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REVIEW *Embryology*

Time-lapse monitoring as a tool for clinical embryo assessment

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ABSTRACT: As elective transfer of a single embryo (eSET) becomes increasingly accepted, the need to improve implantation rates becomes crucial. Selecting the most competent embryo therefore constitutes a major challenge in assisted reproductive technology. Embryo morphology and developmental stage at given time points are closely correlated with developmental competence and assessment of morphological parameters at discrete inspection points thus remains the preferred way of evaluating embryonic potential. Lately, more attention has been given to the assessment of dynamic embryo development as a tool for evaluating embryonic potential. The introduction of time-lapse equipment approved for use on human embryos offers novel clinical opportunities for continuous monitoring of embryos, enabling flexible evaluation of known morphological parameters and potentially introducing new dynamic markers of viability. Due to lack of larger, randomized clinical studies it remains to be elucidated whether embryo selection using dynamic parameters improves clinical outcome and which parameters are of significance. Before such randomized controlled studies are organized, the most promising parameters to evaluate must be identified. This mini-review summarizes the current knowledge about dynamic markers of viability and discusses the potential clinical role of time-lapse analysis in embryo assessment and selection.

Key words: time-lapse monitoring / development kinetics / morphology / embryo selection

Introduction

Despite efforts to optimize current procedures, implantation rates of IVF embryos remain relatively low with a clinical pregnancy rate of ~30% per transfer (Andersen *et al.*, 2008). To maximize the probability of pregnancy, multiple embryos are often transferred simultaneously, which increases the risk of multiple pregnancies and the associated neonatal complications and maternal pregnancy-related health problems (Stromberg *et al.*, 2002; Pinborg *et al.*, 2003; Pinborg *et al.*, 2004; Walker *et al.*, 2004). Elective transfer of a single embryo (eSET) is an efficient method of reducing the risk of multiple gestations. As eSET becomes increasingly applied in clinical practice, the challenge of identifying the single embryo with highest developmental competence in a cohort becomes crucial. Therefore, several areas have been investigated in search of additional markers of viability to supplement current criteria for selection, e.g. aneuploidy screening, O_2 respiration, metabolic profiling and gene expression analysis (Mastenbroek *et al.*, 2007; Ottosen *et al.*, 2007a,b; Jones *et al.*, 2008; Scott *et al.*, 2008; Seli *et al.*, 2010). Although many of these methods are promising, grading systems based on morphology remain the preferred way of assessing embryonic competence. There is a well-documented close correlation between morphological appearance and developmental stage of the embryo at given time points and developmental competence [as reviewed in a recent consensus paper by ALPHA and ESHRE (2011)]. The restricted use of alternative methods may largely be explained by the simplicity and cost-effectiveness of static morphological grading, when compared with most of the alternative methods, and by the lack of documentation for superiority of alternatives. The dynamic nature of cell cleavage and embryo development is, however, well known as demonstrated with respect to fragmentation, evenness of blastomeres, appearance and disappearance of pro-nuclei (PN) and nuclei (Payne *et al.*, 1997; Hardarson *et al.*, 2002; Lemmen *et al.*, 2008) along with the change in blastomere numbers over time due to cell divisions. The inherent limitation in evaluating a dynamic process by a few snapshots at discrete time points is reflected in the recent observation that the result of embryo scoring can change markedly within few hours (Montag *et al.*, 2011). Frequent evaluation outside the incubator enables the assessment of timing of events, but also exposes the embryos to undesirable changes in temperature, humidity and gas composition (Zhang *et al.*, 2010). Using traditional incubators, a conflict therefore exists between the need to obtain a detailed picture of embryo development and the risk of compromising stable culture conditions. Time-lapse monitoring using cameras incorporated in the incubation chamber overcomes this limitation, thus providing the potential benefit of stable culture conditions during inspection, and offering a

promising clinical method of extending and refining morphological evaluation to include dynamic parameters. Several recent studies (Arav et al., 2008; Lemmen et al., 2008; Pribenszky et al., 2010; Wong et al., 2010; Meseguer et al., 2011) have addressed the potential role of time-lapse monitoring in clinical selection of competent embryos. All studies are however descriptive and only a few studies include transfer of human embryos. Yet, the studies provide valuable information with regard to what parameters could be of interest to study when conducting the necessary larger clinical trials. In this mini-review we provide an overview of the current literature concerning the clinical use of time-lapse and the candidate dynamic parameters that has been identified in retrospective studies. The identification of candidate viability markers is a prerequisite for designing the larger prospective, randomized studies that must be conducted in order to evaluate whether time-lapse monitoring can improve pregnancy rates.

Kinetics of fertilization and early development of human embryos

Time-lapse monitoring has been used to study the kinetics of developing embryos in a variety of animal species, with the first reports going as far back as to 1929, when rabbit embryos were evaluated with a cinematographic film (Lewis and Gregory, 1929). Other pioneer studies have examined the behaviour of non-human embryos under different conditions (Massip and Mulnard, 1980; Massip et al., 1982; Grisart et al., 1994; Gonzales et al., 1995; Gonzales et al., 1996). One of the first time-lapse studies of human embryo kinetics described the time course of fertilization and the earliest embryonic development using time-lapse cinematography (Payne et al., 1997), followed by a study with an extended observation period (Mio and Maeda, 2008). The time-lapse sequences have revealed characteristic events in the development of human embryos from cleavage stage via morula to blastocysts with the formation and expansion of a blastocoel, the cyclic expansion and partial or complete collapse of the blastocyst followed by a breach of zona pellucida ending with hatching.

Kinetic markers of viability

The following sections describe candidate viability markers in non-humans and humans. The majority of studies have used achievement of a given developmental stage, e.g. blastocyst formation as end-point, which is not necessarily equivalent to implantation potential. Although implantation potential would be the preferred end-point with regard to clinical selection of embryos, ability to predict blastocyst formation from an early developmental stage could prove valuable with regard to selection of the optimum day for transfer. Transfer of blastocysts yields higher implantation rates than transfer at the cleavage stage. However, the higher implantation rate must be weighed against the potential drawbacks of longer culture, e.g. the higher economic costs, the risk of cancelled cycles and the suspected effects of *in vitro* culture on children born after IVF. Indications have been found that differences between IVF and control children in terms of birthweight, pre-pubertal height and serum IGF levels are an effect of *in vitro* culture, an effect that has been speculated to originate from changes in gene expression and epigenetic alterations (Miles et al., 2007; Katari et al., 2009; Dumoulin et al., 2010). Here we will discuss and review the literature concerning time-lapse studies of development in both human and non-human IVF embryos with regard to embryo viability assessment. Tables I and II provide an overview of time-lapse studies with non-human and human embryos, respectively, whereas Tables III and IV provide an overview of candidate viability markers evaluated in non-human and human embryos, respectively.

Timing and synchrony of cleavage stages in non-human embryos

Arav et al. (2008) related embryo cleavage rates to the ability to develop into blastocysts, concluding that early divisions, especially the first cleavage, were closely synchronized in embryos, with a well-defined duration of division. Timing became less uniform as development proceeded, a finding also reported in several other studies (Grisart et al., 1994; Wale and Gardner, 2010).

Regarding the relevance of early cleavage, Arav et al. (2008) found that the proportion of mouse embryos with a late first cleavage that developed to blastocysts was significantly lower than embryos with a first cleavage within the normal range (33.5–35.5 h after hCG administration). In this study normal range was defined as the time interval during which 50% of all embryos cleaved. In accordance with these findings Pribenszky et al. (2010) described that in mice timing of the first cleavage significantly influenced the probability of reaching the blastocyst stage, with fast cleaving embryos being more likely to

Table I Overview of studies using time lapse to evaluate non-human embryo developmental potential.

Study	No. of embryos/ oocytes included	Species	End-point	Time between image acquisition	Comments
Grisart et al. (1994)	130 zygotes	Bovine	Blastocyst development on Days 5–7	1 min	Start time (t_0): time of insemination
Gonzales et al. (1995)	62 embryos	Hamster	Blastocyst development on Days 5–7	5–20 min	Start time (t_0): pronuclear envelope breakdown
Holm et al. (1998)	392 embryos	Bovine	Compact morula or blastocyst on Day 7	30 min	Start time (t_0): time of insemination
Arav et al. (2008)	230 2-cell zygotes	Mouse	Blastocyst development on Day 5	30 min	Start time (t_0): time of hCG administration
Pribenszky et al. (2010)	345 embryos	Mouse	Blastocyst development on Day 5	10 min	Start time (t_0): midpoint of the dark period (mating)

Table II Overview of studies using time lapse to evaluate human embryo developmental or pregnancy potential.

Study	No. of embryos/ oocytes included	End-point	Time between image acquisition	Starting point and fertilization method	Comments/ observation time
Payne *et al.* (1997)	50 oocytes	Embryo development 68 h after fertilization (good = freezing or transfer; bad = discarded)	1 min	Time of fertilization. Only ICSI embryos	No transfer, observation time 17–20 h
Lemmen *et al.* (2008)	102 fertilized 2 PN embryos. 29 of these were transferred	Number of blastomeres on Day 2 and pregnancy	5 min	Time of fertilization. IVF and ICSI embryos	Transfer on Day 2. Only one zygote from a cohort was evaluated
Wong *et al.* (2010)	100 fertilized embryos	Blastocyst development on Days 5–6 (blastocyst = normal/ non-blastocyst = abnormal)	5 min	Duration of events. Only IVF embryos	No transfer, frozen/ thawn 2 PN embryos
Meseguer *et al.* (2011)	247 transferred embryos	Pregnancy	15 min	Time of fertilization. Only ICSI embryos	Transfer on Day 3, clinical time-lapse instrument

Table III Putative markers of non-human embryo competence.

Event	Author	No of embryos evaluated	Species	Statistically associated
First cleavage/time point of 2-cell stage	Arav *et al.* (2008)	230	Mouse	Yes
	Pribenszky *et al.* (2010)	345	Mouse	Yes
	Holm *et al.* (1998)	335	Bovine	Yes
	Gonzales *et al.* (1995)	62	Hamster	No
Early second division/time point of 3-cell stage	Pribenszky *et al.* (2010)	345	Mouse	Yes
	Holm *et al.* (1998)	335	Bovine	Yes
Interval between second and third division/synchrony in second cell cycle (duration of 3-cell stage)	Pribenszky *et al.* (2010)	345	Mouse	No
	Gonzales *et al.* (1995)	62	Hamster	No
Short first four cell cycles	Holm *et al.* (1998)	335	Bovine	Yes
	Grisart *et al.* (1994)	13	Bovine	Yes
	Gonzales *et al.* (1995)	62	Hamster	Yes
Time interval between 4 and 8 cell stage (duration of third cleavage cycle)	Gonzales *et al.* (1995)	62	Hamster	Yes

develop. No indications were found that the most rapidly cleaving embryos displayed less potential than others. The time point of the second cleavage (2- to 3-cell stage) showed even stronger correlation with developmental potential. Bovine embryos have been described to display same behaviour, with embryos developing into morula–blastocyst completing the first cell-stages significantly earlier than embryos that arrested development before 9-cell stages (Grisart *et al.*, 1994; Holm *et al.*, 1998). Moreover, prolongation of the cell cycle of non-viable bovine embryos was most noticeable in the cell cycle just prior to the cessation of development (Holm *et al.*, 1998). In line with most other studies on early cleavage, this finding suggests that a delayed development in first cell cycle will result in a lower chance of reaching the 4-cell stage, and therefore also a lower percentage of blastocyst formation. A normal first cleavage, on the other hand, did not necessarily imply viability, since bovine embryos that ceased development at later stages proceeded through the first cell cycle with the same speed as non-viable embryos, a finding that challenges early cleavage as an independent predictor of viability.

In contrast to the findings in mice (Arav *et al.*, 2008; Pribenszky *et al.*, 2010), a previous study undertaken on hamster embryos (Gonzales *et al.*, 1995) observed the time intervals of first, second and third cleavage division (1–2 cells; 2–4 cells and 4–8 cells, respectively) and found that the time intervals of the earlier cleavage stages were unable to predict blastocyst development. Instead, they identified the time interval from 4 to 8 cells (third cleavage division) as being predictive of blastocyst formation.

Synchrony in division, meaning the embryo spending only a short time at the 3, 5, 6 and 7 cell stages, has been studied to a smaller extent in animals without any apparent correlation with developmental potential (Gonzales *et al.*, 1995; Pribenszky *et al.*, 2010). In contrast, synchronism of early developmental events has been identified as positively associated with developmental potential in human embryos (Payne *et al.*, 1997; Lemmen *et al.*, 2008; Wong *et al.*, 2010).

Timing and synchrony of cleavage stages in human embryos

Four studies have been published correlating human embryo kinetics using time-lapse analysis to either development or pregnancy potential. In the following sections we will focus on events occurring

Table IV Putative markers of human embryo competence.

Event	Author	End-point	No. of embryos analysed[a]	Statistically associated
Fast PB extrusion	Payne et al. (1997)	Day 3 quality	30	Yes
Synchrony in male and female PN formation	Payne et al. (1997)	Day 3 quality	30	Yes
Fast PN abuttal	Payne et al. (1997)	Day 3 quality	30	Yes
Early disappearance of pronuclei	Lemmen et al. (2008)	Day 2 blastomere number	102	Yes
Duration of first cytokinesis	Wong et al. (2010)	Development to blastocyst	100	Yes
First cleavage/time point of 2-cell stage	Meseguer et al. (2011)	Pregnancy	247	Yes[b]
	Lemmen et al. (2008)	Day 2 blastomere numbers	102	Yes
		Pregnancy	102	No
Fast reappearance of nuclei after first cleavage	Lemmen et al. (2008)	Day 2 blastomere number	29	Yes
		Pregnancy	19	Yes
Synchrony of reappearance of nuclei after first division	Lemmen et al. (2008)	Day 2 blastomere number	102	No
		Pregnancy	10	Yes
Early second division/time point of 3-cell stage	Meseguer et al. (2011)	Pregnancy	246	Yes[b]
	Wong et al. (2010)	Development to blastocyst	100	Yes
Duration of the 2-cell stage	Meseguer et al. (2011)	Pregnancy	246	Yes[b]
Interval between second and third division/synchrony in second cell cycle (duration of 3-cell stage)	Meseguer et al. (2011)	Pregnancy	243	Yes
	Wong et al. (2010)	Development to blastocyst	100	Yes
	Lemmen et al. (2008)	Day 2 blastomere number	102	No
		Pregnancy	17	Trend
Time point of the 4- cell stage	Meseguer et al. (2011)	Pregnancy	243	Yes[b]
Time point of the 5-cell stage	Meseguer et al. (2011)	Pregnancy	228	Yes[b]

[a]Number of embryos where the parameter in question was evaluated. The number does not necessarily correspond to the number of embryos included in the studies (Table III), since the information was not obtained for all embryos.
[b]No difference in median time point, but significant difference in optimal range of cleavage.

before first cleavage, time-points of the subsequent cell divisions and synchronization of the cell cycles, in the order mentioned.

Payne et al. (1997) studied in detail the morpho-kinetic events occurring before first cleavage in a relatively small set ($n = 38$) of ICSI fertilized embryos. The short time elapsed between each image acquisition ($t = 1$ min) allowed for a precise description of the sequence of events after fertilization, along with identifying and recording time-points of a cytoplasmic wave, extrusion of the two polar bodies (PB) and appearance and abuttal of PN. Of these, timing of extrusion of the second PB, synchrony in appearance of PN and abuttal of PN were correlated positively to embryo quality on Day 3 (Table IV). The very accurate and precise description of developmental events that occur in the fertilized oocytes before first cleavage has not been repeated in other studies on human embryos. Wong et al. (2010) studied thawed IVF fertilized embryos ($n = 242$) that were cryopreserved at the zygote stage 12–18 h after fertilization, and could thus not assess events occurring before first cytokinesis. Lemmen et al. (2008) studied timing of disappearance of PN on a larger set of both IVF and ICSI fertilized embryos ($n = 102$), from a clinical programme with later transfer on Day 2 ($n = 29$). However, only 2PN embryos were time-lapse monitored and events occurring before appearance of PN were therefore not evaluated. They showed that early disappearance of PN was correlated with embryo quality on Day 2 (Table IV), but did not assess synchrony in appearance of PN. Meseguer et al. (2011) studied a large set ($n = 247$) of ICSI fertilized embryos transferred on Day 3 using a commercial time-

lapse incubator designed for clinical use, but did not evaluate events occurring before first cleavage. The detailed analysis of the events generated by Payne *et al*. (1997) and Lemmen *et al*. (2008) was enabled by the short intervals between image acquisitions (1 and 5 min, respectively). As most systems that have been introduced clinically acquire images every 10–20 min (Meseguer *et al*., 2011), it would be difficult to obtain and evaluate in detail parameters occurring before first cleavage using these systems, since many of these events have a duration of >20 min and thus may be overlooked or imprecisely evaluated. Therefore, when evaluating and comparing the findings from the four studies, the differences in time intervals between image acquisitions must be recalled (Table IV).

Duration of first cytokinesis, defined as the first appearance of the cleavage furrow to complete separation of the daughter cells has been found to predict blastocyst formation (Wong *et al*., 2010). Embryos reaching the blastocyst stage completed first cytokinesis with a mean time of 14.3 ± 6.0 min. Since most clinical systems acquire images every 10–20 min (Meseguer *et al*., 2011), it is questionable whether duration of first cytokinesis is a useful prognostic parameter using these systems. This parameter has not been evaluated in other human studies, but has previously suggested as a prognostic parameter in bovine embryos, where short duration of cell divisions was shown to be positively correlated with blastocyst development (Ramsing and Callesen, 2006).

Early first cleavage is considered a non-invasive marker of developmental competence, as demonstrated by several groups (Shoukir *et al*., 1997; Sakkas *et al*., 1998; Lundin *et al*., 2001; Giorgetti *et al*., 2007; Terriou *et al*., 2007), although evidence exists that exceptionally early first cleavages can be indicative of underlying abnormalities, and are associated with lower developmental potential (Ziebe *et al*., 1997; Alikani *et al*., 2000; Magli *et al*., 2001). However, most studies have used periodic inspection at fixed time intervals so that the range of timing is too broad opening the possibility of the observations being out of synchrony with embryo cleavage, thereby making the observations imprecise. Lemmen *et al*. (2008) found that early cleavage was positively and significantly correlated with developmental potential (4-cell stage at Day 2), but not with pregnancy outcome. Based on these findings the authors question the importance of early cleavage as a strong separate prognostic parameter, although acknowledging the small number of embryos in the study ($n_{transferred} = 29$). Holm *et al*. (1998) showed on bovine embryos that prolongation of the cell cycle of non-viable embryos is most noticeable in the cell cycle just prior to the cessation of development. Assuming that the findings from this study on bovine embryos can be applied to human embryonic development, it may provide an explanation as to why early cleavage in the study by Lemmen *et al*. (2008) was positively correlated with development but not pregnancy, since embryos were inspected and transferred on Day 2. Thus, embryos that arrested development at the morula or blastocyst stage, and hence failed to implant, may have displayed a normal first cleavage. On the contrary, first cleavage within an optimal range was found to predict pregnancy in a later study with a larger number of transferred embryos ($n = 247$; Meseguer *et al*., 2011). First cleavage was not evaluated in the other two studies on human embryos, since the observation time in the study by Payne *et al*. (1997) was too short and Wong *et al*. (2010) studied thawed embryos that were cryopreserved at the zygote stage.

The duration of the 2-cell stage was identified by Wong *et al*. (2010) as a predictor of blastocyst formation but not as a predictor of pregnancy by Meseguer *et al*. (2011). In this large study, the duration of the 3-cell stage was the only parameter with a median value that differed significantly between implanted and non-implanted embryos.

Timing of the subsequent cleavages up till the 5-cell stage was evaluated in detail by Meseguer *et al*. (2011). They found the distribution of cleavage time points in embryos that subsequently implanted to show a smaller variance than distribution of cleavage time points in non-implanted embryos, indicating that viable embryos display a more uniform pattern of division. Analysing the second, third and fourth cleavages they found no differences between the implanted and non-implanted embryos in median time points. They divided the cleavage time points into quartiles in order to establish optimal time ranges for each cleavage. The found that embryos that cleaved within the optimal time-range for all parameters evaluated showed significantly higher implantation (Table IV). They subsequently used a logistic regression model to select predictive variables for implantation. Using this approach, timing of the fourth cleavage, and the duration of the 2-and 3- cell stages were selected along with a set of negative predictive factors (direct transition from the 1- to the 3-cell stage, uneven blastomere size at the 2-cell stage and multinucleation at the 4-cell stage) to generate a hierarchical selection model. In the proposed model, embryos were first excluded based on clearly abnormal morphology evaluated with traditional static evaluation and secondly using the time-lapse exclusion criteria mentioned. The positive time-lapse criteria were used to place the remaining embryos into ranked categories. The model provides a very promising and concrete tool for selection, enabling randomized studies that prospectively can evaluate whether embryo evaluation using time-lapse monitoring has the potential to improve pregnancy rates.

Synchrony in development has been proposed as a positive predictor of embryo competence. Lemmen *et al*. (2008) reported that embryos resulting in pregnancies displayed a significantly higher degree of synchrony in appearance of nuclei in the first and second blastomeres after first division compared with non-implanting embryos, a parameter not evaluated in other studies. In addition, they found a non-significant trend of synchrony of the two first divisions, i.e. a short duration of the 3-cell stage, being predictive of implantation potential. This finding has been confirmed in larger studies, where synchrony in the first cell cycle has been positively correlated both with blastocyst development (Wong *et al*., 2010) and pregnancy potential (Meseguer *et al*., 2011).

Based on these four studies, it seems that viable embryos proceed fast and synchronously through the first divisions following a uniform pattern of division. Moreover, it appears that time-lapse monitoring can be used to exclude embryos that would be deemed viable using a static evaluation, but which display aberrant cleavage patterns.

Significance of fertilization method and culture conditions

The interpretation of timing and definition of optimal time ranges for cleavage of viable embryos are complicated by the influence of different culture conditions on cell cycle lengths and timing of development. In mice, culture in 20% oxygen significantly delays all stages of embryo development compared with culture in 5% oxygen (Wale and Gardner, 2010). The choice of medium has been shown to influence

cleavage rates for human embryos (Van Langendonckt et al., 2001; Ben-Yosef et al., 2004; Zollner et al., 2004; Sifer et al., 2009). Furthermore, some studies indicate that the fertilization method can influence timing of the first cleavage, with a higher percentage of ICSI than IVF fertilized embryos having early cleavage (Lundin et al., 2001; Giorgetti et al., 2007), although reaching 4-cell stage at the same time (Lemmen et al., 2008). This impact of fertilization method on timing of events in human embryos was not confirmed by Mio and Maeda (2008) who found only small differences between the groups, without elaborating further on the findings. Cryopreservation and thawing do not seem to have a delaying effect on developmental kinetics, although their impact has only been studied by comparing the development of a small number of frozen 2PN embryos to fresh 3PN embryos (Wong et al., 2010).

Fragmentation and evenness of blastomeres

Two distinct patterns of fragmentation have been documented by time-lapse analysis in human embryos (Van Blerkom et al., 2001): definitive fragmentation and transient pseudo fragmentation. The observation that fragments can be resorbed in human embryos has been confirmed subsequently by other time-lapse studies (Hardarson et al., 2002; Lemmen et al., 2008). Moreover, it has been observed that the resorption of fragments occurs mostly in human embryos with normal first cytokinesis and only moderate degree of fragmentation, whereas fragmentation in embryos with abnormal first cytokinesis seldom reverses (Wong et al., 2010). Normal cytokinesis was in this study defined as initiating and completing first cytokinesis in a smooth controlled manner over a narrow time window (14.3 $\pm$ 6.0 min), whereas abnormal cytokinesis was prolonged or consisted of unusual morphological behaviour followed by fragmentation. Also blastomere evenness can improve over time since both fragmentation and blastomere unevenness may be most pronounced during or just after cleavage (Lemmen et al., 2008). As recently shown in mouse embryos (Pribenszky et al., 2010) daily or bi-daily evaluation will underestimate the extent of fragmentation. However, in this study the authors recorded fragmentation as either absent or present, as opposed to reporting the degree of fragmentation, as in most scoring schemes used clinically. Whether the resorption of fragments would have an impact on total embryo score and selection was thus not evaluated. Montag et al. (2011) found that embryo scoring using static parameters can change over time, but since their scoring of morphology on Day 2/3 included many parameters, the individual significance of fragment resorption cannot be estimated. In summary, time-lapse studies have shown that fragment resorption occurs, although the significance and incidence of the event remain to be elucidated.

Blastocyst kinetics *in vitro*

Some studies indicate that there are substantial differences between the course of hatching *in vitro* and *in vivo* (Montag et al., 2000; O'Sullivan et al., 2002). *In vitro* formation of the blastocoel, subsequent expansion and the repeated partial or complete collapse and expansion of the blastocyst ending with hatching have been described in different species including humans using time-lapse equipment (Massip and Mulnard, 1980; Massip et al., 1982; Gonzales et al., 1996; Holm et al., 1998; Mio and Maeda, 2008). Pulsatile movements have been described in a variety of species (Lewis and Gregory, 1929; Gonzales et al., 1996; Holm et al., 1998). The continuing expansion and resultant thinning of zona pellucida has been considered to be the most likely cause of hatching *in vitro*. Some have regarded the pulsatile movements as a normal phenomenon that does not influence the hatching process, while others have reported that the frequent interruption of expansion due to repeated contractions disturbs the hatching process (Massip and Mulnard, 1980; Massip et al., 1982). Cytoplasmic extension of the trophectoderm (trophectoderm projections), suggested as a method of hatching, has been visualized using time-lapse monitoring and shown to be expressed in a variety of mammals, including humans (Gonzales et al., 1996). Time-lapse analysis of mouse embryos after blastomere removal revealed that the number of contractions and expansions in the interval between blastocyst formation and hatching was significantly increased compared with a non-biopsied control group (Ugajin et al., 2010), whereas this finding was not confirmed in a larger study of human embryos biopsied at the cleavage stage (Kirkegaard et al., 2012). Whether blastocyst kinetics can be related to embryonic implantation potential remains to be investigated.

Parameters and nomenclature for clinical embryo evaluation

Time-lapse recording introduces several dynamic morphological parameters for embryo evaluation. However, the data analysis and comparison between studies are complicated due to diverging nomenclature and definitions of events and their timing. Consensus on how to collect and report data is therefore desirable. Division and cleavage cycle are often confused. Based on how the kinetics is most commonly reported in the literature on time-lapse studies we suggest the following designations: a division, or cleavage, is the mitotic event leading to the formation of two cells from one cell, whereas a cleavage cycle refers to the cluster of developmentally consistent cleavages, where the cell number is doubled (Grisart et al., 1994; Gonzales et al., 1995; Wong et al., 2010). The first three divisions/cleavages thus yield a 4-cell embryo from a 1-cell embryo, whereas the first three cleavage cycles yield a 2-cell embryo (first cleavage cycle), a 4-cell embryo (second cleavage cycle) and an 8-cell embryo (third cleavage cycle), respectively.

An objective evaluation of absolute time points for developmental event requires that the starting point of the observation is precisely defined. In animal studies this goal is difficult to achieve, as illustrated by the various starting points in the animal studies listed in Table I which complicate comparison of absolute time points for developmental events. In human studies, time of fertilization is mostly chosen as the starting point for observations. Time of fertilization can be recorded precisely in ICSI embryos as the time of injection, but may be difficult to assess for IVF embryos and frozen/thawed embryos, thereby increasing the measurement uncertainty. To some extent this limitation may be overcome by registering time from first cleavage, or preferably duration of events, rather than absolute time points (Wong et al., 2010). Another solution is to make the analysis on ICSI embryos only (Payne et al., 1997; Meseguer et al., 2011).

It is also crucial that the parameter is well defined and that the evaluation is consistently independent of the person performing the

Table V Proposed clinical parameters for time-lapse analysis.

Proposed parameter	Description	Definitions
First cytokinesis	Time point and duration of the first cytokinesis	Time from appearance of cleavage furrow at the 1-cell stage to complete separation of the two daughter cells by a cytoplasm membrane
Cleavage pattern	Time point for each cell division until compaction stage	A cell division is defined as the first time point when the two daughter cells are completely separated
Synchronicity	Time from beginning of one cleavage cycle till the beginning of the next	First cleavage cycle: Duration of the 1-cell stage. Second cleavage cycle: Duration of the 3-cell stage. Third cleavage cycle: Duration of the 5–7-cell stage
Embryo kinetics	Time points and duration of compaction, morula and blastocyst stages	Compaction is the first time point when fusion of at least two cells is observed and the diameter decreases Morula is the time point when all cells have fused Blastocyst is the first time point when a blastocoel is visible Full blastocyst is the time point when the blastocoel fills out the embryo Hatching blastocyst is the time point when zona breaches and a hatched blastocyst is the first time point when the blastocyst has fully escaped zona pellucida
Blastocoel pattern	Time points, duration and number of collapses of the blastocoel. Extent of collapses	Collapse should be defined as the time point the measured diameter is smaller than the diameter at the previous time point, full recovery to be defined as the time point, when the blastocyst diameter is identical to the diameter just before collapse. Extent of the collapse is the largest diameter minus the smallest diameter, and number of collapses can be defined as the number per 24 h from appearance of a blastocoel until the end of culture or hatching
Nuclei	Time points for appearance and disappearance of nuclei	The first time point a nuclei is visible or non-visible, respectively

evaluation. Whereas observer assessment may be subject to interpretation, an automated computer-based tracking algorithm of developmental events, which becomes feasible with time-lapse monitoring, has the potential to simplify and objectify the analysis, provided that reproducibility of the analysis is ensured (Ramsing and Callesen, 2006; Ramsing *et al.*, 2007; Wong *et al.*, 2010).

Constant surveillance of embryo development will also reveal events with a known relation to poor implantation or early pregnancy loss which is a temporary condition easily overlooked in a static evaluation, e.g. bi- and multi-nucleation.

Table V lists our suggestions for parameters that may be included in a time-lapse evaluation.

Safety of time-lapse analysis

An important issue to consider before implementing time-lapse analysis in a clinical setting is the safety of the instrument. Time-lapse imaging necessitates periodical exposure to light. It has been shown that extensive light exposure may be detrimental to embryo development, and especially that short wavelength light exposure should be minimized (Oh *et al.*, 2007; Ottosen *et al.*, 2007a,b; Takenaka *et al.*, 2007). If the time-lapse solution incorporates moving parts or moving of embryos, then heat accumulation due to motion and friction may theoretically be an issue along with sheer stress to the embryos and the presence of lubricants and fumes from lubricants. The complexity of and access to maintenance, risk of pollution with infectious agents and consequences of potential breakdowns must also be considered. Moreover, the continuous presence of electromagnetic fields found in some time-lapse systems can affect embryo development (Cameron *et al.*, 1985; Beraldi *et al.*, 2003). Stability of the culture conditions is another important factor that must be considered, since it can vary between different designs of time-lapse instruments. Comparisons have been made between embryos, both mouse and human, cultured in conventional incubators and time-lapse instruments, without any adverse effects on development or implantation rate having been demonstrated (Holm *et al.*, 1998; Lemmen *et al.*, 2008; Mio and Maeda, 2008; Nakahara *et al.*, 2010; Pribenszky *et al.*, 2010; Wong *et al.*, 2010). However, these reports have been supplementary observations in descriptive studies. In a recent study, embryo quality, blastocyst and ongoing pregnancy rates were compared in a clinical time-lapse incubator and a standard incubator (Cruz *et al.*, 2011). The study included data from 478 embryos arising from donated oocytes and concluded that incubation in the clinical time-lapse instrument was equivalent to incubation in a standard incubator, although recognizing that the study was not randomized and that donated oocytes may not be representative of oocytes from an infertile population. Our own recent data from a two-centre, randomized, controlled, clinical trial including 676 oocytes comparing the culture of human IVF oocytes in the same clinical time-lapse instrument or in a conventional incubator support these findings (manuscript submitted). However, given the importance of the subject, the safety must be evaluated continuously.

Conclusion

Time-lapse analysis offers the possibility to monitor embryo development continuously, providing a novel non-invasive method to increase the precision and sensitivity of current morphological evaluation and introducing potential dynamic markers. Several putative markers of viability have been suggested based on retrospective studies both on

animals and humans. The studies provide no unambiguous information regarding what parameters are predictive. Several of the parameters identified as predictive for pregnancy or development have been evaluated in human studies using time-lapse systems with short intervals between image acquisition and their applicability to clinical systems with longer time intervals between recordings is questionable. Nonetheless, it seems that human embryos with high developmental potential display a uniform pattern of optimal time ranges for the early cell divisions. Identification of the embryo most likely to develop into a blastocyst by the timing of early developmental events may allow embryos with high developmental potential to be selected for early transfer thus avoiding prolonged *in vitro* culture (Wong *et al.*, 2010). Moreover, it appears that time-lapse monitoring can be used to exclude embryos that would be deemed viable using a static evaluation, but which display aberrant cleavage patterns. To identify embryos with the highest pregnancy potential, a hierarchical predictive model was recently proposed (Meseguer *et al.*, 2011), combining static evaluation with assessment of dynamic parameters. Yet, it remains to be elucidated whether the increased precision of embryo evaluation by time-lapse monitoring improves pregnancy rates. To address this question larger randomized clinical studies are needed.

Authors' roles

K.K. did the literature searches and wrote the initial draft. J.I. and I.A. cross-checked the literature searches and edited the manuscript. All authors approved the final manuscript.

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Conflict of interest

J.I. and K.K. declare no conflict of interest. I.A. works part-time as a scientific consultant for Unisense FertiliTech and holds stocks in the company.

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