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Mini Review

Selecting Developmentally Competent Human Blastocysts: From Basic Morphological Assessment to Morphokinetics and Preimplantation Genetic Testing for Aneuploidy -

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Abstract

The ability to select human embryo with the highest implantation potential remains one of the greatest challenges in the management of *In Vitro* Fertilization patients. Here, we review current methods for embryo selection including blastocyst static morphological grading, analysis of embryo morphokinetic parameters, and preimplantation genetic testing for aneuploidy, and their impact on the success of clinical outcomes. We also discuss emerging non-invasive cell-free embryonic DNA examination of spent embryo culture media. Other experimental methods, such as metabolomics, proteomics, cumulus cells or trophectoderm biopsy sample transcriptomics remain controversial and not yet suitable for routine clinical application. These are thus beyond the scope of this review. Traditionally, embryos with superior morphology have been selected for transfer, but these static parameters have been difficult to measure objectively and to use for prediction of blastocyst implantation potential. The introduction of time-lapse imaging system and morphokinetic markers to the laboratory practice allowed to study embryo cell dynamics during early preimplantation development, enhancing the evaluation of embryo quality. Nevertheless, morphokinetic annotations and predictive algorithms might vary between clinics because of different culture environments, stimulation protocols and embryo developmental hallmarks, which overall limit their prognostic value for assessing embryo viability. Moreover, the use of an objective but invasive technique of preimplantation genetic testing for embryo aneuploidy has been shown to improve the selection of embryos with the most potential to implant and produce a healthy pregnancy. However, not all euploid embryos implant, likely owing to embryonic, endometrial factors, or both. Thus, at present, the combination of blastocyst testing for chromosomal abnormalities, analysis of embryo morphokinetics and morphological blastocyst scoring, *as* integrated approaches, will help to select developmentally competent embryos to maximize chances of successful reproductive outcome. Furthermore, implementing artificial intelligence to the field of Assisted Reproductive Technology may standardize the embryo selection process for a more reliable prediction of embryo quality and fate.

Keywords: Human blastocyst; Standard morphology; Morphokinetics; Preimplantation genetic testing for aneuploidy; Embryo selection; Artificial intelligence

ABBREVIATIONS

ART: Assisted Reproductive Technologies; AI: Artificial Intelligence; BF: Blactocoel Fluid; CGH: Comparative Genomic Hybridization; DNA: Deoxyribonucleic Acid; DNNs: Deep Neural Networks; cfeDNA: cell-free embryonic DNA; EB: Early Blastulation; eSET: Elective Single Embryo Transfer; FET: Frozen Embryo Transfer; ICM: Inner Cell Mass; ICSI: Intracytoplasmic Sperm Injection; IVF: *In Vitro* Fertilization; KIDScore: Known Implantation Data; MN: Multinucleation; NGS: Next Generation Sequencing; NIPGT-A: Non-Invasive PGT-A; PGT-A: Preimplantation Genetic Testing for Aneuploidy; PGDIS: Preimplantation Genetic Diagnosis International Society; qSEA: Quantitative Standardized Expansion Assay; RCT: Randomized Controlled Trial; SC: Standard Culture; RCT: Randomized Clinical Trial; SEM: Spent Embryo Culture Medium; tPNa: Time When Pronuclei Appeared; tPNf: Time When Pronuclei Faded; t2-t9: Time To Reach 2-9 Cell Stage; tSB: Start Of Blastulation Time; tB: Time To Full Blastocyst Formation; tHB: Time of Blastocyst Hatching; TE: Trophectoderm; TLS: Time-Lapse System; TLI: Time-Lapse Imaging; WB: Whole Blastocyst; WGA: Whole Genome Amplification.

INTRODUCTION

The ability to select the embryo with the highest implantation potential remains one of the greatest challenges in the management of *In Vitro* Fertilization (IVF) patients. This is especially important given the worldwide adoption of Elective Single Embryo Transfer (eSET) at the blastocyst stage.

Since the inception of human IVF, methods to evaluate embryo viability have continuously evolved. Various non-invasive and invasive techniques such as metabolomics, proteomics, automated time-lapse algorithm-based embryo examination, Preimplantation Genetic Testing for Aneuploidy (PGT-A) or cumulus cells transcriptomic analysis have been developed to improve embryo selection for uterine transfer [1-3]. Based on recent publications [4,5] and *our own data [6]*, transcriptomic analysis of a trophectoderm biopsies might also be a promising tool for assessing embryo competence *in vitro*. However, considered impact of these approaches on implantation potential and successful pregnancy remains still controversial. In addition, some of these new methodologies appear not yet suitable for routine clinical application. Therefore, at present, static morphological criteria combined with genetic information and embryonic morphokinetics are the most practical approaches to this continuing dilemma in ART programs (Assisted Reproductive Technologies).

Morphological blastocyst characteristics cannot be fully relied upon choosing the embryos which are most likely to implant and result in a successful pregnancy because they are subjective and associated with considerable inter-observer variability. Furthermore, conventional static morphology is not always indicative of high-quality embryos. Nevertheless, there is some evidence that this method is linked to embryo competence [7]. It has been observed that the quality of Inner Cell Mass (ICM) and Trophectoderm (TE), and blastocyst developmental stage correlates with implantation potential in fresh as well as frozen cycles [8-10].

Morphological embryo assessment has been enhanced by the introduction of morphokinetic analysis using Time-Lapse Monitoring System (TLS) [11,12]. Due to this technology, several time points of embryo cellular dynamics can be recorded and analyzed during early development of an embryo *in vitro*. Predicting blastocyst formation with the use of specific early developmental milestones have shown promising results but foreseeing clinical pregnancy using these parameters remains debatable [13].

On the other hand, development of an objective method such as preimplantation genetic testing of the 24 chromosomes for aneuploidy after TE biopsy has been a major breakthrough in the field of ART. It is well documented that the main cause of embryo arrest, implantation failure, and pregnancy loss is the occurrence of aneuploidy [14-16]. The use of PGT-A has been shown to increase the implantation rate with a single embryo transfer, thus reducing the need for multiple embryo transfer to achieve a pregnancy, and the resultant risks of multiple pregnancy [17]. PGT-A has also reduced the miscarriage rate and shortened the time to achieve a pregnancy

[18]. However, not all normal, euploid embryos implant, owing to embryonic and/or endometrial factors.

It is clear by now that despite remarkable advances in IVF procedures, new strategies for improved embryo selection are still needed to optimize the success rates of IVF. The ability to select and transfer single blastocyst with the highest developmental potential plays a crucial role in minimizing the risk of multiple gestation pregnancies along with the associated maternal and fetal complications. This will improve implantation rates, decrease miscarriages and increase the probability of healthy live birth. Sensitive molecular methods of chromosomal analysis may deselect some embryos with detrimental developmental abnormalities and separate those with high potential for successful implantation. The advancement in embryo selection can also significantly reduce time to pregnancy and facilitate the ranking of remaining cryopreserved embryos in order to ensure the subsequent transfer of viable embryos. Unfortunately, all current available methods are not reliable and cannot identify viable from non-viable embryos with 100% accuracy. Here, the present mini-review consist of narrative description of current state of knowledge regarding the most practical approaches for human blastocyst selection and their contribution to the reproductive outcomes.

Standard Morphology and Blastocyst Implantation Prediction

Embryo quality has long been considered an important determinant of successful implantation and pregnancy, but embryonic characteristics have been difficult to measure for use in the prediction of embryo developmental competence.

The conventional blastocyst grading systems that are currently use in IVF laboratories around the world are based on static morphological assessment of three parameters: degree of blastocoel expansion, size and compactness of the ICM, and cohesiveness and number of cells in the TE in accordance with the Gardner [19] and simplified SART [20] scoring systems. Embryo quality is considered a major predictor of implantation and pregnancy, and traditionally, embryos with superior morphology have been selected for transfer.

In early studies, there were attempts to correlate the morphological grading of blastocysts with their implantation potential [10,19]. It has been shown that blastocyst score had a significant effect on implantation and clinical pregnancy rates. When only one top-quality blastocyst (≥ 3AA) was available for transfer, both implantation and pregnancy rates were statistically higher when compared to low-scoring blastocysts (< 3AA) [10].

It has been observed that embryos with better ICM/TE grades and greater blastocoel expansion are associated with a likelihood of higher implantation rates compared to embryos with compromised morphology [8,21]. For example, the recent paper by Minasi, *et al.* [21], has shown that the implantation rates were significantly higher for blastocysts with top quality ICM and TE, compared to blastocysts with poor quality of those parameters (47.2% and 46.5% vs. 17.1% and 26.6% respectively; $p < 0.001$). In contrast, other reports have stated that higher TE grade was the only blastocyst feature that predicted ongoing clinical pregnancy and live birth and could replace ICM scoring in terms of priority [22,23].

Conventional morphological grading has been used as well, to help selection among euploid blastocysts, and appeared to be a useful predictor of superior embryo transfer outcomes. High quality euploids yielded statistically increased implantation and ongoing pregnancy rates over good, average and poor embryos [9]. Moreover, it was suggested that when selecting among poor-quality or average-quality embryos, priority should be given to those with top ICM morphology because this was a better predictor of pregnancy outcomes than good trophectoderm grade or blastocoel expansion [9]. In another study of Irani, *et al.* [24] it has been found that the speed of embryo development to blastocyst stage together with blastocyst morphology was critical for selecting the best euploid embryo. They showed that euploid embryos rated as good quality blastocysts on day 5 resulted in significantly higher implantation and live birth rates after frozen single blastocyst transfer, compared with similar quality blastocysts on day 6 (77.7% vs. 58.7%; $p = 0.02$ and 72.8% vs. 56.5%; $p = 0.03$: respectively).

In contrast to this, some reports demonstrated that standard morphological parameters are relatively poor predictors of implantation and live birth rates in fresh and Frozen Embryo Transfer (FET) cycles. Wirleitner, *et al.* [25] obtained comparable results after transfer of top or non-top-quality blastocysts regardless of cycle type. In another paper, regression logistic analysis also showed that neither blastocyst morphology nor developmental rate was predictive for the implantation potential of euploid embryos [26].

Our group reached the same conclusions, when we evaluated the relationship between morphological grade and chromosomal content of the embryo to determine the importance of prioritizing euploid blastocysts analyzed by PGT for FET [27]. Based on 335 single euploid transfers, we found that standard embryo quality assessment was not a reliable predictor of clinical outcome. The implantation and clinical pregnancy rates were comparable for all euploid embryos regardless of their morphology in all age groups. Moreover, euploid embryos graded as poor were not associated with higher miscarriage rates than embryos graded as average, or good (9.5%, 14% and 19% respectively).

In summary, although it is somewhat useful and still the most used method by embryologists to monitor embryo development and select best embryos for uterine transfer, morphological assessment remains a subjective, unreliable strategy to predict developmental potential. Advanced technologies such as Time-Lapse Imaging (TLI) microscopy with morphokinetic embryo scoring systems, as well as PGT offer solutions to overcome some drawbacks of common standard approaches, and hold the promise to complement or replace conventional morphological evaluation.

Time-Lapse and Blastocyst Formation Prediction

The introduction of a novel technology to clinical IVF practice, such as non-invasive time-lapse imaging allows one to track the timing of developmental events from the zygote to blastocyst stage. It allows to monitor the exact time of specific cell divisions, to measure cell-cycle lengths during embryo growth, and also to receive additional morphological parameters that are often not detected using static morphological observations within conventional incubators. Those events include abnormal cell divisions (i.e. revers or direct cleavage), multinucleation, blastocyst collapse/re-expansion, timing of blastocoel appearance and other embryonic phenomena, some of which are negatively associated with clinical outcomes. At present, there are several different TLI systems used in the embryology laboratories i.e. Embryoscope (Vitrolife), Geri (Genea Biomedx, Miri TL (Esco Medical) Astec CCM-IBIS (Astec), Primo Vision (Vitrolife) and Eva (Merck-Serono) [28-30].

Numerous TLI studies have sought to correlate cellular kinetic markers with specific outcomes, including blastocyst formation, ploidy status and implantation. The vast amount of morphokinetic parameters collected as a result, has been proposed in the form of embryo selection algorithms as possible predictors of IVF treatment outcome [12]. To date, over 20 morphokinetic, developmental events have been recognized, some of which have been identified as possible indicators for implantation potential and aneuploidy.

At least two benefits might be expected from time-lapse technology [31,32]. Firstly, embryos are kept in a less disturbed environment during culture as they are not exposed to changes in gas composition, pH or temperature shifts that accompany daily embryo evaluation under standard conditions, which could adversely affect embryo development [13]. Secondly, additional developmental kinetics and phenotypic markers can be acquired at distinct time-points as compared with standard morphological embryo evaluation.

To verify the usefulness and effectiveness of TLI system in our laboratory, we compared the rate of blastocyst formation in embryos cultured in the EmbryoScope™ with their sibling embryos cultured in a conventional incubation system (1,080 vs 733 embryos) [33]. The number of usable blastocysts (transferred or cryopreserved) that developed in the EmbryoScope™ (VitroLife, Sweden) was significantly higher compared to their sibling embryos that developed in the K-system™ (Cooper Surgical, Denmark) (49.8% and 41.5%; respectively; $p = 0.0005$). Moreover, a significantly higher blastocyst formation rate was observed in the EmbryoScope™ on Day 5 (36.4% and 27.0% respectively; $p = 0.006$), indicating an enhanced growth rate when using TLI incubation technology. Thus, the continuous culture environment provided by TLI systems may favorably influence blastocyst formation rates.

Review of the literature on the use of continuous time-lapse monitoring has shown promising results with the use of specific early developmental milestones to predict blastocyst formation [34-36]. For example, from Scarica, *et al.* [36], pronuclei appearance and their fading, and the time of the first and second division after fertilization (tPNa, tPNf, t2, t4) were significantly faster in embryos that developed to blastocyst stage, but in general, all the early timings up to t5 were associated, if slower, with the risk of developmental arrest and degeneration (time to reach 2-, 4- and 5-cell stage). The t2 was the only early TLI parameter that maintained a significant association with blastocyst formation, suggesting that the very first karyokinesis and cell divisions are critical for the developing embryo.

Time-lapse imaging has also revealed the prevalence of specific dysmorphisms such as multinucleation, occurring especially at the 2-cell stage. Blastocysts derived from these multinucleated embryos have been shown to have lower implantation potential, and decreased probability of live birth [35,37,38]. The present findings on the chromosomal status of blastocysts originating from multinucleated embryos are therefore particularly intriguing. In our TLI studies we showed that euploid and aneuploid blastocysts were equally affected by Multinucleation (MN) at 2-cell stage (40.8% and 46.7%, respectively) suggesting that MN-embryos have the capacity for self-correction and development into euploid blastocysts and healthy babies [39]. Furthermore, there is some time-lapse evidence that while they have reduced developmental potential, embryos exhibiting atypical developmental patterns may result in euploid and transferrable blastocysts and give rise to a normal live birth [30,40].

Despite widely adopted TLI technologies to predict blastocyst formation in IVF laboratories, there is still considerable disagreement regarding which morphokinetic and morphological parameters are useful to predict embryo implantation potential, clinical pregnancy and the ploidy status of the embryo [7].

Time-Lapse and Blastocyst Ploidy Prediction

It has been shown that embryo aneuploidy, a major cause of IVF failure, correlates with specific time-lapse morphokinetic variables, which were used to develop an aneuploidy risk classification [41,42]. According to this simple three classes' model, based on late kinetic markers, two precise time points are especially important: the start of blastulation (tSB) and the time when the embryo reaches a full blastocyst stage (tB) post-insemination. Embryos with early tSB and tB (< 96.2 hpi and < 122.9 hpi) were classified as having low aneuploidy risk, while embryos with late tSB but early tB (≥ 96.2 hpi and < 122.9 hpi) or late tB (≥ 122.9 hpi) were classified as having medium or high aneuploidy risk, respectively. Within these parameters, the analysis of the frequency of positive fetal heartbeats and live birth rates, indicated a significant difference between embryos from low versus medium aneuploidy risk categories, suggesting the clinical relevance of time-lapse imaging in the ploidy assessment ($p < 0.0001$ and $p < 0.01$ respectively).

Several studies which evaluated the effectiveness and potential impact of this risk model, confirmed the Campbell, *et al.* [41,42] finding that a delay in starting blastulation and reaching full blastocyst stage was more often observed in aneuploid embryos compared to euploid ones. A large sample size study ($n = 1,730$) has found that the time for the blastocyst to start to form (tSB), to completely formed (tB), expanded (tEB) and hatched (tHB) was considerably shorter in euploid compared to aneuploid embryos [21].

The report by Desai, *et al.* [35] demonstrated as well, that chromosomal status correlated significantly with late embryo kinetics: a higher euploidy rate was observed for blastocysts with early rather than late tSB (48.2% vs.36.6, respectively). In addition, a drop in euploidy rate to 30% was detected in blastocysts with significantly delayed time of blastocoel expansion. It was also noted that the proportion of euploid embryos was significantly increased with shorter intervals of blastocyst enlargement (tEB-tB less than 13 hours). Thus, the authors concluded that while the early cell cycle kinetics are predictive of embryo development to blastocyst, the late kinetic parameters (tSB, tEB, and tEB-tSB) are clearly associated with likelihood of euploidy. They also suggested that TLI could be a valuable method to enhance the selection of competent embryos with the greatest implantation potential.

Recent, investigation of 188 autologous blastocysts from PGT-A cycles evaluated the rate and size of blastocyst expansion using a new Quantitative Standardized Expansion Assay (qSEA), and have shown that on average the expansion rate in euploid versus aneuploid blastocysts was 52.8% faster ($p = 0.0041$) [43]. It was also revealed that the mean time of initial blastocyst formation was slightly earlier in euploid (105.7 h) than aneuploid embryos (108.1 h), although the difference was not significant. It was suggested that impaired blastocyst expansion may represent an early symptom of aneuploidy, and blastocyst expansion mapping may add value to embryo selection algorithms, in both cycles with and without PGT-A.

Although, several studies indicated a positive correlation between certain time-lapse parameters and blastocyst ploidy status, there were other reports disputing such associations. Conversely, Rienzi, *et al.*

[44] and Zhang, *et al.* [45] using 19 early (tPNf, t2, t3, t4, t5, t8, t9 etc.) and some late morphokinetics (TSB and TB), showed no significant difference between those parameters in euploid embryos compared to their aneuploid counterparts in patients at increased risk of aneuploidy because of advanced maternal age, history of unsuccessful IVF treatments, or both. The data collected also indicated that the "Aneuploidy Risk Classification" model proposed by Campbell et al. [41,42] was not predictive of blastocyst ploidy, thus not suitable for separating normal embryos from chromosomally abnormal ones.

In conclusion, it appears that based on present contradictory findings, certain time-lapse morphokinetic parameters might be associated with embryo ploidy. However, predicting chromosomal abnormalities using these markers alone remains insufficient and cannot yet replace PGT, the most reliable method for aneuploidy assessment.

Time-Lapse and Blastocyst Clinical Outcomes Prediction

Numerous studies have focused on validating the impact of TLI technology on embryo selection in relation to clinical IVF outcomes. These reports remain largely heterogeneous, comparing different patient cohorts, analyzing various morphokinetic evens, and present moderate to low quality evidence owing to inconsistencies across the studies [12,38,46]. Some authors have suggested that time-lapse algorithms have higher predictive power than standard morphology scoring, while others did not show considerable benefits in the ability of TLI to project an embryo's capacity to implantation or result in a live birth after transfer.

A recent, meta-analysis of five early Randomized Controlled Trials (RCT) postulated that ongoing pregnancy and live birth rates were significantly increased by using TLI [47]. For example, one of these randomized trials based on 843 patients, indicated that embryo culture in EmbryoScope™ combined with morphokinetic assessment improved reproductive outcome per treated cycle compared to a standard incubator with conventional morphological scoring (51.4% vs 41.7% ongoing pregnancy rates) [48].

Another RCT published by Goodman, *et al.* [49] compared conventional morphological grading with morphokinetics when all embryos from study and control group were cultured in the closed Embryoscope system (116 vs.119 patients respectively). In the time-lapse kinetic monitoring group, patients had their top-quality embryos determined by morphology, and then the morphokinetic score was used to preferentially rank the best embryos for transfer. Although final outcomes did not reach statistical significance, overall, there was small increase in clinical pregnancy and implantation rates with the use of the additional kinetic markers (74% and 56% vs. 67% and 51%). Thus, time-lapse scores did not emerge as major predictors of clinical outcomes. However, there was a significantly higher implantation rate for embryos which started blastulation by 100 hours post insemination (hpi) when compared to those non-implanted ones (76% vs. 54%; $p < 0.01$); only a tSB of < 100 hours was independently predictive of implantation.

Furthermore, several current retrospective cohort studies used also different approaches to evaluate times-lapse data. In one such reports, Mascarenhas, *et al.* [50] compared live birth rates and perinatal outcomes after fresh and frozen embryo transfer, between time-lapse imaging (n = 1064 patients) and standard culture (n = 818 patients) incubators. The live birth rate in fresh embryo transfers was found to be higher in TLI cycles when compared to conventional culture, (36.8 vs. 33.9%; 95% CI 1.05-1.57 for SC cycles), but not in frozen embryo transfers. The results from TLI incubators were also associated with improved perinatal outcomes and higher mean birth weight after fresh and frozen embryo transfer.

In another retrospective cohort study, twenty-three early and late time-lapse morphokinetic parameters were compared between patients who underwent euploid SET which resulted with (n = 68) or without (n = 61) ongoing pregnancy [51]. There were no significant differences in any embryo kinetics examined between the two groups, with exception of blastulation time. The length of blastulation was considerably shorter in patients with ongoing pregnancies (8.1 ± 3.2 h vs. 9.5 ± 3.4 h; $p = 0.014$). The authors suggested that when more than one euploid blastocyst is available, priority might be given to those with a shorter duration of blastulation.

A recent retrospective observational study in fresh eSET cycles further clarified whether blastulation time can be a useful predictor of IVF outcome [52]. It was found that Early Blastulation (EB) on day 4 of embryo development is a useful predictor of the quality of the following day 5 blastocyst, and it is a simple tool for selecting the best embryo to get a higher pregnancy rate in fresh eSET cycles. The early blastulation group (n = 55) had a higher rate of good blastocyst morphology (84.3% vs. 60.5%; $p < 0.0001$), higher clinical pregnancy rate (56.4% vs. 27.0%; $p = 0.013$), and a lower pregnancy loss rate (3.23% vs. 28.6%; $p = 0.081$) compared to late no-EB on Day 4 group.

Although many papers have pointed out the benefits of using time-lapse systems for embryo culture and evaluation, some authors have questioned their value [13,31,53]. These authors suggested that the available evidence is insufficient to support the use of TLI over conventional evaluation for embryo selection. Racowsky and Martins [53], analyzed 7 RCTs studies and concluded that the ongoing pregnancy rate was possibly worse in TLI compared to standard culture. Also, a Cochrane review by Armstrong, *et al.* [32] concluded that there was no good-quality evidence of differences in live birth or clinical pregnancies between TLI and conventional incubation, and strong randomized controlled trials are still required to validate clinical improvements.

Artificial Intelligence for IVF Prediction

Though time-lapse imaging represents a step toward more objective embryo evaluation, the inter- and intra-evaluator variability among embryologists using conventional morphological grading and/or TLI annotations is well documented [54,55]. The implementation of Artificial Intelligence (AI) for unbiased, automated embryo assessment, which is based on thousands of embryos can potentially more reliably predict embryo quality without human intervention. The algorithms for this analysis involve embryo morphology and morphokinetic parameters required for human annotation [54,56]. Several models with different AI algorithms were already used in ART to improve embryo selection and reproductive outcome, and many of them achieved a satisfactory precision [56].

Recently, Tran, *et al.* [57] created a deep learning model, which was an objective and fully TL automated system to predict the probability of clinical fetal heart pregnancy directly from time-lapse videos without the need for any manual morphokinetic annotation or blastocyst morphology assessment. They performed a retrospective analysis of time-lapse images and clinical outcomes for 10,638 embryos from eight different IVF clinics and demonstrated that a deep learning model has a high level of predictability for the likelihood of implantation.

The objective of another current study was to evaluate the respective performance of two commercially available morphokinetic-based models, KIDScore™ Day 5 versions 1 and 2 developed for Embryoscope™ devices and based on very large multicentric datasets, for the prediction of implantation and live birth after day 5 single blastocyst transfer [58]. Both models had statistically significant but limited predictive power for implantation. The use of these predictive models holds promises as decision-making tools to help embryologists select the best embryo, ultimately facilitating the implementation of SET policy. However, embryologists' expertise remains necessary to make the final decision.

Research in AI based on Deep Neural Networks (DNNs) was also implemented to select highest quality embryos using a large collection of human embryo time-lapse images (about 50,000 images) from a high-volume fertility center in the United States [54]. Using clinical data for 2182 embryos, a decision tree was created to integrate embryo quality and patient age to identify scenarios associated with pregnancy likelihood. Their analysis shows that the chance of pregnancy based on individual embryos varies from 13.8% (age ≥ 41 and poor-quality) to 66.3% (age < 37 and good-quality) depending on automated blastocyst quality assessment and patient age. Nonetheless, this method still has some limitations. For example, the results showed that the designed algorithm cannot successfully identify positive versus negative live birth using embryo morphology or morphokinetics alone.

In summary, the evaluation of the present pioneer studies on implementing AI to IVF clinics, suggest the need for further standardized research to validate the proposed models and their algorithms. It is evident too, that such analysis should include not only embryonic but also clinical and demographic data to increase the predictive accuracy of AI approach system.

In Overall Conclusion

1) The use of time-lapse monitoring, while not able to predict the ploidy status of an embryo, may be useful for identifying the embryo(s) with the highest implantation potential in a cohort of euploid embryos;

2) Although, there are diverse embryo selection algorithms, none of them are universal and can uniformly predict embryo viability and be applicable to different TL devices in different IVF clinics. This may be caused by the fact that existing algorithms do not include confounding factors like patient age, diagnosis, treatment type and environmental laboratory conditions.

3) Artificial Intelligence as a fully automated system, integrating enormous amount of various data could potentially avoid subjective assessment variability and more reliably predict the likelihood of successful clinical outcomes. Automation would also shorten the time required for analysis of TLI.

Preimplantation Genetic Testing for Aneuploidy

Preimplantation genetic testing for aneuploidy (PGT-A) has been a major breakthrough in the field of ART and became an alternative approach for identifying chromosomally normal embryos with the most potential to implant and produce a viable pregnancy [16,59]. Over the last several years this invasive method has undergone many technical developments, including implementation of trophectoderm cell biopsy at the blastocyst stage and introduction of Next-Generation Sequencing (NGS), a genetic analysis with greater accuracy and faster processing [60].

Although, many studies reported an improvement in clinical outcomes, the usefulness of PGT-A as a universal screening test for all IVF patients has yet to be determined [61]. Nonetheless, some recent studies provided important perspective on the value of 24-chromosome testing, suggesting the potential for this procedure to increase eSET utilization. For instance, the results of multicenter RCT showed that compared to standard morphological grading alone, PGT-A increased ongoing pregnancy and live birth rates in women aged 35-40 years after the transfer of a single euploid frozen-thawed blastocyst [62]. The addition of simultaneous testing for aneuploidy in PGT cases for monogenic disorders was found to significantly improve implantation and live birth rates (64.29% vs. 50.38% and 53.06% vs. 36.09%, respectively) after the first single FET cycle for young women carrying genetic diseases, in contrast to the control group which did not receive extra chromosomal screening [63]. Double embryo testing also reduced the miscarriage rate (3.17% vs. 11.94%) and the time interval to a successful pregnancy.

The chromosomal constitution of human embryos appears to have a significant effect on its developmental competency. Evaluation of PGT results revealed that preimplantation embryos might not entirely consist of normal, euploid or abnormal, aneuploid cells, but may contain of these, genetically different cell types within the same embryo. Such embryos are termed mosaic embryos. Since some mosaics produce successful pregnancies, it may be appropriate, with genetic counselling, to consider transferring mosaic embryos when euploid embryos are not available for transfer [64-66].

PGDIS Position Statement on the Transfer of Mosaic Embryos 2019 [67,68] stated that the classification of mosaicism has some ambiguity, and mosaicism levels lower than 20% or higher than 80% within trophectoderm cells tested by NGS cannot be differentiated from technical noise. Therefore, it is recommended that embryos with a lower range value of mosaic cells (< 20%) should be classified as euploids while embryos with mosaic cell > 80% as aneuploids. If the level of mosaicism is between 20-40%, embryos should be classified as mosaic with a low level of mosaicism, and if the level of mosaicism is between 40-80%, the embryo would be considered mosaic with a high level of mosaicism.

The reproductive potential of mosaic embryos, in addition to the degree of mosaicism, can be also affected by other different types of abnormalities such as chromosome gains or losses (trisomy or monosomy), segmental aneuploidy, or by the presence of particular chromosome(s) which may have detrimental consequences for clinical outcome (like chromosome 13, 18 or 21). Moreover, some embryos may have multiple chromosomal aberrations involved in complex mosaicism, and those are especially predicted to have a very low capacity to produce ongoing pregnancies. It is important to note that a single TE biopsy is unable to unmistakably reflect an embryo's overall chromosomal make-up, since it is impossible to know the degree to which TE cells are representative of the ICM that subsequently form the child. Moreover, the capacity of human embryo to self-correct during preimplantation and possibly the post-blastocyst stage may have a positive impact on embryo fate, thus making accurate diagnosis of embryonic viability remains a difficult task indeed. Therefore, all these data should be considered for integration of possible strategies related to the transfer of embryos with mosaicism and segmental aneuploidies [67,69].

Preimplantation genetic screening at the blastocyst stage is a complicated, complex procedure which has three main challenges

associated with trophectoderm biopsy samples: 1) TE biopsy is labour intensive and should only be performed by a highly skilled embryologist [59]; 2) TE biopsy is invasive and may have a negative impact on implantation and clinical pregnancy [70-72]; and 3) TE biopsy is subject to sampling bias and may not accurately represent the genetic constitution of the whole embryo [69,73-76].

The recent discovery of DNA within Blastocoel Fluid (BF) and spent embryo culture medium has led to the development of new technique for Non-Invasive Preimplantation Genetic Testing for Aneuploidy (NIPGT-A) [77,78] which can possibly eliminate the need for invasive trophectoderm biopsy procedure, and may better represent a whole embryo's chromosomal status [79,80].

At present, there are three major ongoing research approaches regarding how to collect a Cell-Free Embryonic DNA (cfeDNA) for non-invasive aneuploidy testing (NIPGT-A) [80]:

1. Blastocoel fluid aspiration using an ICSI pipette [81,82].
2. Spent Embryo Culture Medium (SEM) collection [83,84].
3. Combined spent embryo culture medium and blastocoel fluid (SEM+BF) collection without using an ICSI pipette [85,86].

Studies from our research group as well as others have shown that cfeDNA testing using blastocoel fluid and/or spent embryo culture medium on days 5 or 6 has great potential to detect chromosomal aneuploidy [81-86].

General ploidy concordance rates between cfeDNA from SEM and the corresponding whole blastocysts or TE biopsy have been published as low as 30.4% and as high as 87.5% [80]. However, in our report from 2018, measuring cfeDNA from a mixture of BF and SEM, we received 100% ploidy concordance between cfeDNA and TE biopsy in 19 freshly cultured blastocysts, with 98.2% concordance per single chromosome [85]. In addition, in our recent study on the efficacy and factors affecting accuracy of a non-invasive testing, we enhanced NIPGT-A technique and developed it as minimally invasive test for aneuploidy (miPGT-A) [87]. The overall concordance rate per sample for euploidy/aneuploidy status between miPGT-A and matching TE biopsy samples (n = 145 embryos) was 88/90 (97.8%), and was not different between good 47/48 (97.9%) and moderate/low quality blastocysts 41/42 (97.9%) ($p > 0.05$), suggesting that such improved test has a great potential to be a superior to the present PGT-A technique for analysis of embryo variability and selection for transfer.

Despite some progress in non-invasive genetic screening of human blastocyst several important issues still need to be addressed before the routine clinical implementation of NIPGT technique.

The main factor affecting the accuracy of the testing is the fact that the origin of extra-embryonic DNA in culture media remains unknown [88]. More work also needs to be undertaken to minimize genetic contamination from different sources (maternal or paternal DNA), to standardize culture conditions as well as optimize the Whole Genome Amplification (WGA) protocol for cfeDNA.

In summary, preimplantation genetic testing for aneuploidy by NGS is a valued method to predict blastocyst ploidy status. However, not all euploid embryos implant and such failure can be a consequence of complex problems with a wide variety of etiologies and mechanisms. Multiple causes affecting embryo implantation may not only include embryonic and endometrial factors but also anatomical anomalies, immune and endocrine or pelvic factors [89-91]. Therefore, additional tools and approaches to select the most viable blastocyst among euploid embryo cohort will potentially improve live birth rates in PGT cycles. Morphological and morphokinetic blastocyst assessment within the euploid embryo cohort could help to select the best quality blastocysts to further improve reproductive outcomes in PGT cycles. It seems also that treatment approach toward individual patient cases, depending on unique set of patients' characteristics, could be a new step to improve reproductive results.

CONCLUSION AND FUTURE IMPLICATIONS

Embryo quality has long been considered a major predictor of successful implantation and pregnancy, and within the last 20 years a remarkable progress was achieved in the development of the optimal and reliable strategies for identification of human blastocysts with the highest reproductive potential. The variety of techniques evolved rapidly from non-invasive, static morphological grading system and time-laps analysis of embryo dynamic morphokinetics to the invasive and complex evaluation of embryo genetic constitution. All these methods undeniably introduced unique values to the assessment of embryo quality, their selection for uterine transfer and to the enhancement of IVF clinical outcomes. It became evident that top-quality morphological features as well as certain embryonic kinetic markers of blastocysts, especially rate of blastocyst formation, are clearly associated with normal embryo ploidy and likelihood of higher implantation, pregnancy and live birth rates. The implementation of PGT-A has been a major breakthrough in the field of ART which has improved considerably in accuracy and enhanced selection of chromosomally normal, euploid blastocysts with promising developmental competency. Furthermore, this technique shed light on interesting biological facts' regarding human preimplantation embryos, such as a high frequency in chromosomal abnormalities, formation of mosaics and the exceptional ability of embryos for self-correction; all of which may have a significant impact on embryo fate after uterine transfer. However, despite notable advances in the process of embryo selection, the present strategies appeared to be inadequate to fully predict blastocyst capability to reach the term, as a significant proportion of euploid embryos still fail to implant and reported clinical outcomes are highly variable and contradictory. Therefore, extensive studies are currently performed on novel approaches for optimization and standardization of laboratory techniques that could reliably predict the best embryo to transfer with high probability of singleton delivery. Although, in experimental phase and still controversial, metabolomics, proteomics, cumulus cells or trophectoderm biopsy sample transcriptomics are promising methods which can provide additional and more adequate information regarding blastocyst viability, subsequently aiding better embryo selection.

While the recently developed non-invasive PGT-A technique, based on cell-free embryonic DNA collected from spent culture media and blastocoel fluid, has a unique potential to replace present commonly used invasive genetic embryo evaluation, it still requires further technical refinements and validation before routine use in clinical practice. Finally, emerging AI techniques, are slowly being introduced in assisted reproduction clinics, may represent a comprehensive strategy that may in the near future revolutionize IVF. This computerized approach created due to integration of an enormous amount of different data, combining embryo morphology, morphokinetics and genetics, as well as other clinical and patients' data, hold the promise to be a superior strategy for selecting human

blastocyst with the highest viability and reproductive potential. In summary, more research needs to be undertaken to improve and test the practicality of evolving technologies, and it is imperative to establish their validity as well as reliability for non-invasive embryo selection.

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