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MINI-REVIEW *Developments in reproductive biology and medicine*

Fertilization signatures as biomarkers of embryo quality

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ABSTRACT: Fertilization underpins the vital transition from gametic meiosis to embryonic mitosis. For decades, in human IVF, microscopic observation at a single time point has limited our appreciation of the morphokinetic complexity of this process. More recently, the introduction of time lapse technology—also enhanced by combination with artificial intelligence—has revealed the finest morphokinetic details of the beginning of human development. Overall, a picture has finally emerged in which the precise timing, morphology and geometry of several fertilization events offer clues to predict the fate of the embryo—a key aspect of assisted reproduction. In this scenario, correct unfolding of intra- and interpronuclear rearrangements emerge as a crucial factor to create a platform able to preserve genetic and cellular integrity at the first mitotic cleavage.

Key words: fertilization / oocyte / spermatozoon / meiosis / mitosis / embryo / morphokinetics / time-lapse

Introduction

Fertilization underpins the extremely delicate transition from the meiotic status of the gametes to the mitotic dynamics of the early embryo (Clift and Schuh, 2013; Coticchio and Brambillasca, 2013). IVF has offered a unique opportunity to directly investigate early human development. In the last decade, time lapse technology (TLT) has allowed dynamic and continuous observation of preimplantation development. Developmental and genetic studies have also revealed unsuspected complexities and critical points of the meiosis–mitosis transition (Clift and Schuh, 2013; McCoy *et al.*, 2018). Such knowledge has increased our awareness of the importance of fertilization as a cellular platform shaping the preimplantation embryo. In this review, we provide an update on the morphokinetic events accompanying fertilization, highlighting their importance as biomarkers for early embryo assessment.

Methods

PubMed was used to search the MEDLINE database for peer-reviewed English-language original articles concerning the fertilization stage in human IVF. Searches were performed by adopting 'fertilization', 'polar body', 'pronuclei', 'cleavage', 'blastocyst', 'pregnancy', 'live birth' and 'IVF/assisted reproduction' as main terms, in association with other keywords expressing concepts relevant to the subject (e.g. 'biomarker'). Papers based on static embryo assessment were cited solely for historical purposes. Records were further screened to select TLT investigations carried out since this technology was systematically introduced in human IVF in 2011. This highlighted 346 studies, 47 of which were included and discussed critically in this review (Fig. 1).

Fertilization biomarkers of embryo developmental competence

Second polar body

The time of second polar body (PB2) emission as a potential biomarker of embryo quality has been overlooked for decades. TLT has revealed that PB2 emission occurs earlier in oocytes that are microinjected with testicular spermatozoa (Scarselli *et al.*, 2018; Buran *et al.*, 2019) or artificially activated with Ca^{2+} ionophore prior to ICSI (Martínez *et al.*, 2021). However, the time of PB2 emission is comparable in euploid and aneuploid embryos (Chawla *et al.*, 2015; Minasi *et al.*, 2016). The predictive value of this parameter remains, however, marginal.

Pronuclei

Increasing evidence from TLT suggests that the timings of appearance of pronuclei (PNs) and their juxtaposition and breakdown are

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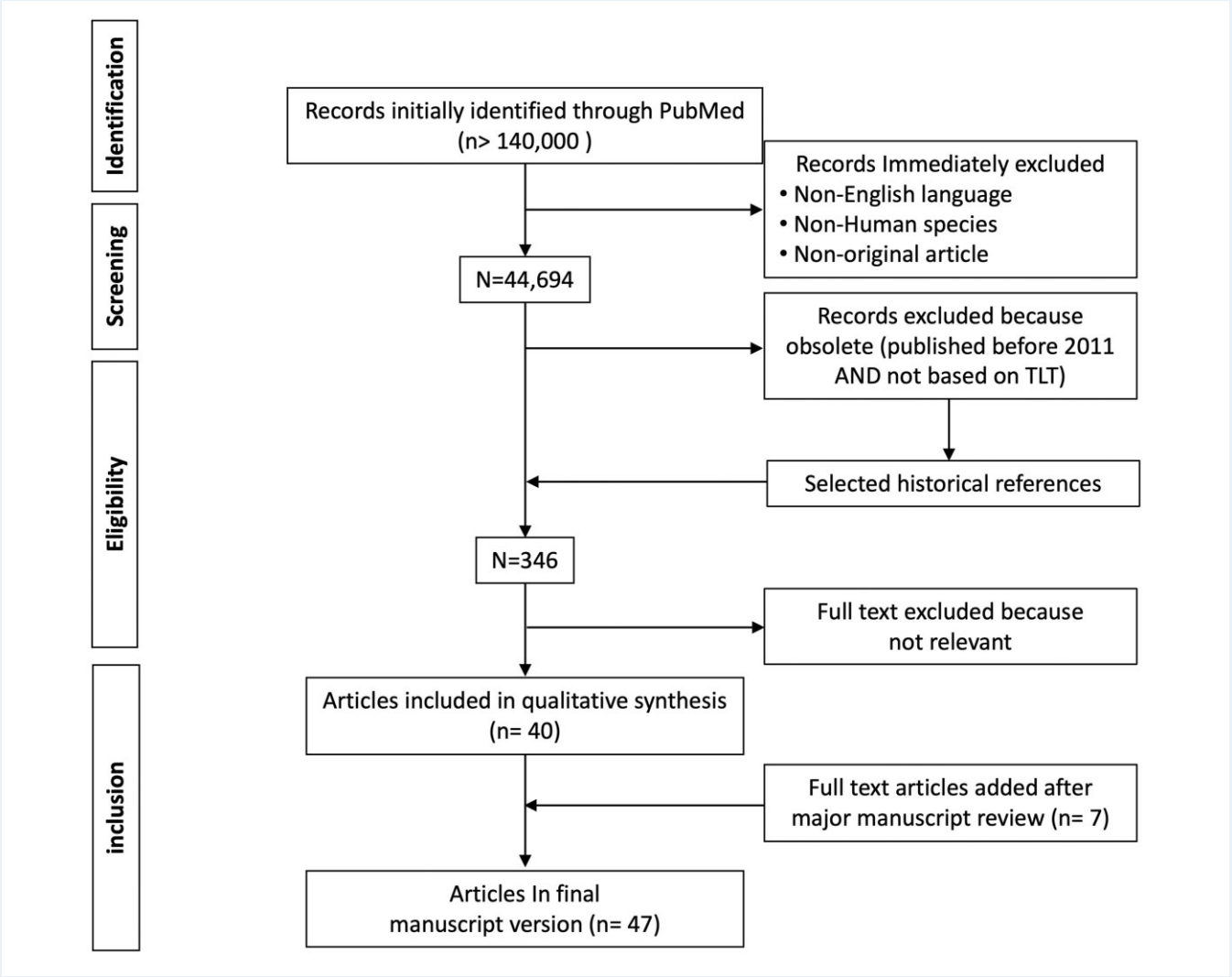

Figure 1. Schematic representation of the literature search for publications relating to fertilization and human embryo quality. TLT, time lapse technology.

associated with embryo quality and subsequent pregnancy outcomes (Payne *et al.*, 1997; Aguilar *et al.*, 2014; Coticchio *et al.*, 2018; Ezoe et al., 2022).

Timing of PN observation

Male and female PNs appear synchronously at ~6 h after ICSI (Coticchio *et al.*, 2018). PN juxtaposition occurs at a mean of 8.5 h post-ICSI. PNs then reposition centrally and disappear synchronously at 24.5 h after ICSI (Coticchio *et al.*, 2018). The timing of PN disappearance is associated with successful embryo implantation (Aguilar *et al.*, 2014). A recent study (Barrie *et al.*, 2021) showed that between 15.0 and 17.5 h post-insemination (hpi) the average number of 2PN zygotes was 98.2%, with the highest proportion detectable at 16.0–16.5 hpi (98.3%). At 18.0–18.5 hpi, the number of visible pronuclei reduced to 95.53% and continued to fall to 87.0% at 19.5–20.0 hpi. This suggests that without TLT and based on current consensus guidelines (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011) at the time of fertilization check (16–18 hpi), over 11% of oocytes would have been scored as unfertilized.

Off-centre PN position

Once juxtaposed, the position of PNs determines the orientation of the first cleavage plane, with a possible significant impact on blastomere size and integrity at the 2-cell stage. A stable off-centre PN position was associated with abnormal cleavage and a 2-fold decrease in the odds of live birth (Coticchio *et al.*, 2018; Barberet *et al.*, 2019) (Fig. 2A and B).

Mutual orientation of polar and PN axes

The position of PB2 extrusion determines the oocyte polar axis. Failure of the polar and PN longitudinal axes to align (Fig. 2A) may increase the rates of developmental arrest, abnormal cleavage,

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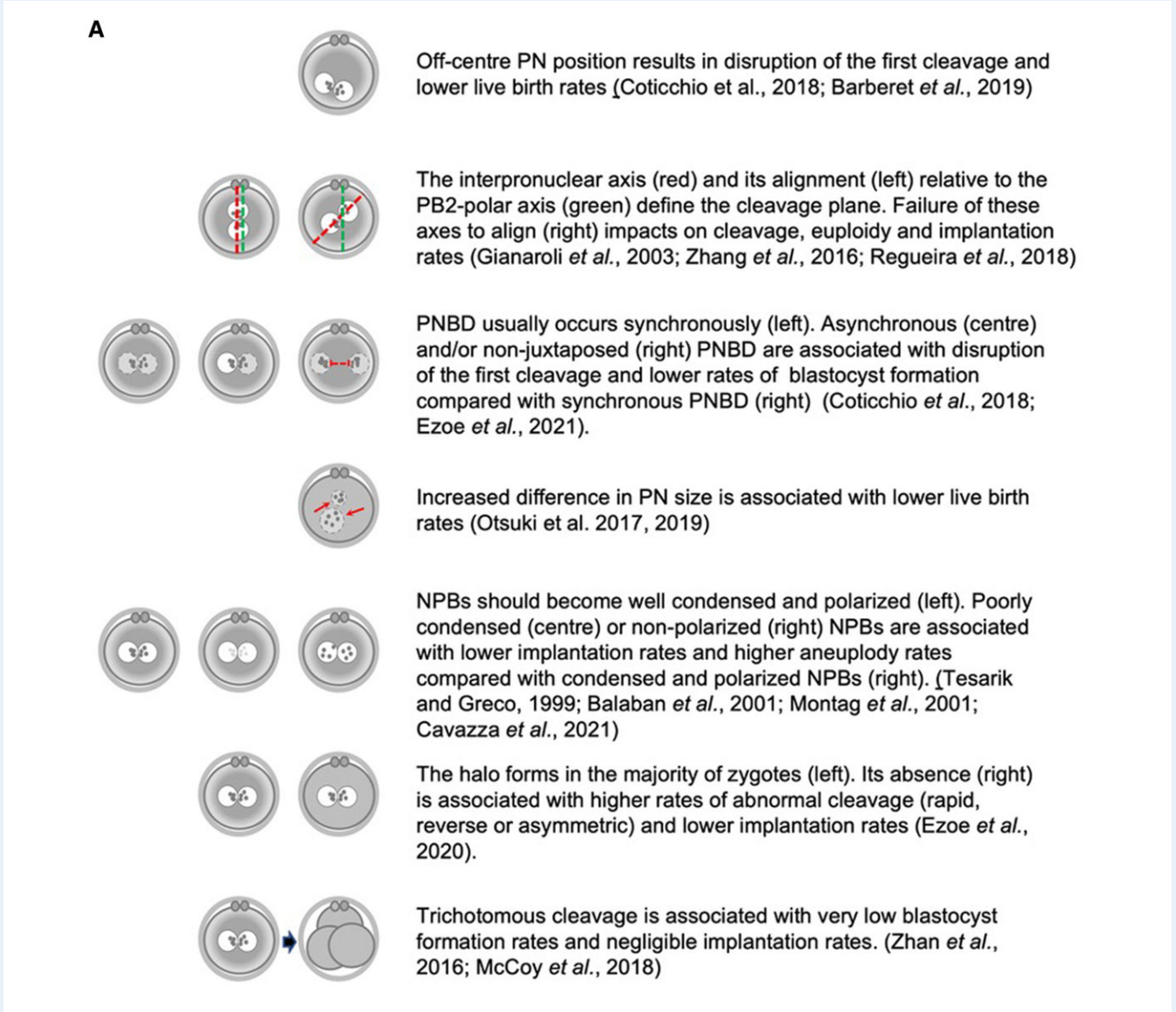

Figure 2. Morphokinetic abnormalities in the pronucleus and nucleolar precursor body. (**A**) Alternative configurations of morphokinetic events of fertilization and their relevance to embryo development and implantation. NPB, nucleolar precursor body; PB2, second polar body; PN, pronucleus; PNBD, PN breakdown. (**B**) Micrographs of abnormal fertilization patterns. Top: PNs positioned off-centre (left) shortly before breakdown (right). Centre: PNs showing no apparent abnormalities (left) before asynchronous PNBD derived from initial isolated disappearance of the PN positioned below (right). Bottom: Non-juxtaposed PNs (left) shortly before breakdown (right). Scale bar: 20 μm.

(continued)

aneuploidy and implantation failure (Gianaroli *et al.*, 2003; Zhang *et al.*, 2016; Regueira *et al.*, 2018).

PN juxtaposition and breakdown

Once formed, PNs migrate to the cell centre, juxtapose, and break down synchronously. In recent studies (Coticchio *et al.*, 2018; Ezoe et al., 2022), non-juxtaposition and asynchronous PN breakdown (PNBD) were significantly associated with aberrations of the first cleavage (Fig. 2A and B), which can seriously impact embryo development (Zhan *et al.*, 2016). Consistently, a lack of PN juxtaposition affects blastocyst cryopreservation rate (Ezoe et al., 2022).

PN size

The male PN is usually larger than its female counterpart, although such a difference tends to decrease before PNBD. Otsuki *et al.* (2017) observed that smaller differences in size between male and female pronuclei were positively associated with live birth rate (Fig. 2A). They also reported that when the female PN was larger than the male PN no pregnancies were achieved. Based on such evidence, they elaborated a formula integrating male and female PN size to predict live birth (Otsuki *et al.*, 2019). PN size also has some potential to predict the developmental ability of 1PN zygotes. Araki *et al.* (2018) showed that blastocyst rate was severely affected in 1PN zygotes with a small

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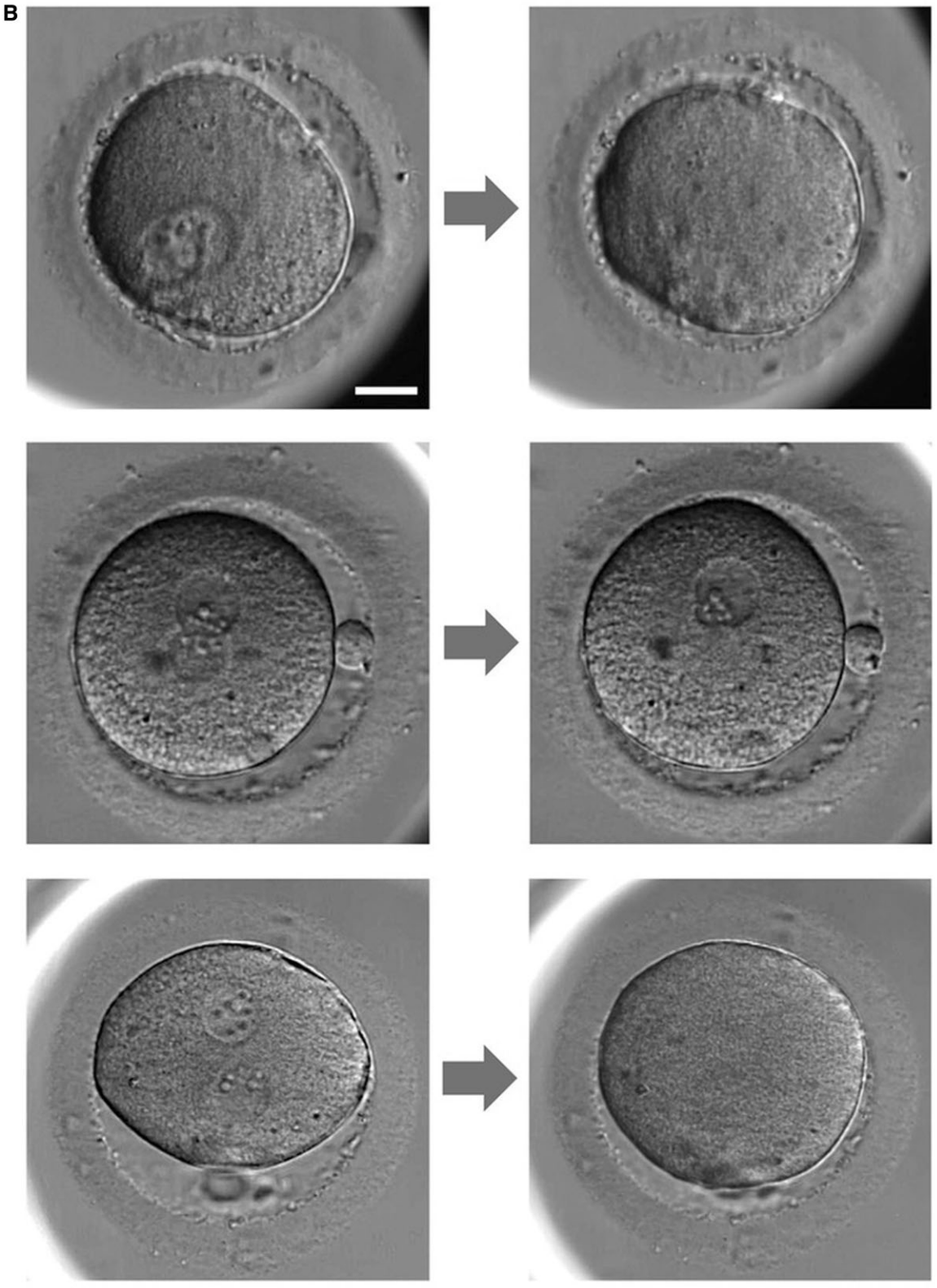

Figure 2. **Continued.**

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PN area. However, in 1PN zygotes with a larger PN area, blastocyst rate was comparable to that of normally fertilized controls (Araki et al., 2018).

Nucleolus precursor bodies

Nucleolus precursor bodies (NPBs) are aggregations of dense fibrillar material of largely unknown composition that are visible in the PN of human fertilized eggs. Spatial patterning of these particles is associated with chromatin rearrangement during fertilization, in preparation for the first cleavage (Fig. 2A). In 1999, for the first time, Tesarik and Greco (1999) observed that embryo ability to cleave and implant was positively correlated with two alternative NPB patterns: a relatively larger, but comparable, number of smaller NPBs homogeneously distributed (non-polarized) in both PNs; and a smaller number, typically 3–7, of larger NPBs clustered (polarized) in the area of juxtaposition between the two PNs. Subsequent studies based on static observation did not confirm conclusively these findings (Balaban et al., 2001; Montag and van der Ven, 2001; Salumets et al., 2001).

In recent years, TLT has given a new impetus to investigations on NPBs. As soon as PNs form, NPBs are visible in scattered arrangements. TLT showed that while the female PN repositions towards the male PN, both female and male NPBs tend to cluster in their respective intranuclear areas where PNs will ultimately juxtapose (Coticchio et al., 2018). TLT also revealed that NPB migration speed is positively associated with the rates of formation of a euploid blastocyst and live birth (Inoue et al., 2021), perhaps reflecting the tendency of NPBs to redistribute and cluster.

Recent investigations provide novel evidence for a relation between NPB patterns and chromatin rearrangements. A recent study (Cavazza et al., 2021) reported that NPB and chromatin distributions spatially coincide; the authors also observed that chromatin—initially decondensed—undergoes progressive condensation and clustering at the area of pronuclear juxtaposition. Hence, transition of NPB distribution from scattered to polarized reflects a parallel large-scale chromatin rearrangement and clustering. Cavazza et al. (2021) also observed that bovine zygotes with uncondensed chromatin in either the male or the female PN at the time of PNBD undergo major perturbations in chromosome segregation at the first mitosis, such as lack of chromosome interaction with the spindle, failed congression, chromosome lagging and formation of micronuclei.

Cytoplasmic movements

When observed by conventional light microscopy, the oocyte cytoplasmic texture is homogeneous and finely granulated. Shortly after ICSI, dynamic subtle perturbations—which escape static observation—can be detected by TLT. Cytoplasmic flares were described as an area with 'glassy cytoplasmic appearance' radiating from the site of formation of the male PN (Payne et al., 1997). Mio (2014) detected the same phenomenon in almost all normally fertilized oocytes inseminated by ICSI or standard IVF. However, the absence of flare was associated with no or abnormal fertilization. In addition, the timing of the cytoplasmic flare precisely predicts the time of appearance of the male PN (Coticchio et al., 2018). The nature of the cytoplasmic flare remains uncertain. However, time of appearance, positional relation with the male PN and shape suggest that it could reflect formation of the sperm microtubule aster.

Cytoplasmic movements represent a possible target for novel biomarkers of embryo quality. In the mouse, Ajduk et al. (2011) found that at fertilization rhythmic cytoplasmic movements detected by particle image velocimetry (PIV) predict zygote developmental potential. A recent study on human fertilized eggs and early embryos indicates that PIV and artificial intelligence analysis of cytoplasmic movements can predict blastocyst development (Coticchio et al., 2021).

Cytoplasmic halo

In most fertilized eggs, assessment of PN formation at 16–18 hpi also reveals a cortical area of reduced cytoplasmic granularity referred to as 'cytoplasmic halo'. The halo is not observed in non-inseminated or non-fertilized eggs. It probably reflects a displacement of organelles—mainly mitochondria—from the cell periphery to the centre to support PN function (Van Blerkom et al., 1995). Scott and Smith (1998) reported for the first time that the halo is predictive of implantation ability. Subsequent observations were consistent with these initial findings (Ludwig et al., 2000; Salumets et al., 2001; Zollner et al., 2002; Ebner et al., 2003). TLT has been instrumental in observing the cytoplasmic halo in a dynamic fashion. Formation of the halo starts at approximately 11 hpi (Coticchio et al., 2018). Its disappearance closely precedes PNBD by <1.5 h and involves a radial redistribution of cytoplasmic material towards the cortex. In a recent large and well-designed study (based on 1009 zygotes and single vitrified blastocyst transfer), Ezoe et al. (2020) provided convincing evidence of the importance of the cytoplasmic halo. They reported much higher rates of abnormal cleavage (rapid, reverse or asymmetric cleavage) in halo-negative zygotes (Fig. 2A). For the first time, they also described that the absence of the halo is associated with gamete characteristics, such as oocyte diameter and sperm quality. Therefore, the cytoplasmic halo has emerged as a significant biomarker of embryo quality.

Morphokinetic intervals

TLT has also generated novel parameters of embryo assessment, such as intervals between morphokinetic events. Aguilar et al. (2014) reported that embryos with higher implantation potential had a shorter interval (between 5.7 and 13.8 h) defined by the times of appearance and disappearance of PNs. Coticchio et al. (2018) assessed the ability of 10 different intervals to predict the number of cells and the degree of fragmentation of Day 3 embryos. They observed that intervals delimited by early fertilization events (such as PB2 emission, cytoplasmic flare and PN appearance) were not predictive of embryo quality. On the contrary, intervals defined by late fertilization events (halo appearance and disappearance, PNBD, first cleavage) were highly predictive of higher embryo quality (Fig. 3). Ezoe et al. (2020) focused on the morphokinetics of the cytoplasmic halo, reporting that the duration of the interval of halo appearance/disappearance is associated with rapid, reverse and asymmetric cleavage. They also observed an inverse relation between the duration of the same interval and rates of development to the stage of expanded blastocyst and ongoing pregnancy rates. Collectively, this evidence indicates that the duration of intervals delimited by later fertilization events may be associated with embryo and blastocyst quality.

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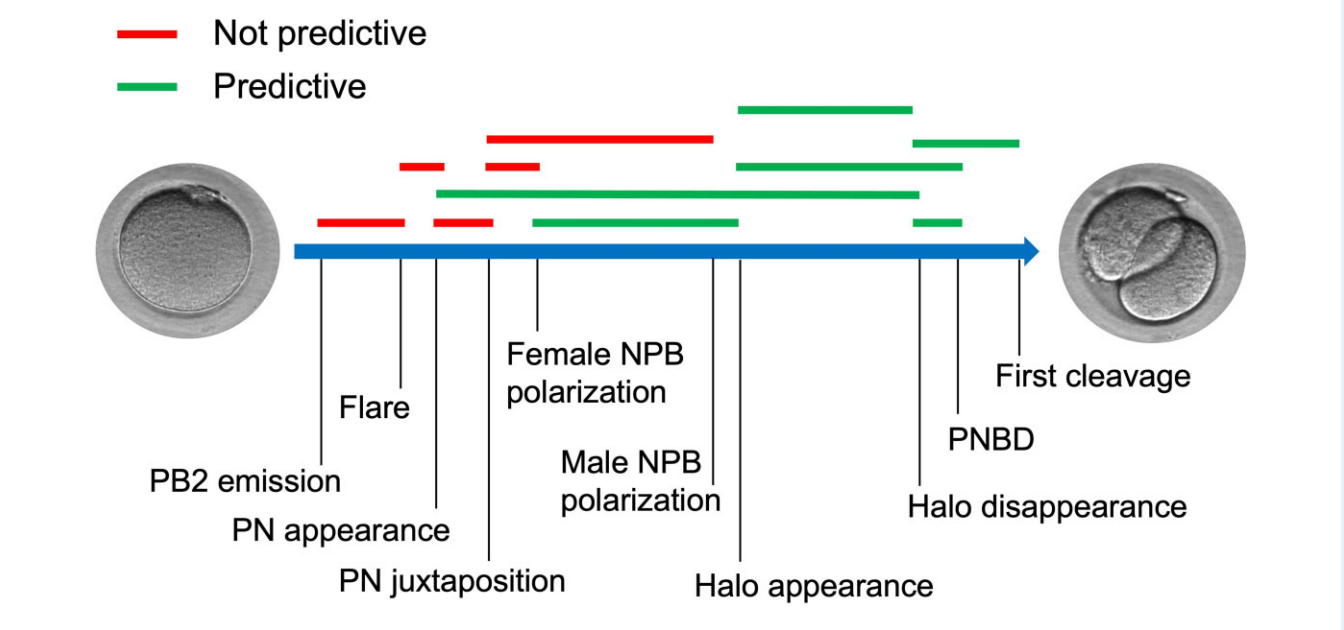

Figure 3. Schematic representation of intervals between fertilization events. Intervals between fertilization events that are predictive (green lines) or not predictive (red lines) of embryo quality and implantation potential are indicated (Coticchio *et al.*, 2018; Ezoe *et al.*, 2020). NPB, nucleolar precursor body; PB2, second polar body; PN, pronucleus; PNBD, PN breakdown.

First cleavage

The transition from meiosis to mitosis is challenging to accomplish safely in an interval of hours. This exposes the fertilized egg to perturbations of the first cleavage. For the first time, Shoukir *et al.* (1997) and Sakkas *et al.* (1998) proposed 'early cleavage'—i.e. occurrence of the first zygote division into two blastomeres within 25 h from insemination by standard IVF—as an independent parameter positively associated with the chances of an embryo producing a clinical pregnancy. This finding was confirmed by subsequent studies (Bos-Mikich *et al.*, 2001; Lundin *et al.*, 2001; Lundqvist *et al.*, 2001; Salumets *et al.*, 2001, 2003; Van Montfoort *et al.*, 2004; Ciray *et al.*, 2005; Giorgetti *et al.*, 2007).

TLT has generated further, more precise data on the timing and morphological correlates of the first embryonic mitosis. Meseguer *et al.* (2011) defined a precise interval of the first cleavage—from 24.3 to 27.9 hpi by ICSI—associated with higher implantation rates. Notably, this observation introduced the previously neglected concept that exceedingly early times of the first cleavage are negatively associated with implantation rates. TLT has also revealed other aspects of the first cleavage. Several observations have described the phenomenon of direct cleavage (DC) by which an embryonic cell divides into three daughter blastomeres via either a single trichotomous mitosis or two consecutive mitoses separated by a very short (<5 h) intervening time. This anomaly can occur at different cleavage stages. At fertilization, it directly forms a 3-cell embryo without an intervening 2-cell stage (Fig. 2A), with a frequency as high as 26% (Zhan *et al.*, 2016). The single trichotomous mitosis is of particular interest because it implies a highly abnormal cytokinesis derived from two intersecting cleavage planes. The developmental consequences of DC at the first mitosis are catastrophic. Zhan *et al.* (2016) observed that blastocyst rates are approximately six to seven times lower compared with controls. In addition, following Day 3 embryo transfer, implantation rates are negligible, and no live births are achieved. Studies based on TLT combined with preimplantation genetic testing for aneuploidy confirmed the crucial nature of the first cleavage for normal development and its sensitivity to disruptive deregulation. It was observed that blastocysts carrying cells affected by multiple and complex chromosome abnormalities often derive from DC occurring at the first mitosis (McCoy *et al.*, 2018). The reported chromosome abnormalities are consistent with segregation mediated by a tripolar spindle. McCoy *et al.* (2018) also observed that DC is strongly associated with a maternal genetic variant spanning the centrosomal regulator polo-like kinase 4 (PLK4), which can induce tripolar mitosis in experimental models.

Conclusion

Single static observation is unable to capture the extreme dynamism of fertilization and its numerous sub-processes. The introduction of TLT and advanced image analysis in IVF have prompted a paradigm shift. They have allowed observation of the continuum and the fine details of morphological changes. Such novel technology has produced a much more precise and informative morphokinetic map of embryo development and unveiled previously unknown phenomena. This is dramatically confirmed by the recent observation that suboptimal timing of static fertilization assessment fails to detect as many as 11% of normal 2PN zygotes (Barrie *et al.*, 2021). Overall, TLT has strengthened the significance of traditional fertilization biomarkers, for example PN dynamics and the cytoplasmic halo. More compellingly, novel

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parameters of assessment have emerged: cytoplasmic movements, NPB velocity, PNBD asynchrony and morphokinetic intervals are some of the more prominent. Strikingly, among them, the timed and detailed observation of the first mitosis has revealed trichotomous cleavage—presumably triggered by the formation of a tripolar mitotic spindle—as a frequent and often devastating event for the destiny of the embryo. Collectively, this evidence calls for a new consensus in human IVF on the significance and practical exploitation of morphokinetic parameters of fertilization as adjuncts to more established criteria of embryo assessment.

Data availability

No new data were generated or analysed in support of this research.

Authors' roles

G.C.: coordination; manuscript design, draft, critical reading and approval; A.B.: manuscript design, draft, critical reading and approval; C.Z.: manuscript design, draft, critical reading and approval; E.M.: manuscript design, draft, critical reading and approval; and I.S.: coordination, manuscript design, draft, critical reading and approval.

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Conflict of interest

The authors have no conflicts of interest to declare.

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