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## Towards the selection of embryos with the greatest implantation potential

Dalia Khalife<sup>a</sup> , Antoine Abu-Musa<sup>b</sup>, Ali Khalil<sup>c</sup> and Ghina Ghazeeri<sup>b</sup>

<sup>a</sup>Department of Obstetrics and Gynecology, Jumeirah American Clinic, Dubai, UAE; <sup>b</sup>Reproductive Endocrinology and Infertility Unit, Department of Obstetrics and Gynecology, American University of Beirut Medical Center, Beirut, Lebanon; <sup>c</sup>Division of Gynecologic Oncology, American University of Beirut Medical Center, Beirut, Lebanon

### ABSTRACT

Choosing the most suitable embryo remains challenging as the standard approach to select top-quality embryos for transfer rely on static morphological assessment. It is completed after fertilisation, on days 3 and 5 post oocyte retrieval and evaluates the size and number of blastomeres, presence of nucleation and percentage of fragmentation for cleavage stage embryos. Because of the limited number of observations during the morphological assessment, morphokinetic development of embryos has been implemented. It shows a broader image of embryo behaviour with precise evaluation of the timing of events. Yet, studies are inconsistent and debatable in predicting the parameters to identify chromosomal abnormalities. Pre-implantation genetic testing detects dysmorphic embryos and correlate their developmental potential to the assessed morphology. However, the clinical utility of PGT-aneuploidy remains controversial. The future relies on newly described scoring systems such as artificial intelligence and non-invasive PGT, yet their application and actual success rate still lacks supportive evidence.

### KEYWORDS

Embryo morphology; pre-implantation genetic testing; time lapse imaging

### 1. Introduction

Choosing the most suitable embryo remains challenging for embryologists who are still relying on static morphological assessment as a standard approach to select top-quality embryos. To overcome the uncertainties of the morphological assessment, the transfer of multiple embryos is routinely practiced, at the expense of increasing the risk of multiple gestations (Bromer and Seli 2008). New advancements in the field have taken place and are now implemented to improve the selection criteria and increase the reproductive outcomes.

### 2. Morphological dysmorphisms

Morphological assessment is completed at separate points in time: after fertilisation and on days 3 and 5 post oocyte retrieval. It is based on the evaluation of the size and the number of blastomeres, the presence of nucleation and the percentage of fragmentation for cleavage stage embryos (Stensen et al. 2010). Extending embryo culture to day 5 is one approach to select the most suitable embryos for transfer as it offers the advantage of deselecting aneuploid embryos. Selection criteria are based on blastocoel expansion, inner cell mass (ICM) and trophectoderm (TE) characteristics (Alfarawati et al. 2011) (Table 1).

As half of embryos conceived through In vitro fertilisation/ intracytoplasmic sperm injection (IVF/ICSI) are aneuploid, the majority may arrest at the pre-implantation stage, may fail to implant or may undergo miscarriage (Scott et al. 2012). The

morphological selection criteria are based on different parameters. The morphological abnormalities encountered at a different stage of development with its prognostic relevance are summarised in Table 2. Cleavage stage embryos with 3 to 4 cells (slow development) or 9 or more cells (fast-growing) are more likely to be aneuploid when compared to those with normal development (Magli et al. 1998). Other investigators have reported that the higher the percentage of asymmetrical blastomeres, cellular fragmentation and multinucleation, the higher is the presence of post-meiotic chromosomal abnormalities (ChrAb) and thus the lower is the implantation rate (IR) (Racowsky et al. 2003; Munné 2006). In a recent study, blastocyst morphology was found to be correlated with the euploidy rate which was not true for cleavage stage embryos (Majumdar et al. 2017).

In view of the data, the developmental competence of embryos is not linked to their viability, as aneuploid cleavage stage embryos can still reach the blastocyst stage. Thus, embryo morphology assessment alone is not sufficient to select chromosomally normal embryos limiting the prognostic value of these observations.

Detecting aneuploid embryos before the day of embryo transfer (ET) is of utmost importance for both physicians and patients in order to counsel on the chances of success. New techniques are now available enabling the detection of the ploidy status in a more extensive manner. Attention has been focused on the visualisation of embryos in a continuous fashion using the embryo scope (or time lapse imaging [TLI])

**Table 1.** Morphological scoring of embryos on day 3 and day 5 (Modified from Gardner and Schoolcraft [1999]).

Cleavage stage embryos			
Cleavage stage grade	Description		
Grade 1 = very good	Even-sized blastomeres, no visible fragmentation, homogeneous cytoplasm.		
Grade 2 = good	Less than 10–20% fragmentation, even or uneven blastomeres, non-homogeneous cytoplasm.		
Grade 3 = average	More than 20% fragmentation, even or uneven blastomeres.		
Grade 4 = poor	More than 50% fragmentation, even or uneven blastomeres.		
Blastocyst stage embryos			
Blastocyst expansion grade	Description	ICM and TE grade	Description
6	Hatched blastocyst: completely escaped the ZP.	A	ICM is tightly packed with many cells, TE is formed by many cells in a cohesive epithelium.
5	Hatching blastocyst: TE cells have begun to herniate through ZP.	B	ICM is loosely grouped with several cells, TE is formed by few cells forming a loose epithelium.
4	Expanded blastocyst: the blastocoel is larger than the early embryo, ZP has begun to thin.	C	ICM has very few cells, TE has very few large cells.
3	Full blastocyst: blastocoel fills the embryo completely.		
2	Blastocyst: blastocoel occupies more than one half of the volume of embryo.		
1	Early blastocyst: blastocoel accounts for less than one half of the volume of embryo.		

ZP: zona pellucida; TE: trophectoderm; ICM: inner cell mass.

for a thorough analysis of embryo morphokinetics (Montag et al. 2011; Hashimoto et al. 2012; VerMilyea et al. 2014; Singh et al. 2019). In other words, cell division and movement patterns are observed over the minutes to differentiate between aneuploid and euploid embryos. Another option is to biopsy the embryo, either on day 3 or on day 5 with extensive cytogenetic analysis.

### 3. Correlation between morphological assessment, aneuploidy and embryonic development

Pre-implantation genetic testing (PGT) has now offered the privilege to detect dysmorphic embryos and correlate their developmental potential to the assessed morphology. It is proposed as the method of choice to select embryos with the highest potential for implantation (Munné et al. 1993). Initial studies lacked randomised controlled trials (RCTs) and were based on the correlation between the presence of ChrAb and morphology on a limited number of chromosomes (13, 16, 18, 21, 22, X and Y) with the use of fluorescence *in situ* hybridisation (FISH). In a retrospective cohort study, PGT-Aneuploidy (PGT-A) performed on 703 embryos showed that severe ChrAb (chromosomes 13, 20 and 21) were not detected after standard morphology assessment (Eaton et al. 2009). Because of the drawbacks of the FISH method, certain aneuploid embryos are classified as euploid due to untested abnormal chromosomes. Thus, one cannot firmly conclude on the euploidy of an embryo as the majority of chromosomes remain unverified.

Consequently, new methods were implemented to study the effect of aneuploidy by testing concurrently the 24 chromosomes. The cytogenetic analysis via Comparative Genomic Hybridisation (CGH) microarray performed on 1213 embryos indicated that good quality embryos on day 3 are aneuploid in 82.4% of cases with 43.4% harbouring a complex abnormality; hence the poor correlation between the morphological appearance and the presence of aneuploidy on day 3. The use of PGT-A with array-CGH has been shown to be

beneficial in terms of improving the delivery rate (52.9 vs 24.2%) and lowering the pregnancy losses (27.5 vs 39%) in patients with advanced maternal age (38–41 years) (Rubio et al. 2017).

In contrast, a stronger correlation to aneuploidy was found on day 5. Fifty-one percent of the fully expanded blastocysts were normal compared to 33% of lower grade blastocysts ( $p = .012$ ) and the frequency of complex abnormality was higher in early blastocysts compared to fully expanded ones (14 vs 6%). When it comes to the correlation of the quality of ICM and TE cells, poor ICM and TE morphology were more evident among aneuploid embryos (Fragouli et al. 2014). These findings were portrayed in an observational study, where only 37.5% of early blastocysts were euploid after CGH screening. In addition, embryos were able to reach the blastocyst stage even in case of severe anomalies but with a lower growth rate (Alfarawati et al. 2011). These findings endorse the fact that chromosomal errors are more prominent after genomic activation. The initial stages of development depend on proteins derived from the oocyte. The activation of the human embryonic genome and expression of genes starts at the stage of 4 to 8 cells of preimplantation development. Therefore, ChrAb impacts the embryonic morphology past day 3 of development (Braude et al. 1988). The high frequency of aneuploidy on day 3 is the mixture of errors at the level of the oocyte and mitotic errors during the first cellular divisions after fertilisation. This is concordant with data published by Fragouli and colleagues who noted a significant decrease in the percentage of aneuploidy from 84% on day 3 to 56% on day 5 (Fragouli et al. 2014). Accordingly, the formation of ICM and TE via cellular compaction and differentiation is disturbed by the presence of aneuploidy. As such, abnormal blastocysts are more likely to display a low-quality ICM and poorer growth of TE cells (Alfarawati et al. 2011). In a consequent RCT, blastocyst biopsy with microarray CGH testing showed significantly higher delivery rates (84.7 vs 67.5%) in the CGH group compared to controls (Scott et al. 2013). Although, the

**Table 2.** Non-invasive morphological assessment of embryos at different stages of development.

	Normal morphology	Morphological abnormalities	Prognostic relevance	References
MII Oocyte	Clear, moderate granulations in cytoplasm, small PVS, intact first PB, colourless ZP	Cytoplasmic: granularity of cytoplasm, aggregation of smooth endoplasmic reticulum, vacuolisation, presence of refractile bodies Extra-cytoplasmic: irregularities in oocyte form, large PVS, presence of debris and inclusions, fragmented first PB, dark ZP Giant oocytes (diameter >200 microns)	Lower IR and PR  Lower quality of zygotes  Embryos from giant oocytes are more likely to be triploid or polyploid	(Munné and Cohen 1998).
Zygote on day 1 (16–18 h after insemination)	Paternal and maternal PN of comparable size, centrally located. Polarised nucleoli and NPB  Presence of cytoplasmic halo (shift of mitochondria and other organelles to the centre of oocyte) Early cleavage (25–27 h after insemination) is reflective of a coordinated cytoplasmic and nuclear maturation (2 blastomeres stage)	Uneven size of PN Asynchronous formation of nucleoli Suboptimal PN orientation  Unequal number of NPB between the nuclei Absence of halo  No early cleavage	Mosaicism Impairment of advanced embryo development Poor morphology (uneven cleavage, fragmentation) Abnormal cell cycle  Lower IR and PR  Lower PR (3.2% compared with 26% with early cleavage)	(Nasiri and Eftekhari-Yazdi 2015).
Embryo on days 2–3 (cleavage stage)	4–5 blastomeres on days 2, 7 or more blastomeres on day 3, absence of multinucleation, fragmentation is 20% or less	20–50% fragmentation reflective of abnormal metabolism, apoptosis or abnormal chromosomal segregation Asymmetrical cellular cleavage = uneven distribution of genetic material Multinucleated blastomeres (more than one nucleus in each blastomere) Other factors: presence of vacuoles, thick ZP	Poor quality embryos with higher rate of chromosomal anomalies/mosaicism → decreased IR	(Kroener et al. 2015).
Embryo on day 5 (blastocyst stage)	Volume of embryo occupied by blastocoel expansion, tightly packed ICM, cohesive TE cells	Non-expanding blastocoel, loosely packed ICM and few TE cells	Lower IR and PR	(Majumdar et al. 2017).

This table shows the predictive value of each morphological assessment at different stages of development (zygote, cleavage stage and blastocyst stage).

Selection of embryos still rely on morphological appearance in most ART units. Descriptions of the PN stage and early cleavage are associated with higher reproductive outcomes. Assessment before transfer on day 3 or day 5 is the most used, yet the link to embryo viability is still imprecise.

MI I oocyte: metaphase II oocyte; NPB: nucleolus precursor body; PVS: perivitelline space; PB: polar body; ZP: zona pellucida; PR: pregnancy rates; PN: pronuclei; IR: implantation rates; ICM: inner cell mass; TE: trophoctoderm.

mentioned studies suggested the link between morphological assessment of blastocyst and aneuploidy, one cannot rely on safeguarding the transfer of an euploid embryo. The recent addition of next-generation sequencing (NGS) methods has improved the efficiency of PGT-A via a precise detection of mosaicism in order to avoid transferring aneuploid embryos. Mosaic embryos have poorer embryo viability as well as poorer pregnancy outcomes compared to euploid blastocysts. Yet, few embryos are still able to implant and should not automatically be excluded but should be given a lower preference to be transferred (Fragouli et al. 2017). Subsequently, further attention has been drawn on the capacity of mosaic embryos to grow into euploid ones. The transfer of blastocyst embryos with <50% of mosaicism (biopsied and NGS performed) in patients who did not have euploid embryos to transfer showed significantly higher

implantation rate (IR), clinical pregnancy rate (CPR) and live birth rate (LBR) per embryo transfer (ET) compared to embryos with >50% of mosaicism (Spinella et al. 2018).

The efficiency of PGT-A compared to the standard embryo morphology while using NGS as a new reliable tool is becoming more popular to predict the pregnancy outcomes of mosaic embryos. A retrospective study on blastocysts biopsied and handled with NGS revealed that complex mosaic embryos with the highest percentage of mosaicism have the lowest pregnancy rates. However, 41% of mosaic embryos carried on ongoing implantation (Munné et al. 2017). A recent RCT named ‘Single Embryo Transfer of Euploid Embryo’ (STAR) displayed the lack of benefit of TE biopsy along with NGS analysis in selecting euploid embryos as it did not enhance the ongoing pregnancy rate (OPR) compared to embryos selected by morphology while making use

of the intention to treat analysis. The failure to attain significant results in the PGT-A group was related to the fact that embryos of poor quality were also tested which may have induced bias in the results (Munné et al. 2019).

As well as the quality of embryos, the timing of embryo biopsy at the time of blastocyst development has been shown to be of utmost importance. The embryo expansion and embryo hatching have been correlated to higher pregnancy outcomes as CPRs and LBRs are significantly higher and pregnancy losses are significantly lower in embryos with full expansion at the time of biopsy. Hatched embryos were also correlated with higher LBRs (Singh et al. 2019). Thus, the clinical utility of PGT-A remains controversial as a wrong diagnosis or biopsy related damage to the embryo may result in embryo wastage.

#### 4. Continuous surveillance of embryos: time-lapse imaging

Because of the few observations during the morphological assessment, several investigators have accepted the challenge to show that morphokinetic development of embryos presents a broader image of the embryo behaviour. It has been shown to be advantageous in providing more information on the timing of events such as fertilisation, the extrusion of polar bodies and the timing of cellular divisions (Fréour et al. 2013; Muñoz et al. 2013). Morphokinetic parameters used to predict the formation of a zygote into a blastocyst were based on the duration of the first cellular division, the time interval between 1 and 2 cell embryo and the synchronicity from 2 to 4 cell embryo. The development to high-grade blastocyst can be estimated in the first 2 days of embryo culture (Kirkegaard et al. 2013), as it is correlated to an early cellular division, to a shorter time of second division (3 to 4 cells) between 9.33 to 12.65 h and shorter time of third division (5 to 8 cells) between 0 to 4 h (Montag et al. 2011; Hashimoto et al. 2012; VerMilyea et al. 2014).

Morphokinetic parameters such as cleavage time (time to division up to 4 cells), the number and size of blastomeres and the presence of multinucleation were also correlated with reproductive outcomes (Meseguer et al. 2011). Implanted embryos were more likely to have an advanced number of blastomeres along with an earlier appearance of nuclei (Lemmen et al. 2008). For instance, the early cleavage status of the embryo yielded significantly higher pregnancy rates (PR) reaching 46% (Van Montfoort et al. 2004). Intriguingly, a direct cleavage from one to three cells of less than 5 h has been significantly associated with lower IR due to higher ChrAb (Rubio et al. 2012). This is in favour of selecting embryos with normal divisional patterns which is in line with a retrospective study showing an increased CPR per ET by 20% compared to embryos cultured in standard incubators (Meseguer et al. 2012). Recently, a retrospective study on 716 embryos was conducted to establish the relationship between blastomere arrangement and LBR. Although the tetrahedral formation of the embryo was found to be associated with the formation of best quality blastocysts for

transfer in 62.9% of cases, it did not predict a higher IR or LBR (Desai and Gill 2019).

Because of the extensive disordered divisions in complex aneuploid embryos, substantial alterations in the duration of the cell cycle, delay in cell compaction and a decreased rate in reaching the blastocyst stage are clearly identified in TLI. Yet, in the case of single meiotic abnormalities, errors seem not to affect the regular morphological scoring and therefore may be missed with TLI (Fragouli et al. 2014; Campbell et al. 2013).

Studies have been then inconsistent in predicting the parameters to identify ChrAb. Henceforward, the association between aneuploidy with the different blastocyst parameters has not been confirmed in a large cohort study on 138 poor prognosis patients with advanced maternal age (Rienzi et al. 2015). A Cochrane analysis portrayed the absence of a significant difference between TLI and conventional incubators with regards to CPR (OR 0.88) and LBR (OR 0.73). Yet, studies were subject to a high incidence of bias (Armstrong et al. 2019). Supposedly, the assumption that aneuploidy may be predicted by the different embryo morphokinetics remains debatable.

#### 5. Future prospects

The performance of assisted reproductive technologies (ART) lies in choosing the best embryo for transfer. Despite the extensive data on the previous cited methods, discrepancies still exist between the different methods of embryo selection. A growing body of evidence suggests the use of web-based systems with Artificial Neural Network (ANN) working on the extraction of descriptors from a given input parameters in order to score embryos and predict the IVF/ICSI outcomes (Siristatidis et al. 2016). Yet, more data and randomised studies are needed to compare the use of artificial intelligence to other scoring systems before offering it as a routine service to patients seeking infertility treatments. The overall performance and accuracy of the system depend not only on the professionalism and training of the medical staff, but it necessitates a thorough statistical evaluation of the data as well (Manna et al. 2013).

Moreover, non-invasive pre-implantation genetic testing (NI PGT) has been developed after the discovery of nuclear DNA in the culture medium. It is an interesting advent to circumvent the invasiveness of embryo biopsy, yet its application is regulated by the illustration of the whole embryo (Stigliani et al. 2013). This method seems to be promising, but its efficacy is still debatable as conflicting results exist on the actual success rate (Babariya 2019). It is controlled by different parameters such as differences in culture media, type of culture, the timing of sampling and culture volume (Kuznyetsov et al. 2017).

In a proof of concept study, a CGH analysis performed on blastocyst culture media for advanced maternal age was compared to polar body analysis resulted in 72.2% of ploidy concordance and only 48.6% of concordance for single chromosomal aneuploidies because of maternal contamination (Feichtinger et al. 2017). Whole-genome amplification

of embryonic DNA showed a high concordance rate per sample between cell-free DNA from culture media and TE biopsies in analogous blastocysts (Valeriy et al. 2019). These results were not in line with previous outcomes that showed a discordance rate as high as 67% (Vera-Rodriguez et al. 2018). Discrepancies were attributed to a result of the mixture of maternal and embryonic DNA that are more complex for mosaic embryos with a lower percentage of embryonic DNA at 8% (Vera-Rodriguez et al. 2018). Future studies must rely on improving the performance of these methods towards specifically identifying DNA set free from embryos to handle a successful assay.

## 6. Conclusion

Blastocyst stage analysis is more valued in terms of morphological screening as it prevents transferring embryos with complex abnormalities. Yet, it is not sufficient to avoid single aneuploidies that are able to generate a pregnancy. TLI has been developed but did not prove to be superior to the standard morphological scoring. Genetic testing of embryos via invasive techniques and the application of CGH microarrays/NGS at the blastocyst stage provides a reliable way to transfer the most suitable embryo and cryopreserve the surplus of embryos, yet its safety and efficacy is yet to be proven. NI PGT is an interesting advent to circumvent the invasiveness of embryo biopsy, yet its efficacy is still debatable. As for artificial intelligence, more trials are needed before offering it as a routine service to patients seeking infertility treatments.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

## ORCID

Dalia Khalife  <http://orcid.org/0000-0003-0354-7311>

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