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ORIGINAL ARTICLE *Embryology*

Time-lapse parameters as predictors of blastocyst development and pregnancy outcome in embryos from good prognosis patients: a prospective cohort study

K. Kirkegaard[1,2,*], U.S. Kesmodel[1,2], J.J. Hindkjær[1], and H.J. Ingerslev[1,2]

[1]Centre for Preimplantation Genetic Diagnosis, The Fertility Clinic, Aarhus University Hospital, Skejby, Brendstrupgaardsvej 100, Aarhus N 8200, Denmark [2]Health, Aarhus University, Vennelyst Boulevard 9, Aarhus C DK-8000, Denmark

*Correspondence address. E-mail: kirstine.kirkegaard@ki.au.dk

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STUDY QUESTION: Do early time-lapse parameters predict which embryos will develop to high-quality blastocysts and does timing of development differ between embryos that implant and those that do not.

SUMMARY ANSWER: Development to high-quality blastocysts could be predicted within the first 48 h of culture, whereas time-lapse parameters could not predict pregnancy.

WHAT IS KNOWN ALREADY: Historical cohort studies on embryos from unselected groups of patients have suggested several putative kinetic markers of viability. Before well-designed randomized studies can be conducted, relevant selection models based on solid data must be developed. So far conclusions from the previous studies are ambiguous.

STUDY DESIGN, SIZE, DURATION: A prospective cohort study conducted from February 2011 to June 2012. A total of 571 ICSI embryos from 92 patients were included in the blastocyst development analysis and 84 single embryo transfers were included in the pregnancy outcome analysis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Embryos from women aged <38 years, with no endometriosis and ≥ 8 oocytes retrieved. University affiliated clinic. Embryos were cultured in a time-lapse incubator till Day 6. Logistic regression analysis was performed with variables selected based on indication.

MAIN RESULTS AND THE ROLE OF CHANCE: Duration of the first cytokinesis, duration of the 3-cell stage and direct cleavage to 3-cells predicted development to high-quality blastocyst. We found no difference in timing between implanted and non-implanted embryos.

LIMITATIONS, REASONS FOR CAUTION: A larger study might detect differences in timing between implanted and non-implanted embryos. The cohort consisted of good prognosis patients only and may not be representative of the entire IVF population.

WIDER IMPLICATIONS OF THE FINDINGS: Our results in context with the lack of consistency in previous studies and the presumed influences of different external factors indicate that a universal algorithm for optimal timing of development might not be feasible. The apparent negative significance of division patterns that differ from the expected may imply that time-lapse will facilitate de-selection of embryos.

STUDY FUNDING/COMPETING INTEREST(S): Funding for the present study was provided by Aarhus University, the Lippert Foundation, the Toyota Foundation, the Aase og Einar Danielsen foundation and by an unrestricted grant from MSD and Ferring. The authors declare no conflict of interest.

TRIAL REGISTRATION NUMBER: The study was registered at ClinicalTrial.gov with accession number NCT01139268.

Key words: assisted reproduction / human / time-lapse / blastocyst development / pregnancy

Introduction

Elective single embryo transfer is increasingly promoted in clinical practice as an efficient strategy of avoiding multiple pregnancies, which makes accurate prediction of embryo quality and pregnancy potential a daily challenge. Presently, embryos are selected for transfer using grading systems based on morphology (Steer *et al.*, 1992; Pickering *et al.*, 1995; Ziebe *et al.*, 1997; Hardarson *et al.*, 2001; Scott *et al.*, 2007; Racowsky *et al.*, 2010). Morphological assessment has several limitations. Improved methods for selecting the embryo with the best reproductive potential are expected to yield a significant improvement of the relatively low pregnancy rates following ART. Time-lapse monitoring enables a detailed evaluation of morphology, including dynamic parameters, and can therefore be considered a refinement of the present assessment (Montag *et al.*, 2011). The development of clinical time-lapse incubators has enabled safe, continuous monitoring of human embryos cultured for treatment purposes (Cruz *et al.*, 2011; Kirkegaard *et al.*, 2012a,b,c) and, based on cohort data, time-lapse evaluation and incubation are proposed to improve pregnancy outcome (Meseguer *et al.*, 2012).

Historical cohort studies on embryos from unselected groups of patients have suggested several putative kinetic markers of viability (Lemmen *et al.*, 2008; Wong *et al.*, 2010; Meseguer *et al.*, 2011; Cruz *et al.*, 2012; Dal Canto *et al.*, 2012; Hashimoto *et al.*, 2012). Although such data inarguably provide important information that may guide the direction of further research, recent experiences with other methods of embryo assessment, such as near infrared spectroscopy and preimplantation genetic diagnosis with aneuploidy screening, have shown that promising results are not always translated into improved pregnancy rates when tested in randomized trials (Staessen *et al.*, 2004; Mastenbroek *et al.*, 2007; Hardarson *et al.*, 2008; Schoolcraft *et al.*, 2009; Debrock *et al.*, 2010; Hardarson *et al.*, 2012; Vergouw *et al.*, 2012). Before well-designed randomized studies can be conducted, relevant selection models based on solid data must be developed. So far conclusions from the previous studies are ambiguous.

The aim of this study was therefore firstly to test proposed time-lapse parameters during the first 48 h of culture that could serve as predictors of development to high-quality blastocyst in good prognosis patients and to test whether the thereby identified parameters could predict pregnancy. The second aim was to analyse whether timing during the entire culture period differed between embryos destined to implant and to fail. The prospectively recruited study cohort represented a subgroup of infertile patients, who were expected to produce several embryos of high quality and would therefore likely benefit from improved embryo selection.

Methods

Study design and participants

Embryos from infertile patients were recruited as a prospective cohort at the Fertility Clinic, Aarhus University Hospital, between February 2011 and July 2012. Patients were asked for participation if the woman was aged <38 years and had no diagnosis of endometriosis. Their embryos were included if written informed consent was achieved and ≥ 8 oocytes were retrieved. Eligible patients could contribute to the study with one treatment cycle only. Data related to patient characteristics were obtained for the current treatment cycle. In total, 161 IVF and ICSI patients were recruited. The present paper reports the outcome for the 92 infertile patients with ICSI fertilized embryos cultured exclusively under 5% O_2.

Ethical approval

Written informed consent was obtained from all participants before inclusion. Patients consented to blastocyst culture (Day 6), time-lapse imaging, analysis of the spent culture media and blastocyst biopsy. The Central Denmark Region Committees on Biomedical Research Ethics and the Danish Data Protection Agency approved the study. The study was registered at ClinicalTrial.gov with accession number NCT01139268.

IVF, embryo culture and embryo assessment

Ovarian stimulation and oocyte retrieval were performed according to standard procedures as previously described (Kirkegaard *et al.*, 2012a,b,c). Following retrieval, oocytes were fertilized using conventional ICSI procedures and immediately after injection placed in individual wells (EmbryoSlide, Unisense Fertilitech, Aarhus, Denmark) in a tri-gas time-lapse incubator (EmbryoScope, Unisense Fertilitech, Aarhus, Denmark) (Kirkegaard *et al.*, 2012a,b,c) under oil at 37°C, 5% O_2 and 6% CO_2 in sequential culture medium (Sydney IVF Fertilization/Cleavage/Blastocyst Medium, COOK®, Sydney, Australia). Media change was performed in the morning of Days 3 and 5. A trophectoderm (TE) biopsy was obtained following laser opening of zona pellucida from the embryo that was intended for transfer, if the procedure was logistically feasible. Both zona opening and biopsy were performed on Day 5. The biopsy was obtained for research purposes only. Fourteen ($n = 14$) transferred embryos were biopsied.

Categorization of embryo quality on Days 2 and 3 was based on the number of blastomeres, fragmentation and multi-nucleation evaluated at 44 and 68 h, respectively. A good-quality embryo (GQE) was defined on Day 2 (44 h) as an embryo with four blastomeres, no multi-nucleation and $<20\%$ fragmentation. On Day 3 (68 h) a GQE had seven or eight blastomeres, no multi-nucleation and $<20\%$ fragmentation. Blastocysts were graded (on Day 6) according to the Gardner criteria; in brief based on the expansion of the blastocoel cavity (1–6), number and cohesiveness of the inner cell mass (ICM) and TE (A-C) (Gardner *et al.*, 2004). In the morning of Day 6 after oocyte retrieval, a single embryo was selected for transfer, based on the morphological evaluation. Day 6 culture and transfer was motivated by the requirement for regeneration of the biopsied embryo before transfer (Kokkali *et al.*, 2005). Embryo assessment was performed without removing the embryos from the EmbryoScope. Final decision on which embryo to transfer was made after morphological evaluation in an inverted microscope at $\times 200$ magnification.

In the present study, time-lapse recordings and kinetic parameters were not used for embryo assessment or selection.

Outcome assessment

The end-points of the study were development to high-quality blastocysts on Day 6 and clinical pregnancy. One person only assigned the blastocyst scores. For the purpose of this study, blastocysts were accordingly grouped to low (1–3 irrespective of ICM or TE score and 4–6 BB, BC, CB and CC) or high quality (4–6, AA, AB or BA). Biochemical pregnancy rate was confirmed by serum β-hCG measurement 16 days after oocyte retrieval. Clinical pregnancy rate was registered as the number of ongoing pregnancies per embryo transfer, based on the presence of fetal heart activity visualized by ultrasound 8 weeks after embryo transfer.

Time-lapse monitoring and annotation

Images were recorded automatically every 20 min in seven planes (15 μm intervals, 1280 × 1024 pixels, 3 pixels per μm, monochrome, 8-bit <0.5 s per image, using single 1 W red LED). Embryos with two pronuclei

(PN) completing the first cleavage were annotated manually according to definitions previously described (Kirkegaard *et al.*, 2012a,b,c). The time point for the first image recorded of the following events was recorded and analyzed for the embryos in focus (in order of appearance): appearance of first PN, syngamy/abuttal of the two PN, PN breakdown, first cytokinesis, first division, appearance of nuclei after first division, multi-nucleation at the two cell stage, division to 3-, 4-, 5-, 6-, 7- and 8 cells, morula, early-, full- and hatching blastocyst. If evaluation of specific events was not possible due to unfocused imaging, oil drops or technical problems such as no recording, these data points were treated as missing data. Duration (hours) of events such as first cytokinesis, cellular stages and cleavage divisions was calculated. Embryos in which one or more of the blastomeres showed no further cleavage were categorized according to the number of cell cycles observed in the healthy blastomeres (i.e. an embryo with only six blastomeres, where two blastomeres had arrested development, would be defined as having completed the third cleavage cycle. Time points refer to the exact time where an image was recorded and are reported as hours after fertilization or hours of duration, where appropriate.

Statistical analysis

All statistical analyses were performed in the statistical package STATA for Mac, version 11.0 (StataCorp, USA). Two-sided *P*-values <0.05 were considered significant.

Blastocyst development

Blastocyst development was analyzed as a binary outcome (high quality or low quality/arrested development) and odds ratios were obtained with the use of logistic regression. Each of the variables was selected before the analysis based on indications from prior research. We included only events expected to occur within the first 48 h of development. The time-lapse variables identified in the literature as potential predictors of development were time points of PN breakdown, duration of the first cytokinesis, division to 2-, 3- and 4-cell embryos along with the duration of the 2- and 3- cell stage (all continuous variables) and direct cleavage to three cells ($<5/\geq 5$ h) and multi-nucleation (MN/no MN) at the two cell stage as dichotomous variables (Lemmen *et al.*, 2008; Wong *et al.*, 2010; Cruz *et al.*, 2012; Dal Canto *et al.*, 2012; Hashimoto *et al.*, 2012). To evaluate the strength of the prediction, we added known and potential confounders such as age, number of previous cycle, infertility cause, number of GQEs Day 2/3 and BMI. Multi-collinearity was tested with scatter plots and variance inflation factor (VIF) test. Logistic regression was performed on data from embryos that developed beyond the 4-cell stage and where data on all six parameters were available. Data were treated as dependent in the model clustered by patients. To test the parameters ability to predict pregnancy, odds ratios for pregnancy outcome were obtained with logistic regression of the parameters predicting high-quality blastocyst development in combination with known and potential confounders as independent variables.

Clinical pregnancy, overall timing

Timing of events in the pregnant and non-pregnant groups was analyzed as two independent samples from a normal distribution and the hypothesis of no difference tested with Student's *t*-test. The estimates are reported as means with 95% CI and medians with 95% CI if transformation to natural logarithms was necessary for achieving normal distributed data. Assumption of normality was checked with histograms and QQ plots. Data that were not normally distributed were tested with the Wilcoxon rank-sum test and estimates reported as medians and range. For categorical data Fisher's exact test was used to test the hypothesis of no difference between the two groups.

Results

In total, 1191 oocytes were retrieved from 92 patients. Baseline data for these 92 patients are listed in Table I. According to the protocol, we excluded 271 immature oocytes and 349 oocytes that were not fertilized normally ($PN \neq 2$) or did not complete the first division, which left 571 embryos from 92 patients for the blastocyst development analysis (Fig. 1). Patient and cycle characteristics for the pregnant and the non-pregnant groups are listed in Table II.

Blastocyst developmental potential

One-hundred and forty ($n = 140$) embryos developed into high-quality blastocysts. The distribution of the classification is listed in Supplementary data, Table SI. Test for collinearity showed low tolerance (VIF >10) of PN breakdown, duration of the 2-cell stage, duration of the 3-cell stage and division to 2-, 3- and 4 cells. Calculations of the durations of the 2- and 3- cell stages are based on division to 2-, 3- and 4 cells, and the time from PN breakdown till first division was practically constant and thus a measure of the same parameter. We excluded time points of division to 2-, 3- and 4 cells, since registration of duration of events, rather than absolute time points overcomes the limitation of imprecise starting points, which make the parameters useful not only for ICSI embryos, but for IVF embryos as well. Duration of the first cytokinesis, duration of the 3-cell stage and direct cleavage to 3 cells predicted development to high-quality blastocyst (Table III). The three time-lapse parameters possessed comparable predictive value to cumulative FSH dose, number of GQEs on Days 2 and 3, but better predictive value than the remaining potential confounders tested (Table IV). Figure 2 displays the ROC curve for the three time-lapse variables combined. Area under the curve (AUC) was higher for the three variables combined than for the individual parameters (Table V). Using age, other potential confounders and the predictors of development to high-quality blastocyst as independent variables in a logistic regression analysis, only age predicted pregnancy outcome (Table VI). Direct cleavage to 3 cells (duration of the 2-cell stage <5 h) could not be included in this model, but Fisher's exact test revealed no difference between the pregnancy and non-pregnancy groups ($P = 0.30$).

Table I Baseline and cycle characteristics for the blastocyst development analysis.

Number of patients	92
Number of cycles	92
Number of previous cycles/patient	1 (0; 3)
Maternal age (years)	30 (20; 37)
Maternal BMI (kg/m^2)	22.4 (18.0; 36.4)
Cumulative FSH dose (IU)	1575 (675; 4125)
Oocytes retrieved/cycle	12 (8; 34)
Number of GQE on Day 2/cycle	2 (0; 8)
Number of GQE on Day 3/cycle	2 (0; 8)
Number of cycles with embryo transfer	84
Embryos cryopreserved/cycle	2 (0; 8)

Continuous data are presented as medians and range. GQE, good quality embryo.

Table II Baseline and cycle characteristics in the pregnant and non-pregnant groups.

	Pregnant	Non-pregnant	*P*-value
No of patients	26	58	
Maternal age (years)	28.8 ± 3.6	30.6 ± 3.3	0.03
Cause of infertility			
Maternal (anovulation, tubal factor)	3	2	0.09
Paternal	23	49	
Other (incl. unexplained)		7	
Number of previous cycles	1 (0; 3)	1 (0; 3)	0.36
Maternal BMI (kg/m^2) (median; range)	22.75 (18.7; 32.9)	22.4 (18.0; 36.4)	0.56
Cumulative FSH dose(IU) (median ± SD)	1575 (675; 4125)	1600 (700; 3825)	0.74
Number of retrieved oocytes/cycle (median ± SD)	12 (8; 22)	13 (8; 34)	0.69
Number of mature oocytes/cycle	9 (3; 17)	10 (4; 29)	0.62
Number of GQE on Day 2	2 (0; 8)	2 (0; 7)	0.83
Number of GQE on Day 3	2 (0; 8)	2 (0; 6)	0.71
Number of embryos cryopreserved	2 (0; 8)	2 (0; 6)	0.21
Number of embryos biopsied/group	8	6	0.53

Continuous data are expressed as mean ± SD, or median and range if the assumption of normality was not fulfilled. Categorical data are presented as number of cases. For testing differences between the two groups Fisher's exact test was used for categorical data and Student's *t*-test for continuous normal distributed data and Wilcoxon rank-sum test for non-normal distributed data. GQE, good quality embryo.

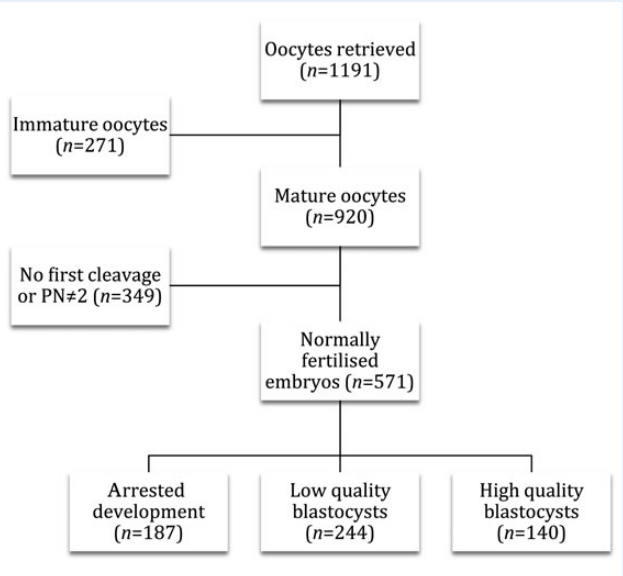

Figure 1 Cohort flowchart. ICSI fertilized embryos cultured at 5% O_2.

Table III Logistic regression analysis of time-lapse predictors for development into a high-quality blastocyst.

Parameter	OR (95% CI)	*P*-value
PN breakdown	0.94 (0.88; 1.01)	0.09
Duration of the first cytokinesis (h)	0.36 (0.16; 0.83)	0.02
Duration of the 2-cell stage (h)	0.89 (0.77; 1.04)	0.14
Multi-nucleation at the 2-cell stage (yes/no)	0.89 (0.49; 1.59)	0.70
Duration of the 3-cell stage (h)	0.88 (0.80; 0.97)	0.01
Direct cleavage to 3 cells[a] (yes/no)	0.11 (0.02; 0.69)	0.02

The OR was obtained with a single logistic regression analysis where all six parameters were included. OR, odds ratio.
[a]Duration of the 2-cell stage <5 h.

Implantation potential

Eight ($n = 8$) patients had no embryo transferred, and 84 single transferred embryos were therefore available for the pregnancy outcome analysis. The distribution of blastocyst quality qualification is presented in Table VII. The mean age was lower for patients in the pregnancy group compared with patients in the non-pregnancy group, but none of the other potential confounders differed between the two groups (Table II). None of the mean time points of cellular divisions or embryonic stages differed between the pregnant and the non-pregnant groups (Fig. 3A and Supplementary data, Table SII). Among the duration of events only first cytokinesis differed between the pregnant and the non-pregnant groups (Fig. 3B and Supplementary data, Table SII).

Discussion

We conducted a prospective cohort study of embryos from patients positively selected by good prognosis factors. We found that development to high-quality blastocysts could be predicted within the first

48 h of culture by a short duration of the first cytokinesis, duration of the 3-cell stage and absence of direct cleavage to 3 cells (duration of the 2-cell stage <5 h). However, in a logistic regression model using age, other potential confounders and the above predictors of development to high-quality blastocysts as independent variables, only age predicted pregnancy outcome. Furthermore, we found no difference in overall timing between implanted and non-implanted embryos.

Blastocyst developmental potential

Unreliable embryo selection within the first 2–3 days of culture has increasingly favored blastocyst transfer, a strategy that has proved efficient in particular for younger patients with a high number of GQEs after 2–3 days of culture (Blake *et al.*, 2007). Some studies, however, have suggested a potential influence of *in vitro* culture on the offspring in terms of epigenetic modifications (Katari *et al.*, 2009; Dumoulin *et al.*, 2010; van Montfoort *et al.*, 2012), a risk that would encourage the shortest culture possible. Other studies have suggested that prolonged culture can create problems with regard to fetal outcomes (Kallen *et al.*, 2010; Dar *et al.*, 2013). The aim of predicting development is therefore to be able to perform early embryo transfers with a more precise selection of viable embryos, and thereby to avoid the costs and unknown consequences of prolonged *in vitro* culture. To fully pursue this strategy we therefore did not evaluate parameters that would occur after the first 48 h of culture. The assumption underlying the approach of this and similar studies (Wong *et al.*, 2010; Cruz *et al.*, 2012; Dal Canto *et al.*, 2012; Hashimoto *et al.*, 2012) is that blastocyst development serves as a suitable surrogate end-point of pregnancy potential, which is justified by a reported correlation between blastocyst quality and pregnancy outcome (Ahlstrom *et al.*, 2011; Hill *et al.*, 2013).

Historical cohort studies on blastocyst developmental potential have been performed on embryos from groups of unselected patients. Duration of first cytokinesis has been evaluated in one previous study only (Wong *et al.*, 2010), where the parameter was found to predict the development to the blastocyst stage with high sensitivity and specificity. Compared with the present study, the study by Wong *et al.* (2010) had the advantage that images were recorded with shorter time intervals (5 min), which allowed for a more accurate assessment. This difference underlies the decision of not evaluating this particular parameter in similar studies (Cruz *et al.*, 2012). Notably, the interval proposed by Wong *et al.* (2010) (<33 min) is longer than the interval between image recordings in the present study (20 min), which in our analysis seems sufficiently short to predict formation of high-quality blastocysts.

Table IV Logistic regression analysis of potential confounders for development into a high-quality blastocyst.

Parameter	OR (95% CI)	*P*-value
Age (years)	0.95 (0.88; 1.03)	0.25
Maternal BMI (kg/m^2)	0.97 (0.92; 1.01)	0.13
Number of previous cycles	0.78 (0.54; 1.13)	0.19
Cumulative FSH dose (100IU)	0.96 (0.93; 0.99)	0.01
No of GQE on Day 2	1.15 (1.02; 1.30)	0.02
No of GQE on Day 3	1.17 (1.05; 1.32)	0.006

OR were obtained by performing a logistic regression analysis with the three predictive time-lapse parameters and one additional parameter. This analysis was repeated for each parameter to test the strength of the prediction compared with the time-lapse parameters. GQE, good quality embryo; OR, odds ratio.

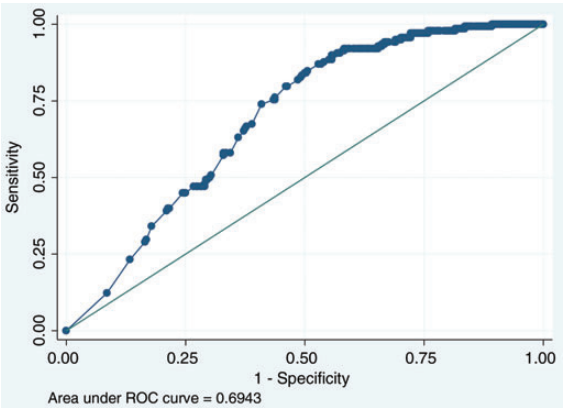

Figure 2 ROC curve for prediction of blastocyst development by duration of first cytokinesis, duration of the 3-cell stage and direct cleavage to a 3-cell embryo.

Table V AUC for parameters predicting blastocyst development.

Parameter	AUC	95% CI
Duration of the first cytokinesis (h)	0.63	0.58; 0.67
Duration of the 3-cell stage (h)	0.63	0.57; 0.67
Direct cleavage to 3 cells[a] (yes/no)	0.58	0.56; 0.61
Combined parameters	0.69	0.65; 0.74

AUC, area under the receptor operator characteristic curve.
[a]Duration of the 2-cell stage <5 h.

Table VI Logistic regression analysis of predictors of pregnancy.

Parameter	OR (95% CI)	*P*-value
Duration of the first cytokinesis (h)	0.84 (0.45; 1.57)	0.59
Duration of the 3-cell stage (h)	0.84 (0.59; 1.22)	0.36
Age (years)	0.84 (0.73; 0.98)	0.03
Number of previous cycles	1.2 (0.62; 2.4)	0.56
Number of GQE on Day 2	1.0 (0.78; 1.3)	0.98
Number of GQE on Day 3	1.1 (0.83; 1.4)	0.57
Total FSH dose(100 IU)	0.99 (0.93; 1.1)	0.82
Cause of infertility (categorical)	0.34 (0.05; 2.2)	0.25

OR were obtained by performing a logistic regression analysis with the two time-lapse parameters and one of the parameters listed below them. This analysis was repeated for each parameter. GQE, good quality embryo; OR, odds ratio.

Table VII Distribution of quality classification among the transferred embryos.

	Pregnant	Non-pregnant
High quality (4–6; AA, AB, BA)	17	36
Low quality (4–6, BB, BC; CB, CC)	9	22

P-value (Fisher's exact test of no difference between the groups): 0.81.

Wong *et al.* (2010) furthermore proposed duration of the 2-cell stage (7.8–14.3 h) and duration of the 3-cell stage (0–5.8 h) as predictors of development to the blastocyst stage regardless of embryo quality. A similar study subsequently evaluated embryo quality (Hashimoto *et al.*, 2012), where, among parameters evaluated in the present study, only the duration of the 3-cell stage was found to distinguish high-quality from low-quality blastocysts.

In both studies, the analysis was conducted on surplus frozen/thawed 2PN embryos. The conclusion that cryopreservation has no impact on embryo kinetics was drawn by time-lapse analysis of a small subset

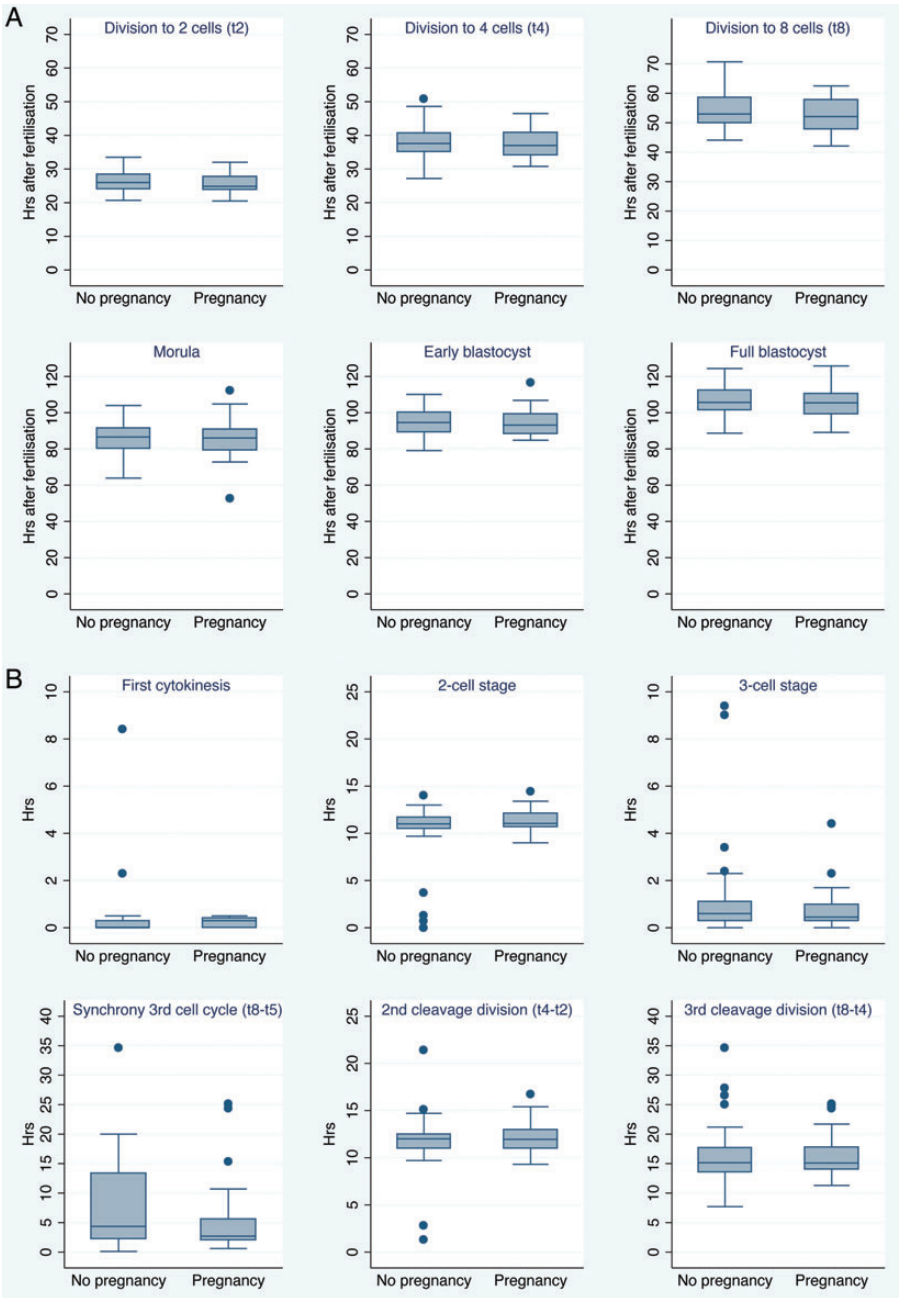

Figure 3 Time points (**A**) and durations (**B**) of selected embryonic stages in the pregnant and non-pregnant groups.

($n = 10$) of 3PN embryos and remains to be confirmed on a larger number of normally fertilized embryos (Wong *et al.*, 2010).

A larger study of fresh surplus, non-transferred embryos from a group of unselected patients showed that timing and duration of all divisions from the 2- till the 8-cell stage differed between expanded and non-expanded blastocysts (Dal Canto *et al.*, 2012), thus confirming previous data showing that the duration of both the 2- and 3-cell stage is predictive of development (Wong *et al.*, 2010). Discrimination between expanded and non-expanded blastocysts was not precisely defined, and the end-point did not include scoring of TE and ICM morphology as usually recommended in blastocyst quality assessment (ALPHA/ESHRE) (ALPHA Scientists, 2011). We believe that the limitations in the above studies concerning cryopreservation, usage of surplus embryos and sub-optimal defined morphological end-points have been overcome in the present study. Thus, our findings consolidate the duration of the first cytokinesis and duration of the 3-cell stage, but not duration of the 2-cell stage, as predictive markers of development to high-quality blastocysts.

Cruz *et al.* (2012) studied donated oocytes ($n = 834$) and identified predictors of development to high-quality blastocysts on Day 5/6 from the subset of embryos ($n = 293$) that developed into blastocysts. Since embryos from fertile oocyte donors have been shown to differ in timing of the first cell divisions compared with embryos from infertile patients (Bellver *et al.*, 2013), the results from that study are not entirely transferable to ART patients. Of the parameters included in the present study identification of duration of the 3-cell stage and direct cleavage to 3 cells were in agreement with our findings. Furthermore, duration of the 2-cell stage was neither in the present study nor in the study by Cruz *et al.* (2012) found to differ between high- and low-quality embryos, in contrast to the findings by Wong *et al.* (2010). Direct cleavage to 3 cells, arbitrarily defined as a duration of the 2-cell stage <5 h, has been suggested as a strong negative predictor of both pregnancy and blastocyst development (Meseguer *et al.*, 2011; Cruz *et al.*, 2012; Rubio *et al.*, 2012). Since direct cleavage to 3 cells is thus derived from duration of the 2-cell stage, this particular parameter presumably holds predictive value, in particular with regard to identifying less competent embryos.

The three time-lapse parameters, that in our analysis were identified as predictive for development to high-quality blastocyst, were used in a logistic regression analysis where potential confounders were included. The time-lapse parameters had a predictive value that equaled the predictive value of number of GQEs on Days 2 and 3. The strength of the prediction, however, not high as AUC for the individual time-lapse parameters was all below 0.70 (Table V).

Implantation potential

Blastocyst development *per se* is not a meaningful end-point for a tool for embryo selection. Predictive parameters identified using blastocyst development and quality as a surrogate end-point should therefore ultimately be evaluated with regard to prediction of clinical pregnancy. We tested whether the three parameters identified to predict formation of high-quality blastocysts in the present trial (duration of first cytokinesis, duration of the 3-cell stage and direct cleavage to 3 cells) would differ between implanted and non-implanted embryos from the same cohort. In a logistic regression model using age and other potential confounders in combination of the predictors of development to high-quality blastocyst as independent variables, only age predicted pregnancy outcome. Notably, age was the only potential confounder that, among the registered baseline data, differed between the two groups. Although we cannot preclude that a larger study would detect differences in timing, we notice that age did, nonetheless, predict pregnancy outcome, even though the cohort included younger patients only. Furthermore, the size of the cohort ($n = 84$) was in principle sufficiently large to test up to five parameters using the targeted logistic regression approach. This leads us to conclude with reasonable strength that the parameters predicting development to high-quality blastocysts do not predict pregnancy in this population. Increasing evidence suggests that a significant proportion of blastocysts are aneuploid (Liang *et al.*, 2013) and that the relation between morphology and aneuploidy is weak (Alfarawati *et al.*, 2011). Furthermore, a recent time-lapse study found no correlation between aneuploidy at the blastocysts stage and timing of the cleavage stages (Campbell *et al.*, 2013). Although the patients in our study might be younger than the populations studied, this might offer a plausible explanation to the observed lack of correlation.

Our comparison of a large number of non-selected parameters suggested that duration of first cytokinesis was different between the pregnancy and non-pregnancy groups. Caution should be taken, however, since >20 parameters were tested, thus making it plausible that the result is merely a product of chance with the chosen significance level. Moreover, the median value of first cytokinesis was higher in the pregnant group, which contrasts with the expected finding.

Previously, PN breakdown, multi-nucleation at the 2-cell stage and synchronous appearance of nuclei after the first division have all been correlated with pregnancy outcome. In our study none of the three parameters differed between the implanted and the non-implanted groups, and neither PN breakdown nor multi-nucleation at the 2-cell stage predicted blastocyst development. Synchronous appearance of nuclei after first division was identified as pregnancy predictor in a small material ($n = 19$) (Lemmen *et al.*, 2008). Both PN breakdown and absence of multi-nucleation were reported to predict pregnancy potential in larger studies than the present (Meseguer *et al.*, 2011; Azzarello *et al.*, 2012).

Direct cleavage into 3 cells has previously been reported to have a strong negative correlation with implantation (Meseguer *et al.*, 2011; Rubio *et al.*, 2012). We found that the absence of direct cleavage to 3 cells predicted development to high-quality blastocysts, but found no significant difference between implanted and non-implanted embryos. Notably, none of the embryos displaying direct cleavage did, however, implant. Direct cleavage to 3 cells constitutes a deviation from the normal cell cycle and uneven cleavage has previously been associated with underlying chromosomal aberrations (Hardarson *et al.*, 2006). However, a recent study did not find that aneuploid embryos differed from euploid embryos in timing of the pre-compaction stages (Campbell *et al.*, 2013).

The external validity of our study may be affected by the Day 6 transfer policy, as blastocyst transfers in most clinics are performed on Day 5. A negative correlation has been reported between Day 6 transfer and pregnancy rates (Dessolle *et al.*, 2011), which suggests that delayed transfer affects the outcome. Notably, the Day 6 transfer policy was, in that particular study, implemented only on embryos that on Day 5 showed insufficient blastulation and were thus of lower quality compared with the embryos that were transferred on Day 5. The difference in outcome between non-elective and elective Day 6 transfer has been confirmed by Elgindy and Elsedeek (2012), who however found no difference in pregnancy outcome when Day 6

culture was a general strategy (Elgindy and Elsedeek, 2012). In the present study, Day 6 transfer was a general strategy motivated by the intention of TE biopsy. A potential impact of the delayed transfer would therefore apply to all embryos in the study, and thus not cause any bias. Only a small subset of embryos were biopsied, which might potentially introduce an effect modification. A stratified analysis showed no difference in the pregnancy outcome between biopsied and non-biopsied embryos. Without the concern of effect modification, the biopsied embryos were included in the analysis,

In summary, previous studies have suggested various time-lapse parameters as candidate predictors of development and implantation, but not with consistency. Our study was unable to reproduce previous findings with regard to prediction of pregnancy. Although, it is possible that our study did not have enough power to detect such differences, the size of the cohort allows us to conclude that the parameters predicting development to high-quality blastocysts do not predict pregnancy in this population. The fact that the cohort consisted of good prognosis patients only, may be important. Plausible explanations for the diverging conclusions are in our opinion most likely to be found in the distinct differences in the population of embryos studied, the parameters evaluated and the end-points chosen as described. Moreover, several recent studies have suggested that treatment-related factors and culture conditions influence timing of divisions (Ciray *et al.*, 2012; Kirkegaard *et al.*, 2012a,b,c; Munoz *et al.*, 2012). It may be speculated that both the reported and presumed influences of different stimulation protocols, culture media, oxygen tension, maternal factors, etc. will complicate the development of an universal algorithm for optimal timing of development. In conclusion, our findings indicate that the positive predictive value of timing might be lower than previous trials have suggested. In contrast, the apparent negative significance of division patterns that differ from the expected, such as direct cleavage to a 3-cell embryo, may imply that time lapse will facilitate de-selection of embryos that on the day of transfer appear to be normal.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

K.K. and J.I. designed the study. K.K. and J.H. collected the data. K.K. and U.S.K. performed the data analysis. K.K., U.S.K. and J.I. interpreted the findings. K.K. wrote the first draft. All authors critically reviewed and approved the final version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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