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The clinical use of time-lapse in human-assisted reproduction

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***Abstract*:** A major challenge in the assisted reproduction laboratory is to set up reproducible and efficient criteria to identify the embryo with the highest developmental potential. Over the years, several methods have been used worldwide with this purpose. Initially, standard morphology assessment was the only available strategy. It is now universally recognized that besides being a very subjective embryo selection strategy, morphology evaluation alone has a very poor prognostic value. More recently, the availability of time-lapse incubators allowed a continuous monitoring of human embryo development. This technology has spread quickly and many fertility clinics over the world produced a remarkable amount of data. To date, however, a general consensus on which variables, or combination of variables, should play a central role in embryo selection is still lacking. Many confounding factors, concerning both patient features and clinical and biological procedures, have been observed to influence embryo development. In addition, several studies have reported unexpected positive outcomes, even in the presence of abnormal developmental criteria. While it does not seem that time-lapse technology is ready to entirely replace the more invasive preimplantation genetic testing in identifying the embryo with the highest implantation potential, it is certainly true that its application is rapidly growing, becoming progressively more accurate. Studies involving artificial intelligence and deep-learning models as well as combining morphokinetic with other non-invasive markers of embryo development, are currently ongoing, raising hopes for its successful applicability for clinical purpose in the near future. The present review mainly focuses on data published starting from the first decade of 2000, when time-lapse technology was introduced as a routine clinical practice in the infertility centers.

Keywords: assisted reproductive technology, embryo culture, embryo development, embryo morphology, embryo selection, morphokinetics, time-lapse technology

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Introduction

Historically, *in vitro* embryo development was assessed by means of morphological evaluations performed at specific time points. This procedure is not able to identify subtle variations in embryo development and is extremely operator dependent. For these reasons, in 2011, a consensus was drawn with the aim to standardize embryo morphology evaluation across different laboratories.[1] Specific criteria concerning embryo characteristics, such as pronuclei morphology, stage-specific blastomere number and symmetry, multinucleation, blastocyst expansion, and quality of trophectoderm and inner cell mass, were established. Despite that, standard morphology evaluation alone remains a very limited embryo selection strategy.[2]

Since the introduction of time-lapse technology (TLT) *in vitro* fertilization (IVF) laboratories, a large amount of scientific studies has been quickly published worldwide.[3] Several types of TLT equipment became available, allowing the real-time observation of human embryo development without disturbing culture conditions and generating a wealth of morphokinetics data. In a few years, a very high number of models promising to be able to identify the embryo with the highest probability to develop, to be euploid or to implant, have been

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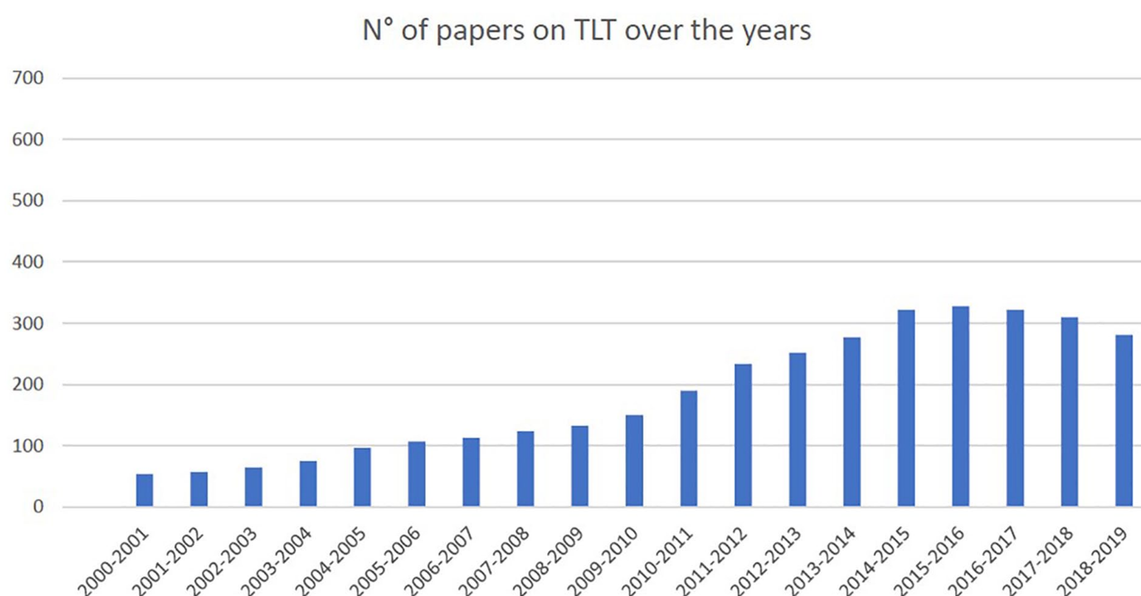

Figure 1. Number of publications on time-lapse technology reported in PubMed® in the last decade.

proposed. Despite the ever-growing interest in this field, the use of this technology to improve clinical outcomes remains controversial.[4–6]

The present review aims to summarize the state of the art concerning the clinical use of TLT in the field of human reproduction. The interest in the applicability of TLT in the field of human embryo development is still intense, and the number of publications on this topic in the last decade has remained constant (Figure 1).

Time-lapse system

A TLT system consists of an incubator with integrated microscope and cameras connected to an external computer. Alternatively, the optical system can be placed inside a standard incubator (SI). Embryo images are captured at defined time intervals (ranging from 5 to 10 min) and on different focal planes (up to 11) for the entire duration of the culture. The acquired stills are collected into a video which allows a detailed morphological and morphokinetic evaluation of each embryo's ongoing development. Several types of TLT systems are commercially available, and some of them are equipped with automated or semi-automated software that simplifies the annotation procedure.

Morphokinetic parameters

The most widely recorded morphokinetic parameters are the second polar body extrusion (tPB2); pronuclear (PN) appearance (tPNa) and fading (tPNf); and cellular division from 2- to 9-cell or more (t2, t3, t4, t5, t6, t7, t8, t9+). The time from PN fading to the first mitotic division is defined as t1. The rounds of cleavage are also considered: times between 2- and 3-cell stages (cc2, second round calculated as t3-t2), between 3- and 5-cell stages (cc3, third round calculated as t5-t3) and between 5- and 9-cell stages (cc4, fourth round calculated as t9-t5). In addition, TLT allows to assess the synchrony of cellular divisions defined as s2 (calculated as t4-t3) and as s3 (calculated as t8-t5). Finally, the timing of starting compaction (tSC), morulae formation (tM), starting blastulation (tSB), full blastocyst stage (tB or tFB), blastocyst expansion (tEB), and hatching (tHB) are annotated. The time of intracytoplasmic sperm injection (ICSI) is usually considered as the starting time point (t0), and values are expressed as hours post insemination (hpi). In some studies in which embryos obtained by means of both ICSI and standard IVF are enrolled, different reference time points, such as PNa or PNf, are identified to perform more accurate comparison of embryo development.[2,7]

Time-lapse versus SIs

One of the advantages of TLT is the ability to examine embryos in real time, without perturbing culture conditions, thereby facilitating the identification of subtle developmental abnormalities. In addition, the technology makes it possible to standardize both culture and embryo assessments by means of consultation and teaching among embryologists. Several studies investigated the clinical outcomes of medically assisted reproductive (MAR) cycles, comparing TLT to SIs.[4] A prospective randomized study analyzed blastocyst development and clinical outcomes of 64 single-embryo transfers performed in good prognosis patients to assess if the use of TLT compared to SI incubators can be advantageous in the clinical use. No statistical differences were found in terms of blastocyst quality, implantation, and pregnancy rates between the two groups.[8] The randomized control trial by Park and colleagues[9] confirmed this result, finding comparable embryo development, morphological quality, implantation, and pregnancy rates in 1979 and 1000 injected oocytes cultured in TLT or SI systems, respectively. Conversely, improved clinical pregnancy (65.7% vs 39%, $p < 0.001$), ongoing (55.7% vs 31.3 %, $p < 0.001$), and live birth (45.7% vs 28.4%, $p = 0.01$) rates were obtained in 239 ICSI cycles when embryos were assessed by means of TLT compared to morphological evaluation alone.[10] Embryo quality at Day 3 was found to be worse in TLT compared to SI systems (top-quality embryos were 55.8 ± 6.4% vs 81.2 ± 4.1% in TLT and SI, respectively; $p = 0.0005$). The sample size of this study, however, was very small (only 76 embryos were included in the analysis) and no differences were found in terms of implantation and clinical pregnancy rates.[11] A prospective study performed on 843 infertile couples found increased ongoing pregnancy in the TLT (51.4%; 95% confidence interval [CI], 46.7–56.0) compared to the SI group (41.7%; 95% CI, 36.9–46.5). Similar results were observed for implantation rates (44.9%; 95% CI, 41.4–48.4 vs 37.1%; 95% CI, 33.6–40.7), while the miscarriage rate was lower, when embryos were cultured in TLT (16.6%; 95% CI, 12.6–21.4) compared to SI (25.8%; 95% CI, 20.6–31.9) systems.[12] Conversely, in the study by Park and colleagues[9] the abortion rate was found to be significantly higher in the TLT group than in the SI cohort (33.3 vs 10.2%, $p = 0.01$, respectively). Clinical pregnancy and implantation rates were comparable also in another randomized trial performed on 235 patients with embryos cultured either in TLT or SI systems, leading the authors to conclude that the addition of morphokinetic data did not improve clinical outcomes.[13] Recently, Kalleas and colleagues[14] compared live birth rates obtained after undisturbed, low-oxygen embryo culture in the TLT system or state-of-the benchtop incubator. The development of 243 and 203 embryos cultured in the two systems were analyzed, respectively. The chance of live birth resulted significantly increased (43% vs 34.5%; odds ratio [OR] = 1.43; 95% CI; 0.96–2.13) and the early pregnancy loss reduced (5.8% vs 13.8%; OR = 0.37; 95% CI; 0.19–0.74) in TLT compared to benchtop incubators. Finally, a higher proportion of 4-cell and 8-cell embryos were obtained in day-2 and day-3 cultures in TLT compared to benchtop incubators. A very similar study performed on 386 patients showed higher percentages of day-2 top-quality embryos (40.4% vs 35.2%) and frozen embryos (29.5% vs 24.8%) with the use of TLT compared to benchtop incubators while no differences were found between the two culture systems in implantation, miscarriage, clinical, and ongoing pregnancy rates for fresh embryo transfers. Cumulative data, however, are not available.[15]

Automated image analysis

Several studies examined potential advantages of stable culture combined with the use of automated image analysis and its effect on biological and clinical outcomes. A prospective, multicenter clinical study performed on 1825 embryos obtained from 160 infertile IVF patients showed that computer-automated cell-tracking software, combined with standard morphology evaluation on day 3, significantly improves the experienced embryologist's ability to identify embryos with the highest chances to develop to blastocyst stage (from 18.3 ± 23.3% to 68.2 ± 1.7%, $p < 0.05$). In addition, it has been noted that the agreement among embryologists in embryo assessment was more consistent and the variability was reduced.[16] Applying the same technology on embryos obtained from 205 patients treated in six clinics, VerMilyea and colleagues[17] found that embryos showing medium or high scores resulted in improved implantation and pregnancy rates, compared to lower scores (37% and 35% versus 15%; $p < 0.0001$ and $p = 0.0004$, respectively). Another study carried out on 319 patients, analyzing the efficacy of automated TLT

systems combined with standard morphology, detected enhanced implantation (30.2% vs 19.0%) and clinical pregnancy (46.0% vs 32.1%) rates after day-3 embryo transfers using TLT technology compared to standard morphology alone.[18] In addition, high-quality embryos selected according to TLT criteria showed a significantly higher implantation rate compared to low-quality ones (44.7% vs 20.5%, respectively). This outcome, however, remains controversial. For example, a study by Kaser and colleagues[19] on 163 patients showed that the use of image analysis software in addition to conventional morphology evaluations did not improve pregnancy rates on day 3 or day 5.

Clinical outcomes

In order to clarify the efficacy of the use of TLT on clinical outcomes, a systematic review including five randomized controlled trials performed on 1637 patients was carried out.[20] In this study, TLT was associated with improved ongoing clinical pregnancy (51.0% vs 39.9%; OR: 1.542; $p < 0.001$) and live birth (44.2% vs 31.3%; OR 1.668; $p = 0.009$) rates, as well as with reduced miscarriage rates (15.3% vs 21.3%; OR: 0.662; $p = 0.019$) compared to standard morphology assessment. The authors, however, pointed out a significant heterogeneity among the studies included in the analysis, such as patient population, day of transfer, time-lapse devices, culture conditions and that the quality of the evidence ranged from moderate to low. Thus, while pointing to possible benefits of TLT applied to MAR cycles in improving clinical outcomes and reducing the time to pregnancy, they highlighted the need for further studies. Similarly, analyzing the outcomes of 1882 cycles, Mascarenhas and colleagues,[21] observed an improved live birth rate for fresh transfers when embryos were cultured in TLT with respect to SI incubators, although cumulative live birth rate was similar between the two systems. In addition, a lower risk for preterm birth and low birthweight was associated with TLT compared to SI cultures. The meta-analysis by Magdi and colleagues[22] enrolling 2057 patients, observed an improvement in live birth (OR = 1.43; 95% CI, 1.10–1.85; $p = 0.007$) and a decrease in early pregnancy loss (OR = 0.71; 95% CI, 0.52–0.97; $p = 0.03$) when TLT was compared to standard evaluation of embryo development, while no significant differences were found for implantation, ongoing, and clinical pregnancy rates. The authors, however, remarked that the quality of the evidence is low and that outcomes should be evaluated with caution due to the statistical and clinical heterogeneity of the data. The meta-analysis by Chen and colleagues[23] including 10 control trials, 4 of them randomized for oocytes and 6 for women, found no differences between TLT and SI control groups concerning blastocyst, ongoing pregnancy and live birth rates. The quality of evidence was moderate and low or very low for oocyte-based and woman-based reviews, respectively.

The Cochrane review published in 2019[4] compared the use of TLT technology, with or without the aid of automated software, to SIs combined with conventional morphological assessment of embryo development, to clarify if the former can lead to improved pregnancy, live birth, and miscarriage rates. A total of eight randomized control trials on 2303 women were included in the analysis, finding the quality of evidence from very low to moderate for all the comparisons performed. In addition, authors reported a high heterogeneity of the collected data: trials included both IVF and ICSI insemination methods, the use of autologous or heterologous oocytes, frozen and fresh or single and multiple embryo transfers as well as different days of embryo transfer. Finally, information about cumulative pregnancy rate is entirely missing. For all these reasons, the authors concluded that the evidence is insufficient to choose between the two incubation systems, confirming the results of the previous Cochrane published by the same authors on the same topic.[24]

Algorithms

Blastocyst formation and implantation models

Several studies have proposed different models based on morphokinetic assessments of embryo development, aiming to establish an efficient embryo selection strategy. The first algorithm promising to predict blastocyst formation based on cell divisions until day-2 of culture was proposed in 2010.[25] Analyzing a total of 242 embryos, the authors found that the duration of the first cytokinesis, the time between first and second mitoses and the synchronicity in the formation of the four granddaughter cells are strictly correlated with the progression to blastocyst stage. In 2011, Meseguer and colleagues[26] developed the first model able to predict the likelihood of embryo

implantation. The authors retrospectively analyzed the morphokinetic development of 247 transferred embryos with known implantation data (KID), identifying 10 categories of embryos with increasing implantation potential. This hierarchical classification was mainly based on the following embryo features: (a) morphological screening; (b) absence of exclusion criteria (i.e. abrupt division from 1 to 3 or more cells, 2-cell stage asymmetry and multinucleation at 4-cell stage); (c) t5; (d) duration of s2; and (e) duration of cc2. Subsequently, the morphokinetic development of 528 transferred KID embryos was analyzed with the aim to retrospectively validate the model.[27] However, the distribution of implantation rates was more heterogeneous than that proposed in the published algorithm. In addition, it was found that the model performed differently with cleavage or blastocyst-stage embryo transfers. Thus, the authors concluded that the model has low efficiency in predicting embryo implantation potential, hypothesizing that in-house algorithms built on a center's own data could prove more accurate. In 2015, a new version of Meseguer's model was proposed by the same group.[28] This second multicentric retrospective study was divided in two parts. First, a new algorithm based on 1289 transferred embryos, among which 754 were KID, was generated. Next, analyzing the results obtained after the transfers of 1122 KID embryos, the algorithm's efficiency in predicting implantation was tested. Compared to the previous study, the authors found that most of the previously identified morphokinetic features were confirmed, although their relevance changed: t3 became the most important variable, followed by cc2 and t5; s2 turned out to be irrelevant, while all exclusion criteria were confirmed. The implantation rate significantly decreased from the first to the fifth selection category. Thanks to the multicentric design, this study demonstrated the applicability of the model to different centers, at least those belonging to the same group, as they share the same procedures and protocols. Again, two research groups tested independently this new algorithm on their own data.[29,30] In both studies, preimplantation genetic testing (PGT) results obtained after the biopsy of, respectively, 167[29] and 256[30] blastocysts were analyzed. Basile's model failed to discriminate between normal and abnormal embryos in both data sets. The ability of the deselection criteria in identifying implantation potential was tested in a study performed on 270 KID embryos obtained with both ICSI or IVF, using pronuclear fading as the reference starting time point.[31] This model, combining qualitative and quantitative embryo parameters, found that direct cleavage from 1 to $\geqslant$3 cells, reverse cleavage, that is, when two or more blastomeres merge, less than six intercellular contact points at 4-cell stage as well as poor embryo quality on day 3 and $<$8 cells at 68 hpi allow to rank embryos into seven categories, with decreased implantation potential. On the contrary, multinucleation at 2- or 4-cell stages was not found to be associated with implantation outcome. The efficiency of this model was then prospectively tested on 66 KID embryos cultured in two different media.[31]

Other studies evaluated the correlation between several cellular time points and the likelihood of an embryo to develop until blastocyst stage or until implant. From the TLT analysis of 244 embryos with known final destiny (implanted, not implanted, or arrested), it was found that s3, t1, t2, t4, t8, tPNa, and tPNf were predictive of blastocyst formation but not of implantation, which significantly correlated only with cc3 value.[32] A combination of morphological and morphokinetic evaluations of 274 KID embryos obtained in 165 egg donation cycles showed that synchrony at 2-cell stage as well as a normal first-cleavage pattern combined with good standard morphology evaluations can be predictive of increased blastocyst formation, even if they are not correlated with implantation outcome.[33] Dal Canto and colleagues,[34] analyzing the development of 459 embryos found that shorter t7 and t8 times distinguish embryos reaching blastocyst stage from blocked ones and that only t8, but not t7 values also correlated with implantation.

The efficiency of six previously developed models in predicting implantation[28,32–36] was retrospectively tested on a set of 977 KID embryos, obtained by means of both standard IVF or ICSI.[37] The positive and negative predictive values of each model varied from 38.29% to 44.28% and from 61.10% to 76.19%, respectively. The sensitivity and the specificity ranged from 16.70% and 98.67% and from 2.67% to 85.83%, respectively. Overall, these outcomes indicate a poor prognostic value of all proposed models and point out the need for the development of in-house algorithms, taking into account internal procedures.

In 2016, Petersen and colleagues[38] drew up a new promising model claiming to be able to discriminate embryos according to their probability to reach blastocyst stage. This retrospective study was performed on 3275 KID embryos transferred on day 3 in 24 IVF centers. First, six annotations, that is, 2PN, tPNf, t2, t3, t5, and t8, were used to build the decision tree, ranking the embryos in five groups according to their implantation potential. Subsequently, the developed algorithm, named KID-score, was tested on morphokinetic data obtained from a different group of 11,218 embryos in 31 clinics and cultured until day 5. As a multicentric study involving embryos cultured with low or atmospheric oxygen, with different culture media and obtained with both standard IVF or ICSI, this algorithm promises to be applicable independently of culture conditions and fertilization method.

More recently, a multicenter study analyzing the development of a total of 830 euploid blastocysts demonstrated that the combination of time of morulation with trophectoderm morphological evaluation is a good indicator of live birth.[39] This study was divided into two steps. During the training phase, 511 vitrified-warmed euploid blastocysts obtained in two IVF centers were used to define the features associated with live birth after a single-embryo transfer. During the validation phase, 319 vitrified-warmed euploid blastocysts obtained in three IVF centers were used to test the consistency and reproducibility of the previous model. It was found that euploid blastocysts showing tM < 80 hpi and high trophectoderm quality lead to a significantly higher live birth rate than those with tM > 80 hpi and low-quality trophectoderm (55.2% vs 25.5%, respectively). The authors concluded that this outcome is reproducible across different centers under specific culture conditions, such as individual culture in single-step medium at 37°C, 6% CO_2, and 5% O_2.[39]

Generally, the majority of the studies highlighted that faster embryos have an improved chance to become blastocysts. Storr and colleagues[40] analyzed the concordance among several proposed algorithms in selecting the best embryo to transfer as well as the agreement between these decision trees and the embryologist's choice. High variability and low concordance were found in both analyses. Overall, the poor consistency obtained in independently developed models warns against generalizing the applicability of a single algorithm, highlighting the need for in-house validation before clinical use.[40] Accordingly, Zaninovic and colleagues, comparing the morphokinetic development of more than 20,000 embryos from two different large data sets, concluded that TLT is still not ready to be universally used, highlighting the need to create *ad hoc* models, based on specific laboratory and clinical characteristics.[41]

Aneuploidy risk

One of the first model based on morphokinetic evaluation of human embryos, which promises to be able to perform a classification of aneuploidy risk, was proposed in 2013 by Campbell and colleagues.[35] A total of 98 biopsied blastocysts, obtained on day 5 or on day 6 of culture, were enrolled in this retrospective study. It was observed that tSC, tSB, and tB were delayed in aneuploid compared to euploid blastocysts. No significant differences concerning either the first cell cycles or multinucleation or irregular division patterns were found. The authors concluded that this non-invasive approach could have been proposed to patients instead of PGT, allowing infertility centers lacking this skill to select the embryos non-invasively. Subsequently, Campbell's aneuploidy risk model was applied by others on their own data sets, in order to test its ability to distinguish embryos on the basis of their ploidy.[30,42,43] Kramer and colleagues[42] retrospectively analyzed the morphokinetic development of 149 blastocysts obtained by means of either IVF or ICSI and biopsied in day-5 or day-6 cultures. The proposed model, however, failed to discriminate aneuploid embryos: the timing of blastocele formation and blastocyst expansion differed significantly from the predicted values. In addition, the authors found high variability among patients of the embryos' developmental times, arguing that morphokinetic clinical selection is probably not accurate enough.[42] The outcome of the longitudinal cohort study by Rienzi and colleagues[43] is in agreement with this conclusion. The correlation between morphokinetic characteristics and embryo ploidy was tested on 455 biopsied blastocysts from poor-prognosis patients, that is, ideal candidates for PGT, finding no statistical correlation between aneuploidy and the most commonly tested embryo developmental timings. In addition, Campbell's aforementioned model was also tested, and again it failed in predicting embryo

ploidy.[43] Finally, Zhang and colleagues[30] applied Campbell's model to the same 256 blastocysts used to test the Basile's model, and again euploid and aneuploid embryos showed similar patterns of distribution.

Other studies evaluated the correlation between ploidy and morphokinetic behavior of the embryos.[6,7,44] The analysis of 185 blastomeres obtained from 45 disassembled 4-cell human embryos showed that euploid embryos have stricter cell-cycle parameters compared to aneuploid ones and that fragmentation can contribute to chromosomal abnormality.[45] The mean timing points tPNf, t2, t5, cc2, and cc3, as well as the duration of the 3-cell stage (t5-t2) were found to be significantly different according to the ploidy in 496 biopsied cleavage-stage embryos, although no statistical differences were observed for tPB2, tPNa, t3, t4, and s2.[46] Vera-Rodriguez and colleagues[47] evaluated all blastomeres from 85 human embryos until day 3 of culture. The timing between PNf and the beginning of the first cell division resulted in the most relevant feature in distinguishing euploid and aneuploid embryos, the former being significantly shorter. This outcome is in accordance with another study performed on 159 zygotes evaluating the assessment of pronuclear morphology and dynamicity as predictors of live births.[36] All zygotes were scored according to six different previously published models, taking into account their shape, size, and position as well as the number, distribution, and polarization of nuclear precursor bodies. All assessments were repeated at three different time points after fertilization. The timing of pronuclear breakdown was identified as the ideal stage for the evaluation, occurring significantly later in embryos leading to live births.[36] Minasi and colleagues[48] analyzing data from 1730 biopsied blastocysts found that embryo ploidy is positively correlated with top-quality trophectoderm and inner cell mass, high degree of expansion, and reduced time to initiate blastulation, but not with cell division until the cleavage stage. Another analysis performed on 767 biopsied blastocysts, showed that early embryo development is predictive only of blastocyst formation but not of euploid rates, while tSB, tEB, and the tEB–tSB interval correlated with chromosomal status.[49] Another retrospective study performed on 416 biopsied blastocysts showed that t9, tM, tSB, tB, and tEB are delayed in aneuploid compared to euploid blastocysts, when taking into account only patient-related factors.[50] In contrast, a logistic regression analysis performed on 485 biopsied embryos identified t3 and t5–t2 as the best predictors of chromosomal normality.[51] Kimelman and colleagues[52] analyzed 2292 embryos from 524 patients and observed significantly longer timings for each morphokinetic variable for blastocysts obtained on day 6 compared to day 5 of culture. Importantly, there were no statistical differences in aneuploidy rates between the two groups. Both t7 and t8 values were independent predictors of euploidy ($p < 0.015$ and $p < 0.014$, respectively), after adjusting for the day of blastocyst formation and biopsy. Transferring day-5 blastocysts, however, led to improved pregnancy and live birth rates, compared to day-6 embryos ($p = 0.0033$ and $p = 0.0359$, respectively) in cycles without PGT. These differences disappeared when PGT was performed. A systematic review of 13 studies found high heterogeneity concerning the study design, patient population, stage of biopsy, statistical approach, and outcome measures, and none of the morphokinetic parameters, alone or combined, were strongly associated with embryo ploidy. The authors concluded that, to date, the predictive ability of TLT in identifying euploid embryos is poor and therefore morphokinetic should not be applied to screen embryo ploidy.[44]

Confounding factors

In recent years, an increasing number of studies reported how the embryo's morphokinetic development can be influenced by many confounding factors related to patient's features or clinical and biological procedures.[2,27–30,37–43]

Female age and stimulation protocol

Analyzing the developmental behavior of 1507 embryos, Kirkegaard and colleagues[53] noted that several patient-related features affect morphokinetic parameters, more at the blastocyst than embryo stage. In particular, tEB was significantly slower in older compared to younger women (0.29 hr/year; 95% CI, 0.03, 0.56), in association with increased follicle-stimulating hormone (FSH) dosage (0.12 hr/100 IU FSH; 95% CI, 0.01, 0.24) and with the number of previous attempts (1.2 hr/attempt; 95% CI, 0.01, 2.5). Similarly, tEB was delayed when high doses of FSH were administered

(0.14 hr/100 IU FSH; 95% CI, 0.03, 0.27) and with increasing numbers of previous IVF cycles (1.4 hr/attempt (0.10, 2.7)). Embryos obtained by means of ICSI procedures performed the first cell division faster compared to IVF ones (23.6%; 95% CI, 26.4, 20.77), while no differences were found for subsequent cleavages. Univariate regression analyses showed that female age, total FSH dosage, blastocyst expansion, inner cell mass quality, and tFB were predictors of live birth. After adjusting for age, previous IVF cycles, and cumulative FSH dosage, however, tFB did lose statistical significance. Overall, it was found that up to 31% of morphokinetic timing variability could be related to embryo origin, although no single variable seems to exert a specific effect.[53] The study by Mumusoglu and colleagues[50] pointed out that several patient-related characteristics, such as body mass index (BMI), total FSH dosage, time of infertility, number of previous IVF cycles, antral follicle count, ovarian stimulation protocol, and estradiol level on the day of the trigger can significantly influence embryo morphokinetic development. The effects of FSH dosage and of estradiol concentration on the development of 2132 embryos were analyzed.[54] It was found that cleavage rate is inversely proportional to FSH dosage and that estradiol concentration levels significantly affect embryo development, especially at the blastocyst stage. In contrast, no association was found between serum progesterone levels and embryo development. Another study performed by the same authors on 2817 embryos found faster embryo cleavage rates in patients treated with gonadotropin-releasing hormone (GnRH) antagonist + agonist compared to patients treated with GnRH agonist + human chorionic gonadotropin (hCG). This effect was significant in the first stages of development, disappearing as embryo development proceeded.[55]

Lifestyle

Female smoking was also reported to negatively affect embryo development. Evaluating a total of 135 infertile couples in 23 of which the female partner was a smoker, it was found that most cell divisions were significantly delayed in the smokers' group.[56] Salvarci and colleagues[57] in a study enrolling 257 couples reported that the number of retrieved, mature, and fertilized oocytes and the number of transferred embryos as well as pregnancy and ongoing pregnancy rates was significantly lower in smoking compared to nonsmoking women. In addition, variability in morphokinetic development was found for PNa, PNf, t2, t8 and t9+ between the two groups. The embryo development of nonsmoking women compared to smokers, as well as in GnRH agonist compared to GnRH-antagonist groups, was found to be more "in range" in the study by Siristatidis and colleagues[10] on 239 women.

BMI is another factor that has been reported to influence embryo morphokinetics, although with controversial results. Analyzing the development of 218 injected oocytes, Leary and colleagues[58] found that gametes retrieved from overweight or obese women are smaller, and the derived embryos reach morula stage faster compared to those obtained from normal-weight patients. In contrast, a recent study including 1528 patients reported that t5 was longer in overweight women and t5 with t8 was longer in obese compared to non-obese patients.[59] Conversely, BMI was not noted to affect embryo development timing points in an analysis performed retrospectively on 89 patients.[60]

Male factor

Sperm source and quality of male gametes were reported to impact morphokinetic embryo development. A study on 10 couples with 125 injected oocytes by means of intracytoplasmic morphologically selected sperm injection (IMSI) found that embryos obtained by the injection of type-I spermatozoa reached 4-cell and blastocyst stages earlier compared to low-quality ones.[61] Similar outcomes were reported by Neyer and colleagues,[62] who found improved top-quality blastocyst rates when first-class spermatozoa were injected. Paternal effects on the development of 165 embryos were also detected by Wdowiak and colleagues.[63] In particular, lower degrees of DNA fragmentation were associated with faster blastocyst formation and improved pregnancy rates. A positive correlation was found between sperm count values and cc2, t4, t6, t7, while non-progressive motility was positively correlated with t2, t3, and t4. A negative correlation between sperm chromatin quality and morphokinetic parameters such as cc2 and t5 was found in a study of 40 infertile patients, which demonstrated that abnormal sperm characteristics as well as chromatin alterations can impair embryo development.[64] Sperm origin (i.e. freshly ejaculated or surgically retrieved) did not seem to have any

effect on embryo development according to the analysis performed on 604 ICSI cycles in which only few kinetic values were found to differ, although a notable overlapping of their intervals was revealed between the two groups.[65] Desai and colleagues,[66] analyzing the outcome of 93 ICSI cycles, found marked differences between the cc2 and tSB time points according to sperm source and significantly fewer number of high-kinetic-quality embryos obtained from testicular compared to ejaculated spermatozoa.

Insemination method

Several biological features related to laboratory procedures were also found to have an influence on embryological morphokinetic development. For example, the insemination method was reported to affect embryo development. In particular, Dal Canto and colleagues[34] found that the first cleavage of embryos obtained with standard IVF was delayed compared to those obtained by means of ICSI (28.6 ± 2.6 hpi vs 27.0 ± 3.1 hpi, $p = 0.0005$). However, during the subsequent cell divisions the difference gradually disappeared. In accordance with these data, a study performed on 102 fertilized oocytes found that ICSI embryos had a shorter 2-cell stage compared to standard IVF.[67] More recently, Bodri and colleagues[68] analyzed embryo development in 238 cycles and found that IVF embryos had significantly slower development during the first few cleavage stages compared to ICSI embryos (from +1.5 to +1.1 hr from PNf to t4). In contrast, at the blastocyst stage, IVF-derived embryos showed faster developmental rates than those observed in ICSI embryos (from +3.3 to 4.1 hr). After setting the PNf as starting time point, however, the differences in developmental times at cleavage stage disappeared, while those at blastocyst stage further increased, revealing faster growth rates in IVF compared to ICSI embryos (from +3.2 to 5.7 hr). On the basis of these data, the authors concluded that the differences in developmental times between standard IVF and ICSI embryos artifactual.[68]

Culture media and oxygen concentration

The choice of the culture medium is a crucial factor in laboratory management. Essentially, two types of culture media are commercially available from different vendors: single-step and sequential media. The former is based on the "let the embryo choose" hypothesis and all components are simultaneously present. The latter refers to the "back to nature" theory, which attempts to mimic embryo physiology. Thus, all substances reflect those present in its natural environment.[69] To date, evidence for one strategy being superior to the other is still lacking.[70] In one of the first papers studying the effects of culture media on embryo morphokinetic, published in 2012 by Ciray and colleagues, 446 injected oocytes from 51 couples were prospectively randomized between single-step and sequential media, and the development was analyzed until the 5-cell stage was reached. Embryos cultured in the single-step medium had shorter tPNf and faster cleavage divisions from t2 to t5 compared to those in the sequential-medium group. No differences in clinical outcomes, however, were found between the two groups.[71] Comparable results were obtained in another study through the analysis of 160 ICSI embryos cultured in the two different media.[72] The authors found faster tPNf and t2 in the single-step medium while the cc2 was shorter in the sequential one, highlighting that the medium used for the culture can influence embryonic developmental timings. In agreement with these findings are the results of a larger study performed on 972 and 514 cycles in which embryos were cultured in single-step versus sequential media, respectively.[73] Blastocyst rates were higher, both for oocyte (n = 2211/5841, 37.9% vs 1073/3216, 33.4%; $p < 0.01$) and for cycle (mean blastocyst rate: 38.7% ± 29.7% vs 34.3% ± 29.4%; $p = 0.01$), in single-step compared to sequential media. Delivery rate and neonatal outcomes, however, were comparable. In two further prospective studies performed on 21[74] and 75[75] patients undergoing autologous[74] and heterologous[75] cycles, no statistical differences were found between the two culture media for all analyzed developmental timing points. Hardarson and colleagues[76] observed that in terms of number and quality, the total blastocyst rate was similar in the two groups.

Oxygen concentration was also found to impact embryo development.[77] In an observational study, embryos were cultured in (a) 20% oxygen throughout; (b) 20% for 24 hr followed by 5% oxygen, or (c) 5% oxygen throughout (26, 28, and 30 IVF cycles, respectively). It was observed that, compared to cultures performed partially or

exclusively at 5%, the higher oxygen concentration led to delayed t8 stages, thus impairing embryo development until blastocyst stage. As a result of the aforementioned studies, it is clear that when morphokinetic data are analyzed, every IVF clinic should take into account all the variables describing their own patient population and laboratory procedures.

Future perspectives

Currently, research projects involving deep-learning models are ongoing. By analyzing videos through multiple focal planes from thousands of KID embryos, deep-learning-assisted TLT should allow to create a prediction model without pre-existing assumptions.[6] The deep-learning model developed by Tran and colleagues,[78] built on the analysis of 10,638 time-lapse videos, had the ability to predict fetal heart pregnancy. This model was reproducible across different laboratories and procedures, without the need for manual annotations or morphology assessments. Another study found that an artificial intelligence approach based on the analysis of around 50,000 images from 877 and 887 good- and poor-quality embryos, respectively, was able to predict blastocyst quality. The decision tree developed on the basis of those observations was associated with pregnancy outcomes.[79] Kragh and colleagues[80] proposed a fully automated method, based on deep learning, able to predict trophectoderm and inner cell mass quality. This model, built on 8664 blastocysts, performed better than individual human embryologists in predicting embryo quality and implantability.[80] Finally, studies combining morphokinetics with other investigations, such as proteomic, metabolomic, oxidative status of spent culture media, cumulus cells' gene expression patterns and metabolic imaging,[81–85] with the aim to enhance TLT predictivity are continuously published.

Conclusion

Time-lapse cinematography, continuously monitoring embryos during the whole culture, allows experts to detect even slight variations in development, which would not otherwise have emerged. Since this technology became commercially available, many IVF clinics equipped their laboratories with TLT systems and a myriad of scientific papers, aiming to identify the morphokinetic parameter(s) correlated to good prognostic biological and clinical outcomes, have been published. To date, however, conclusive results are still missing and a general consensus is lacking.

Nevertheless, several features of embryo development have been more generally recognized as poor-prognosis factors and have been proposed by some authors as embryo deselection criteria. Examples are blastomere multinucleation or asymmetry, and direct or irregular cleavages.[7,26,37,49,86–89] Some studies, however, reported successful implantation and pregnancy outcomes as well as euploid blastocyst formation even in the presence of one or more of these abnormal morphological and morphokinetic behaviors, although at lower rates compared to normally developing embryos.[49,86–89] For this reason, to discard embryos only on the basis of their morphokinetic development could be hazardous. Prolonging the culture at least until blastocyst stage should be preferred.

Although the clinical advantage of TLT technology has yet to be demonstrated, there are other important benefits in its usage. First of all, the opportunity to perform stable culture without perturbing the optimal embryo environment during the daily assessments. In addition, it is possible to observe and record the whole sequence of cleavages and check them whenever necessary, possibly consulting colleagues and thus reducing the subjectivity of morphological evaluations. For the same reason, TLT allows to safely improve the skills of novice embryologists in the application of embryo evaluation criteria. Finally, after the introduction of TLT in IVF centers, a wealth of data has been collected, leading to a deeper knowledge of preimplantation embryo development. In conclusion, TLT is a powerful technology and further studies, aimed to increase the standardization and the reproducibility across different centers, should be promoted.

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Author contributions

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