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TIME-LAPSE EMBRYO IMAGING TECHNOLOGY AND MORPHOKINETIC PROFILING: A TOOL FOR BLASTOCYST FORMATION PREDICTION

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Resum

En els darrers anys, ha sorgit la necessitat d'una eina precisa de selecció no invasiva d'embrions, a causa de l'elevada prevalença d'embarassos múltiples, com a resultat de la baixa competència de la selecció embrionària basada en l'avaluació morfològica. D'aquesta manera, s'ha fet un gran esforç per trobar marcadors morfo-cinètics universals relacionats amb els resultats de la FIV (formació de blastocistes, implantació, taxa de nadons vius) per tal millorar la pràctica diària de l'embriòleg. No obstant això fins ara ni un algoritme universal ni cap paràmetre morfo-cinètic s'han aplicat universalment a la rutina diària de la clínica. En aquest projecte es va realitzar una anàlisi retrospectiva de 181 embrions procedents de cicles de donant per avaluar si algun paràmetre de desenvolupament embrionari primerenc està associat a la formació de blastocists. Es van observar diferències morfo-cinètiques estadísticament significatives entre els embrions que es van desenvolupar en etapes de blastocist d 'aquells que no van poder arribar a aquesta etapa. En efecte, després de transformar les variables morfo-cinètiques en les seves distribucions logarítmiques, a més de definir categories en cadascuna d'elles, es van trobar 23 variables que eren rellevants. Mitjançant procediments de regressió logística, es proposen tres models, definits fins a l'etapa de 2 cèl·lules, l'etapa de 4 cèl·lules i l'etapa de 8 cèl·lules del desenvolupament de l'embrió humà com a eines convincents en el monitoratge embrionari mitjançant un sistema de lapse de temps relacionat amb els cicles de donants amb un percentatge d'èxit de 72,7%, 79,8% i 84,3%, respectivament.

Paraules clau: sistemes de time-lapse, desenvolupament embrionari, selecció d'embrions, morfo-cinètica.

Abstract

In the past few years, a need for an accurate non-invasive embryo selection tool has emerged due to the still high prevalence of multiple pregnancies, as a result of the low competence embryo selection based on morphological assessment. In this way, a great effort has been made to find an universal morphokinetic markers related to IVF outcomes (blastocyst formation, implantation, live birth rate) in order to enhance embryologist's daily practice. Nevertheless, neither a universal algorithm nor a morphokinetic parameter has been universally applied in daily clinic routine. In this project, a retrospective analysis of 181 embryos from donor cycles was performed so as to assess whether any parameter of early embryo development is associated with blastocyst formation. Statistically significant morphokinetic differences were observed between embryos that developed into blastocyst stage from those that failed to reach that stage. In effect, after transforming morphokinetic variables into their logarithmic distributions, as well as defining categories in each of them, 23 variables were eventually found to be relevant. By conducting logistic regression procedures, three models, defined till 2-cell stage, 4-cell stage and 8-cell stage of human embryo development are proposed as compelling tools in embryo monitoring by time-lapse system concerning donor cycles with a success percentage of 72.7%, 79.8% and 84.3%, respectively.

Keywords: time-lapse systems, embryo development, embryo selection, morphokinetics.

INTRODUCTION

The birth of a healthy newborn is the maximum success of an in vitro fertilization (IVF) treatment; and this fact has been described as depending on the patient endometrial status as well as on the accurate embryo development to the same extent. Along these lines, a great scientific research has been done to improve them in order to increase implantation success, decrease miscarriage rates and improving the detection of abnormal embryos, always considering single embryo transfer as the widely recognized standard in IVF practices. Thus, the optimization of non-invasive embryo selection methodologies is one of the main needs in current IVF laboratories.

The development of time-lapse technology in human embryo incubators for daily clinical practice has introduced us to a new era of embryo evaluation, known as morphokinetics (Meseguer et al. 2011)which may further improve accurate selection of viable embryos. The objective of this retrospective study was to identify the morphokinetic parameters specific to embryos that were capable of implanting. In order to compare a large number of embryos, with minimal variation in culture conditions, we have used an automatic embryo monitoring system. METHODS Using a tri-gas IVF incubator with a built-in camera designed to automatically acquire images at defined time points, we have simultaneously monitored up to 72 individual embryos without removing the embryos from the controlled environment. Images were acquired every 15 min in five different focal planes for at least 64 h for each embryo. We have monitored the development of transferred embryos from 285 couples undergoing their first ICSI cycle. The total number of transferred embryos was 522, of which 247 either failed to implant or fully implanted, with full implantation meaning that all transferred embryos in a treatment implanted. RESULTS A detailed retrospective analysis of cleavage times, blastomere size and multinucleation was made for the 247 transferred embryos with either failed or full implantation. We found that several parameters were significantly correlated with subsequent implantation (e.g. time of first and subsequent cleavages as well as the time between cleavages; where not only the need to disrupt the culture conditions so as to assess the embryo stage in its development has disappeared, but also new events in embryo development have come into sight, which were totally missed with conventional embryo evaluation, such as direct cleavage, internalization of fragments, blastocyst collapse, among others, which, in turn, have been seen to impact on embryo's viability (Prados et al. 2012).

This dynamic embryo monitoring has generated a vast amount of information about human early development, having been used by many researchers as a potent embryo selection tool through the development of algorithms as a possible predictors of the most important IVF outcomes. While some ambitious ones have been focused on the prediction of implantation, others have chosen blastocyst formation or aneuploidy prediction (Pribenszky, Nilselid, and Montag 2017). However, none of them has been yet widely accepted nor consolidated for clinical practice, as embryo selection algorithms have been subject to considerable controversy owing to the great variety of confusion factors being described in the literature for affecting morphokinetic embryo parameters between IVF clinics, such as culture conditions (Kaser and Racowsky 2014), inter and intraobserver variability (Kovacs 2014), patients features, etc, to the extent that these algorithm value has been discussed in recent publications to their unproven prospective clinical benefits (Gallego, Global, and Meseguer 2019; Racowsky, Kovacs, and Martins 2015). As a consequence, none of them has been yet widely accepted nor consolidated for clinical practice.

Hence, the main aim of this project is to assess whether morphokinetics are predictive enough of viable human blastocyst formation in the time-lapse system incubator EmbryoScope™ to give evidence for the application of morphokinetics as a non-invasive method in regard to embryo selection. Provided that significant differences are found, the involved parameters will be used to perform a first in-house model, which predicts the probability that an embryo, at early stages of development, evolves into a viable blastocyst, far-sighted to develop a more compounded predictive algorithm.

MATERIAL AND METHODS

Patient Management and inclusion criteria

A retrospective selection of the cases from April 2014 to December 2017 was performed under strict inclusion criteria so as to avoid cofounding variables. Firstly, women undergoing oocyte donation were selected according to their age, hormonal stimulation protocol and stage of embryo transfer, to be less or 35 years old, pituitary down-regulation achieved by using either a GnRH agonist or antagonist (excluding leuprorelin acetate) ovarian FSH stimulation, ICSI as a fertilization technique and prolonged embryo culture (Day 5). The exclusion criteria included women of more than 35 years old, LH activity products, IVF standard fertilization technique, oocyte vitrification, PGD, TESE (testicular sperm extraction), embryo culture less than Day 5, disruptions in time-lapse due to biologist's handling.

In fact, it has been seen how these timings of development are affected by maternal age, interobserver variability and clinical features (Gallego, Global, and Meseguer 2019) for that reason, we focused on donor cycles as a first step of in-house embryo selection algorithm development, highlighting that the proper development of them is a step by step pathway so as to accurately progress in the morphokinetic field, as well as the importance of examine concrete groups of patients, as the predictive variables for them may differ between age and pathology groups.

A total of 245 embryos at blastocyst stage were first included in the project. However, 64 were excluded for having cuts in the video, IVF standard fertilization or lack of proper visibility, among others. At the end, a total of 181 embryos were retrospectively annotated, classified into 93 as non-viable embryos and 88 as viable.

Oocyte retrieval and embryology

Ultrasound-guided oocyte collection was performed transvaginally under sedation. Examination of the follicular fluid was performed so as to seek for oocyte-cumulus complexes. After their collection, the complexes were cultured in well dishes, with each well containing 25µL culture medium covered with 0,45mL paraffin oil. In the matter of sperm preparation, it was performed using a specific gradient separation followed by swim- up technique. The oocytes destined for ICSI were denudated using enzymatic digestion with hyaluronidase enzyme (Hyase 10X, Vitrolife, Sweden) and mechanical digestion by pipetting. This technique was performed maximum at 2 hours after collection. ICSI was performed at X400 magnification using an Olympus microscope. Subsequently, oocytes were placed in individual culture drops and incubated in the time-lapse incubator EmbryoScope™ at 37ºC, 7% CO_2 and 7% O_2.

Viable embryos were those which met the morphological criteria to be vitrified or transferred to the woman's uterus, while non-viable ones were those cultured till Day 5 but did not meet those criteria or arrested in development.

Time-lapse embryo imaging system

Low intensity red light (635nm) from a single light-emitting diode (LED) with short illumination times of 30 ms per image is used in EmbryoScope™ so as to diminish embryo light exposure and to avoid short wavelength light damage. Furthermore, the image interval on the EmbryoScope™ was set to 10 minutes with seven focal planes. Images were collected immediately after ICSI.

Table 1. Summary of mean and standard deviation (SD) values of single morphokinetic variables between viable (V) and nonviable embryos (NV). Results are expressed mean ± SD in *hpi (hours post insemination).*

	NV	V
tPB2	2.701 ± 1.399	2.401 ± 0.747
tPNa	9.209 ± 3.704	7.719 ± 1.957
tPNf	23.875 ± 6.379	22.096 ± 3.252
t2	27.944 ± 8.918	24.667 ± 3.364
t3	37.542 ± 10.032	35.938 ± 4.449
t4	40.371 ± 12.611	37.130 ± 4.903
t5	48.343 ± 11.797	48.475 ± 6.732
t6	52.692 ± 13.170	50.373 ± 6.615
t7	54.596 ± 11.801	52.431 ± 7.962
t8	60.608 ± 15.347	55.373 ± 9.785

Time-lapse evaluation of morphokinetic parameters

A single observer checked retrospectively the images for the required morphokinetic parameters in order to eliminate the interobserver variability. Additionally t0 was defined as the mid-time point from when the injection begins and ends for one patient's pool of oocytes. The morphokinetic variables annotated at EmbryoViewer™ included time of second polar body extrusion (tPB2), time of pronuclei appearance (tPNa), number of pronuclei (PN), time to pronuclear fading (tPNf), time to *n* cell divisions (t_n), time of starting compaction (tSC), time of morula formation (tM), time of start blastulation (tSB), time of full blastocyst (tB), time to expanding blastocyst (tEB) and time of hatching blastocyst (tHB). However, only variables included until 8-cell stage (t8) were finally taken into account. From these annotations, different parameters were calculated, concretely, all the possible interval between tPB2 to t8 (t8-tPB2, t8-tPNa, t8-tPNf, t8-t2...).

Moreover, abnormal events were also registered directly, such as direct cleavage. Regarding time of cleavage, it was defined as the first acquired image where the newly formed blastomeres are entirely separated by confluent cell membranes. Specifically, the annotated parameters were made in hours after ICSI microinjection. Finally, the data was exported by the incubator's software, Embryoviewer™.

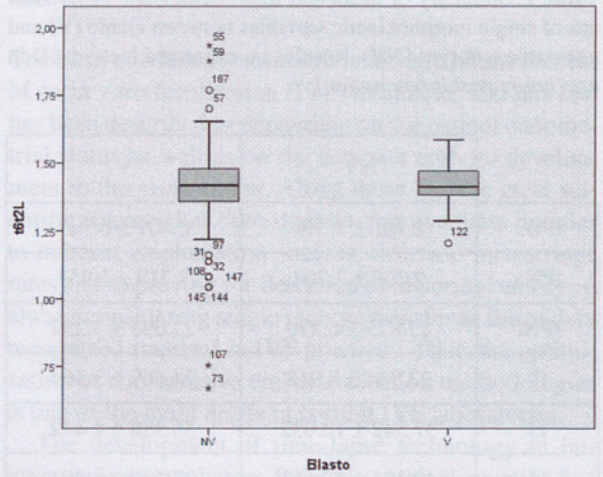

Figure 1. Example of a morphokinetic variable distribution between viable (V) and nonviable (NV) blastocysts where data dispersion can be observed.

Statistical Analysis

First of all, for each morphokinetic variable, descriptive statistics and graphical analyses were calculated in order to compare the sample distribution of viable and nonviable embryos groups. Due to the apparent asymmetry of all variables, a log-transformation was applied to achieve more symmetrical distributions.

To assess the actual distinctness between the variability of the two groups, a Levene's test for equality of variances was first performed. According to the result obtained in Levene's test, and in order to test the two population means, a classic t-test or corrected t-test for unequal variances (Welch two sample t-test) was done. Moreover, the Mann- Whitney U test was used to assess the median. Owing to the distribution of the data, it was decided to codify categories for each morphokinetic variable, using the quartiles as the cut off points (Meseguer et al. 2011)which may further improve accurate selection of viable embryos. The objective of this retrospective study was to identify the morphokinetic parameters specific to embryos that were capable of implanting. In order to compare a large number of embryos, with minimal variation in culture conditions, we have used an automatic embryo monitoring system. METHODS Using a tri-gas IVF incubator with a built-in camera designed to automatically acquire images at defined time points, we have simultaneously monitored up to 72 individual embryos without removing the embryos from the controlled environment. Images were acquired every 15 min in five different focal planes for at least 64 h for each embryo. We have monitored the development of transferred embryos from 285 couples undergoing their first ICSI cycle. The total number of transferred embryos was 522, of which 247 either failed to implant or fully implanted, with full implantation meaning that all transferred embryos in a treatment implanted. RESULTS A detailed retrospective analysis of cleavage times, blastomere size and multinucleation was made for the 247 transferred embryos with either failed or full implantation. We found that several parameters were significantly correlated with subsequent implantation (e.g. time of first and subsequent cleavages as well as the time between cleavages. Of these categorical variables, a Chi-square test was performed. Additionally, binary variables were calculated in order to assess whether an embryo undergoes every of the analyzed stages, labelled as indicators (e.g the indicator of t2 stage is It2).

Besides, all the significant and marginally significant morphokinetic parameters found in variable categorization were organized in time and divided in three stages (stage 1 until t2, stage 2 until t4 and stage 3 until t8) so as to determine the contributing strength of them, potentially associated with blastocyst formation (Figure 2). Subsequently, logistic regression procedures were used to examine if the significant variables were also powerful enough to predict human blastocyst formation for each of the defined stages. Then, predicted probabilities for each embryo were calculated for the three models, and a final embryo viability categorization was determined in each of them establishing 5 categories (A, B, C, D and E) according to the embryo's probability to develop into a viable blastocyst. The significance level was established as α=0.05, so a p-value less than 0.05 was considered statistically significant. Besides, p-values between 0.05 and 0.1 were taken into account as "marginally significant". All statistical analyses were performed with SPSS version 23.0.

Table 2. Embryo viability categorization for model 3. A final embryo viability categorization was determined in each model) according to the embryo's probability to develop into a viable blastocyst. Note: nonviable (NV), viable (V), model 1 (M1), model 2 (M2), model 3 (M3).

	Blastocyst				
	NV		V		Total
Embryo viability M3	n	%	n	%	
A	6	10.5%	51	89.5%	48
B	8	28.6%	20	71.4%	33
C	4	44.4%	5	55.6%	13
D	12	60.0%	8	40.0%	30
E	52	98.1%	1	1.9%	49
Total	82	49.1%	85	50.9%	173

RESULTS AND DISCUSSION

Descriptive analysis of morphokinetic variables

Table 1 shows a brief descriptive of outcomes obtained in single variable analysis between viable (88 embryos) and nonviable embryos (93 embryos). When comparing the two studied groups after the logarithmic transformation, some variables were found to differ from both mean and standard deviation (SD), while others just present dissimilarities in mean or SD suggesting the complexity of the present data Indeed, most distributions of the distinct morphokinetic parameters (and the pairwise differences variables) from viable blastocysts are characterized by significantly smaller variances than the distributions of variables from the nonviable group (Figure 1).

Altogether, these results demonstrated the existence of morphokinetic differences during development between embryos that manage to reach the blastocyst stage from those that fail to reach it, in precise studied group as donor cycles are, and the potential of morphokinetics as a complementary tool of embryo selection.

Morphokinetic variable categorization

A specific categorization was adopted by each significant morphokinetic variable according to its distinct cut-off points. From them, the percentage of viable and nonviable blastocyst per category was estimated, demonstrating how embryos that failed to reached the blastocyst stage widely laid in extreme categories (go faster or slower), while timing of those which reached the blastocyst stage were comprised in central categories. Moreover, among the 3 stages established, 25 morphokinetic variables were eventually found statistically significant (Figure 2). Additionally, 19 variables were identified as marginally statistically significant and therefore included in the subsequent analyses.

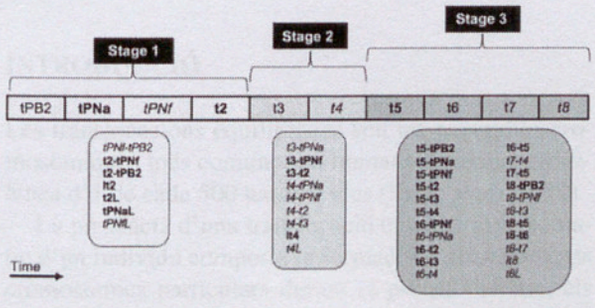

Figure 2. Representation of annotation modelling. Three divisions are done in the whole set of morphokinetic variables comprised in embryo development, from tPB2 to t8. Variables are categorical unless an "L" is found. Parameters in bold are found to be statistically significant ($p<0.05$) and those shown in italics are marginally significant ($0.05 \leq p<0.1$).

Blastocyst formation prediction of the proposed model

Logistic regression models have been fitted through heuristic procedures including the 44 morphokinetic variables for embryo development, as represented in Figure 2, leading to a great variety of predictive models with distinct correct classification percentages. The number of morphokinetic variables along with their success percentage in classification were prioritized to perform model selection.

Concerning the variables involved in stage 1, model 1 included 5 variables: tPNa, t2-tPB2, tPNfC, t2L, tPNfL, resulting in a 72.7% of correct classification. Turning to *stage 2,* the model included a group of 3 more variables as the most powerful until 4-cell embryo: t3-tPNf, t4, t4-t2 and some interactions between them, reaching a 79.8% of prediction success. In regard to the model until *stage 3,* additionally parameters were involved, t5-t4, t6-t2, t6-t3, t7-t4, leading to a success prediction of 84.3%. Furthermore, predicted probabilities of embryo viability were estimated for each of them, considering an embryo as viable if its probability was greater than 0.5 and nonviable if it is below.

Once predicted probabilities in each model were calculated, categories were established in each model regarding embryo's probability to become a viable blastocyst, defining 5 categories: high probability to be viable (A), moderate probability to be viable (B), indeterminate (C), high probability to be nonviable (D), moderate probability to be nonviable (E) (Table 2), obtaining appropriate percentages of correct classification, especially in extreme categories.

In fact, as the model progress (M1, M2, till model 3), the indetermination represented as "'C" category decreases.

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