarian reserve, and previous ovarian response. Human chorionic gonadotropin at 5000 IU or 6500 IU (Merck Sharp and Dohme or Merck Serono) was administered when at least two leading follicles had reached a mean diameter of ≥18 mm. Transvaginal oocyte retrieval was scheduled 34–36 h after triggering of oocyte maturation.

Oocyte retrieval and ICSI

Oocyte cumulus complexes were washed and cultured in Quinn's Advantage Fertilization Medium (QAFM; SAGE, Trumbull, CT, USA) at 37 °C under 5.5% CO_2 and 5.0% O_2 before denudation. Oocyte denudation was performed at approximately 2 h after retrieval through pipetting in bicarbonate and N-hydroxyethylpiperazine-N-ethanesulfonate-buffered medium (ASP; Vitrolife, Vastra Frolunda, Sweden) with hyaluronidase solution (Sigma–Aldrich, St. Louis, MO, USA). The conventional ICSI procedure was performed in QAFM under a × 200 magnification microscope.

Embryo culture and time-lapse recording

Following ICSI, oocytes were individually placed into microwells (LifeGlobal, Guilford, CT, USA) and were cultured in Quinn's Advantage Sequential Medium (SAGE). All embryos were cultured to the blastocyst stage (Day 5 or 6 post insemination) in a time-lapse incubator (CCM-IVF; ASTEC, Fukuoka, Japan), with the culture conditions of 37 °C, 5.5% CO_2, 5.0% O_2, and balanced N_2. The culture medium was changed on Day 3 (at least 70 h post insemination [hpi]). Images of each embryo were obtained every 10 min. The precise timing of completing each ICSI procedure was recorded individually by a technician and was regarded as the starting time of each embryo set in the time-lapse system.

Regarding conventional morphology assessment, the eight-cell stage was verified during the period of 68 ± 1 hpi by using the Veeck grading system [8]. The blastocyst stage was verified during the period of 116 ± 2 hpi by using the Gardner grading system [9]. Good embryos were defined as grade I to II embryos with 6–10 blastomeres. Good blastocysts were defined those with trophectoderm and inner cell mass both rated higher than grade B.

Table 1
Definitions of variables used in analysis.

Variables	Definition
Morphokinetic parameters	
tPNF	The timing of both pronuclei had faded
t2	The timing of division to 2-cell stage (Complete the 1st cleavage)
t3	The timing of division to 3-cell stage
t4	The timing of division to 4-cell stage (Complete the 2nd cleavage)
T2_PNF (t2-tPNF)	Duration of the period as 1-cell
T3_PNF (t3-tPNF)	Duration of the period from pronuclear fading to 3-cell stage
T4_PNF (t4-tPNF)	Duration of the period from pronuclear fading to 4-cell stage
cc2 (t3-t2)	The time of second cell cycle (Duration of the period as 2-cell)
s2 (t4-t3)	The time of synchrony of second cell cycle (Duration of the period as 3-cell)
Morphology dynamic parameters	
Fragmentation > 50%	Over 50% scattered fragments after division
Fragmentation 10–50%	10–50% scattered fragments after division
Direct cleavage	Direct cleavage from one cell to three or more blastomeres
Reverse cleavage	Blastomeres fusion after division
Uneven blastomeres	The largests blastomere being over 20% larger than the smallest blastomere
Big fragmentation	Big fragment develops after division
Delayed division or developmental arrest	The blastomere with division delayed compare with others or did not enter next cell cycle while other blastomere kept going on
Distorted cytoplasm movement	A series of distorted cytoplasm movements during cell division

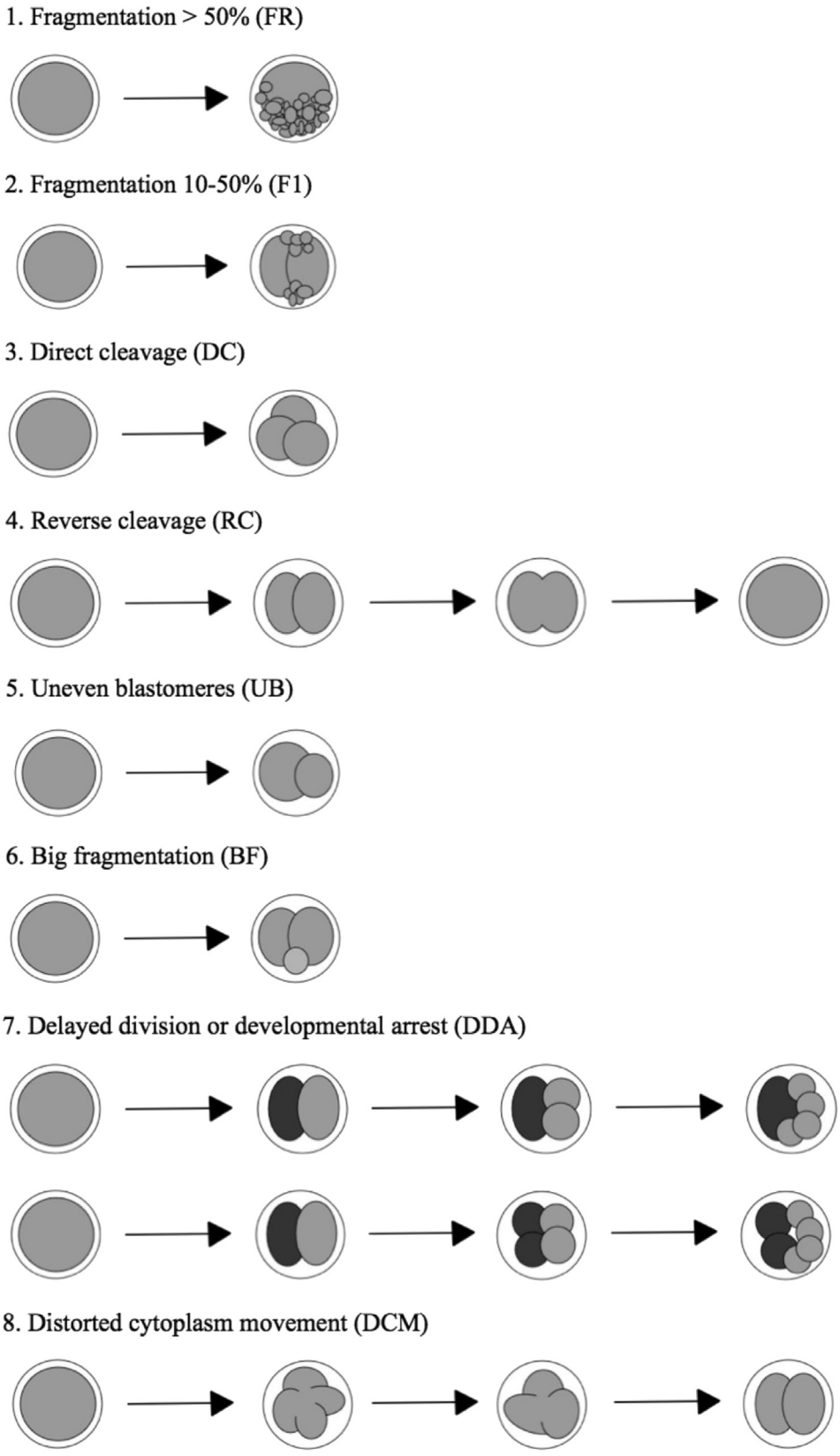


Fig. 1. Abnormal division behavior in the initial three cleavages for assessing embryonic development through time-lapse imaging system.

Table 2
Effect of cleavage patterns on embryonic development.

Variables		Total	Blastocyst				p-value
			No		Yes		
			N	(%)	N	(%)	
	Total	138	65	(47.1)	73	(52.9)	
FR							
1st FR	No	134	61	(45.5)	73	(54.5)	0.047
	Yes	4	4	(100.0)	0	(0.0)	
2nd FR	No	130	59	(45.4)	71	(54.6)	0.148
	Yes	8	6	(75.0)	2	(25.0)	
3rd FR	No	121	53	(43.8)	68	(56.2)	0.038
	Yes	17	12	(70.6)	5	(29.4)	
DC							
1st DC	No	110	46	(41.8)	64	(58.2)	0.014
	Yes	28	19	(67.9)	9	(32.1)	
2nd DC	No	115	51	(44.4)	64	(55.7)	0.147
	Yes	23	14	(60.9)	9	(39.1)	
3rd DC	No	136	64	(47.1)	72	(52.9)	1.000
	Yes	2	1	(50.0)	1	(50.0)	
RC							
1st RC	No	130	60	(46.2)	70	(53.9)	0.475
	Yes	8	5	(62.5)	3	(37.5)	
2nd RC	No	133	62	(46.6)	71	(53.4)	0.666
	Yes	5	3	(60.0)	2	(40.0)	
3rd RC	No	113	48	(42.5)	65	(57.5)	0.021
	Yes	25	17	(68.0)	8	(32.0)	
DDA							
1st DDA	No	138	65	(47.1)	73	(52.9)	
	Yes	0					
2nd DDA	No	126	58	(46.0)	68	(54.0)	0.415
	Yes	12	7	(58.3)	5	(41.7)	
3rd DDA	No	92	33	(35.9)	59	(64.1)	<0.001
	Yes	46	32	(69.6)	14	(30.4)	

FR = fragmentation>50%; DC = direct cleavage; RC = reverse cleavage; DDA = delayed cleavage or developmental arrest.
p-values were calculated using the Chi-square test or Fisher's exact test.

Table 3
Multiple regression analysis of morphological dynamic parameters adversely effecting developmental potential.

Variables		Blastocyst (D5)		Multiple analysis		
		n/N	(%)	OR	95% CI	p-value
3rd FR	No	68/121	(56.2)			
	Yes	5/17	(29.4)	0.27	0.07–0.97	0.044
3rd RC	No	65/113	(57.5)			
	Yes	42607	(32.0)	0.3	0.10–0.93	0.038
3rd DDA	No	59/92	(64.1)			
	Yes	14/46	(30.4)	0.27	0.10–0.70	0.008

FR = fragmentation>50%; RC = reverse cleavage; DDA = delayed cleavage or developmental arrest; OR = odds ratio; CI = confidence interval.

The annotations of each variable recorded by the time-lapse system in this study are defined in Table 1. The morphological dynamic parameters focused on eight abnormal division behaviors, adapted from previous studies [12,15,29], in the initial three cleavages. Both morphological dynamic and morphokinetic parameters were recorded for analysis.

Statistical analysis

The investigated embryos were divided into two groups depending on whether they reached the blastocyst stage. The studied parameters were compared to determine the differences between the two groups.

The morphokinetic parameters were divided into four quartiles of categorical variables, as previously described [13]. The blastocyst formation rate was compared between each quartile of the morphokinetic parameters.

Continuous variables were analyzed using Student's *t*-test, and categorical variables were analyzed using the Chi-square test or Fisher's exact test. Multiple regression analysis was used to adjust the interaction effect of the confounding factors. All statistical analyses were performed using SAS software version 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

Effect of abnormal division behaviors on embryo developmental potential

The morphological dynamic parameters focused on eight abnormal division behaviors in the initial three cleavages (Table 1 and Fig. 1).

Embryos with fragmentation >50% (FR), DC, RC, and delayed division or developmental arrest (DDA) had a significantly lower developmental potential compared with other embryos (Table 2 and Supplementary Table 1). The Chi-square test revealed that the cleavage patterns of first and third FR, first DC, third RC, and third DDA significantly adversely affected blastocyst formation ($p < 0.05$). After adjustment for the interaction effect by using logistic regression, the finding was still significant (Table 3). Other FR, DC, RC, and DDA parameters also exerted adverse effects, although not statistically significant.

The prevalence of embryos with FR, DC, RC, and DDA among different age groups is shown in Table 4. No significant difference was observed between women aged ≥35 years and those aged <34 years.

Effect of morphokinetic parameters on embryo developmental potential

The morphokinetic parameters used in this study are shown in Table 1 and Fig. 2. The four quartiles of each investigated parameter and the blastocyst formation rate are presented in Table 5 and Supplementary Table 2. The blastocyst formation rates were significantly lower in the fourth quartile of tPNF, t2 and t4 than in the first three quartiles. The blastocyst formation rate was significantly higher in the third quartile of t3 than in the other quartiles.

The mean t3 was significantly different between embryos with and those without DC (35.4 h versus 38.5 h, $p = 0.026$), whereas no difference was observed in the mean t2 and t4 between the two groups (Table 6).

Table 4
Comparisons of the incidence of abnormal division behaviors among the age groups.

Age	Total	FR		p-value	DC		p-value	RC		p-value	DDA		p-value
		No	Yes		No	Yes		No	Yes		No	Yes	
		n (%)	n (%)		n (%)	n (%)		n (%)	n (%)		n (%)	n (%)	
<35 years	66	57 (86.4)	9 (13.6)	0.966	42 (63.6)	24 (36.4)	0.584	52 (78.8)	14 (21.2)	0.371	43 (65.2)	23 (34.9)	0.877
≥35 years	72	62 (86.1)	10 (13.9)		49 (68.1)	23 (31.9)		52 (72.2)	20 (27.8)		46 (63.9)	26 (36.1)	

FR = fragmentation>50%; DC = direct cleavage; RC = reverse cleavage; DDA = delayed cleavage or developmental arrest.
p-values were calculated using the Chi-square test or Fisher's exact test.

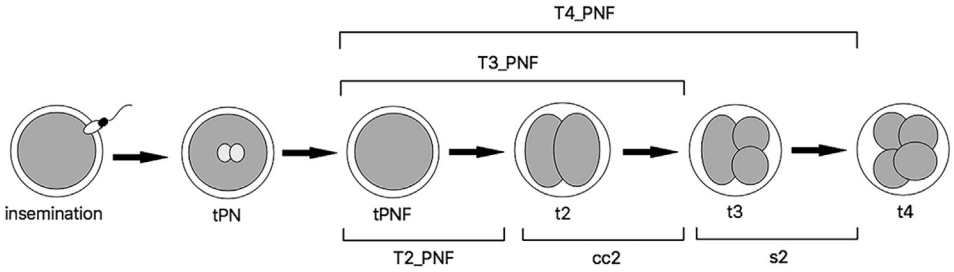


Fig. 2. Graphic description of morphokinetic variables used in analysis. tPN = the timing of pronuclei appearance; tPNF = the timing of both pronuclei had faded; t2 = the timing of division to 2-cell stage; t3 = the timing of division to 3-cell stage; t4 = the timing of division to 4-cell stage; cc2 = t3-t2, the time of second cell cycle; s2 = t4-t3, the time of synchrony of second cell cycle; T2_PNF = t2-tPNF, duration of the period as 1-cell; T3_PNF = t3-tPNF, duration of the period from pronuclear fading to 3-cell stage; T4_PNF = duration of the period from pronuclear fading to 4-cell stage.

Table 5
Each morphokinetic parameter according to quartile from 138 fertilized embryos.

Parameter	Q1		Q2		Q3		Q4	
	Limit (hpi)	Blastocyst (%)	Limit (hpi)	Blastocyst (%)	Limit (hpi)	Blastocyst (%)	Limit (hpi)	Blastocyst (%)
tPNF	≤21.8	54.3[a]	21.8–23.9	67.7[a]	23.9–26.4	65.7[a]	>26.4	23.5[a]
t2 (Complete the 1st cleavage)	≤24.8	55.6[a]	24.8–26.8	67.9[a]	26.8–29.1	68.8[a]	>29.1	31.3[a]
t3	≤33.7	55.6[a]	33.7–36.7	43.3[a]	36.7–39.6	80.7[a]	>39.6	38.7[a]
t4 (Complete the 2nd cleavage)	≤35.2	62.5[a]	35.2–38.3	58.1[a]	38.3–41.3	65.6[a]	>41.3	32.3[a]

hpi = hours post insemination; tPNF = the timing of both pronuclei had faded; t2 = the timing of division to 2-cell stage; t3 = the timing of division to 3-cell stage; t4 = the timing of division to 4-cell stage.
[a] Value with different superscripts within each row indicates statistical significance ($p < 0.05$).

Table 6
Comparisons of the means of morphokinetic parameters between embryos with and those without DC.

Variables	Embryos with DC			Embryos without DC			*p*-value
	n/N	mean (hours)	(sd)	n/N	Mean (hours)	(sd)	
t2	37/128	28.8	(5.6)	91/128	27.4	(5.3)	0.185
t3	39/124	35.4	(7.7)	85/124	38.5	(6.0)	0.026
t4	37/126	40.6	(10.2)	89/126	39.7	(6.3)	0.640

DC = direct cleavage; t2 = the timing of division to 2-cell stage; t3 = the timing of division to 3-cell stage; t4 = the timing of division to 4-cell stage.
p-values were calculated using Student's *t*-test.

Thus, we defined the fourth quartile of tPNF (>26.4 hpi), t2 (>29.1 hpi), and t4 (>41.3 hpi) as out of range, and the first three quartiles as within range for inclusion in the hierarchical predictive model.

The comparison of morphokinetic events among the age groups is shown in Table 7. The prevalence of embryos with favorable morphokinetic event (tPNF, t2, and t4 all within range) and those with detrimental morphokinetic event (any of tPNF, t2, and t4 out of range) are not significant difference between women aged ≥35 years and those aged <34 years.

Table 7
Comparison of the difference of morphokinetic events among the age groups.

Variables	Total N = 117	Age		*p*-value
		<35 years n (%)	≥35 years n (%)	
tPNF/t2/t4 all within range	81	36 (44.4)	45 (55.6)	0.096
Any of tPNF/t2/t4 out of ranges	36	22 (61.1)	14 (38.9)	

tPNF = the timing of both pronuclei had faded; t2 = the timing of division to 2-cell stage; t4 = the timing of division to 4-cell stage.
p-value was calculated using the Chi-square test.

Hierarchical predictive model for embryo assessment

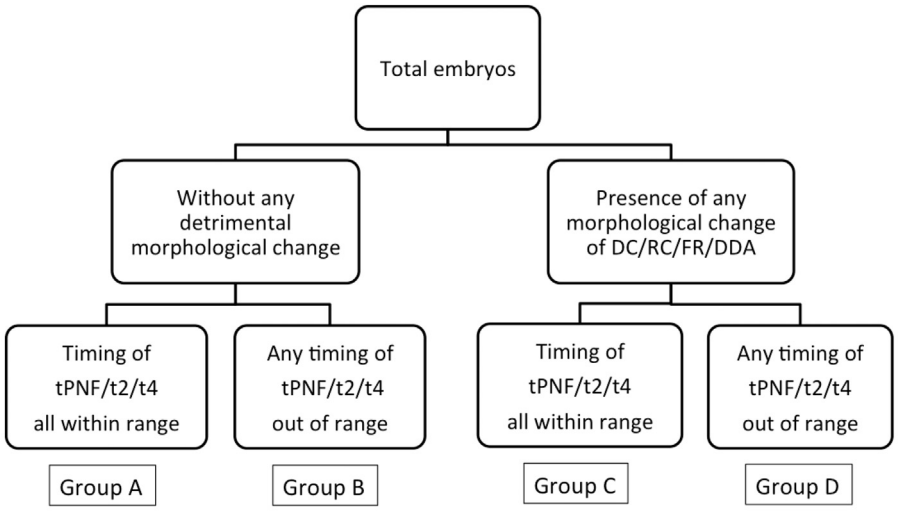
According to previous observational studies of cleavage patterns and morphokinetic assessment for determining the embryo developmental potential, a hierarchical model was developed for blastocyst formation prediction (Fig. 3). Our results showed morphological changes of FR, DC, RC, and DDA had more detrimental effect on blastocyst formation than morphokinetic parameters of tPNF, t2, and t4 (Odds ratio [OR] 0.18, 95% confidence interval [CI] 0.08–0.42, $p < 0.001$ versus OR 0.28, 95% CI 0.12–0.64, $p = 0.002$) (Supplementary Table 3). Thus, the embryos were categorized by the presence of detrimental morphological changes (FR, DC, RC, and DDA) in the first level. Subsequently, the embryos were grouped by tPNF, t2, and t4 all within range or any of tPNF, t2, and t4 out of range in the second level. According to this hierarchical model, all embryos were assigned to groups A-D. The blastocyst formation rates of groups A, B, C, and D were 80.0%, 77.8%, 53.7%, and 22.2% ($p < 0.001$). The good blastocyst rates of groups A, B, C, and D were 60.0%, 44.4%, 14.6%, and 11.1% ($p = 0.007$).

Discussion

This study proposes a concise and feasible time-lapse model for predicting the embryo developmental potential. All predictive markers were analyzed to assess their effect on blastocyst formation. The most influential markers were used for hierarchical classification.

Our data demonstrated that the fourth quartiles of tPNF, t2, and t4 were associated with the lowest blastocyst formation rate. This result was consistent with previous studies [18,19] that embryos with slower cleavage, compared with those with faster cleavage, have been suggested to exhibit more developmental potential.

Embryos with DC morphology, which was determined to be detrimental to blastocyst formation in our study, reached the three-cell stage earlier during cytokinesis. Our study found a significant difference in the mean t3 in embryos with or without DC



%	Group A	Group B	Group C	Group D	*p*-value
Blastocyst formation rate	80.0 (32/40)	77.8 (7/9)	53.7 (22/41)	22.2 (6/27)	<0.001
Good blastocyst rate	60.0 (24/40)	44.4 (4/9)	14.6 (6/41)	11.1 (3/27)	0.007

Fig. 3. Hierarchical predictive model for embryo assessment. Hierarchical predictive model for embryo assessment based on: (1) presence or absence of detrimental morphological changes of fragmentation >50% (FR), direct cleavage (DC), reverse cleavage (RC), and delayed cleavage or developmental arrest (DDA); (2) the timing of both pronuclei had faded (tPNF), the timing of division to 2-cell stage (t2), and the timing of division to 4-cell stage (t4). Good blastocyst = blastocyst with trophectoderm and inner cell mass both rated higher than grade B. *p*-value was test for trend.

morphology, whereas no difference was observed in the mean t2 and t4. The aforementioned results suggested that the parameter of t3 may be influenced by the incidence of DC in the selected group. Considering this point, we did not include t3 or any other parameter calculated using t3 (such as cc2, s2, and, T3_PNF) as a reference in the hierarchical model, though the third quartile of t3 showed significantly higher blastocyst formation rate than in the other quartiles.

When tPNF was used as a reference starting time point, as described by Liu et al. [30], t2_PNF showed a similar blastocyst formation rate in the four quartiles, and t4_PNF showed only marginally significant differences in the blastocyst formation rate between the third and fourth quartiles in our study (Supplementary Table 2). This statistically non-significant result may be due to embryos that reached the two- or four-cell stage late also exhibit delayed pronuclear fading.

Yang et al. [12] first classified the observed cleavage patterns into eight categories and proposed a hierarchical model according to the developmental potential of each cleavage pattern. Our study showed that embryos with DC, FR, and DDA morphological changes had a low developmental potential, consistent with the results of Yang et al. [12]. In addition, our study assessed one analytic parameter, RC, that was not evaluated by Yang et al. [12]. Our results showed that RC had detrimental effects on blastocyst formation, consistent with the results of a previous study [29].

Cellular fragmentation is a crucial determinant in conventional morphology assessment. A high degree of fragmentation leads to the loss of cytoplasmic organelles, such as mitochondria [31], and further induces necrotic effects in the surrounding blastomeres [32], which causing development arrest and low embryo developmental potential. The cause of DC or RC remains unknown. DC is believed to be related to the formation of multipolar spindles, which cause the abnormal segregation of chromosomes during cleavage [33]. Moreover, embryos with DC have a higher likelihood of an abnormal ploidy status or chromosome number abnormalities [34]. One study reported that RC is associated with euploidy by using array comparative genomic hybridization (array-CGH) [35]. However, RC entails the refusion of two already cleaved blastomeres, which presumably provides complete replication of all chromosomes; array-CGH may not be able to detect the abnormalities.

In this study, neither the occurrence of detrimental morphological changes (DC, RC, FR, and DDA) nor the prevalence of detrimental morphokinetic event (any of tPNF, t2, and t4 out of rang) was associated with the women's age. Several studies have discussed the factors affecting morphokinetic parameters [20–25]. However, few studies have discussed the factors affecting morphological dynamic parameters. Liu et al. [29] showed that RC is not associated with the woman's age and demonstrated that RC is more frequently observed in GnRH antagonist protocols than in GnRH agonist protocols.

The current study has some limitations. First, it has a small sample size. Second, the hierarchical model is designed for blastocyst formation prediction; whether the study results are transferable to the implantation potential requires further investigation. Third, changing the medium disturbs the continuous capture of images for a few minutes, which might prevent the third RC morphological change, if any, from being recorded; particularly the incidence of RC was the highest after completion of the third cleavage in this study.

Our results provide alternative information for embryo selection by using the time-lapse imaging system, which will be beneficial for achieving selective single embryo transfer. Further prospective investigation is required to evaluate whether the model can achieve a higher live birth rate compares to the conventional morphology assessment.

Conflicts of interest statement

The authors have no conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.tjog.2017.12.013.

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