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Focused time-lapse analysis reveals novel aspects of human fertilization and suggests new parameters of embryo viability

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STUDY QUESTION: Can focused application of time-lapse microscopy (TLM) lead to a more detailed map of the morphokinetics of human fertilization, revealing novel or neglected aspects of this process?

SUMMARY ANSWER: Intensive harnessing of TLM reveals novel or previously poorly characterised phenomena of fertilization, such as a cytoplasmic wave (CW) preceding pronuclear formation and kinetics of pronuclear chromatin polarization, thereby suggesting novel non-invasive biomarkers of embryo quality.

WHAT IS KNOWN ALREADY: In recent years, human preimplantation development has been the object of TLM studies with the intent to develop morphokinetic algorithms able to predict blastocyst formation and implantation. Regardless, our appreciation of the morphokinetics of fertilization remains rather scarce, currently including only times of polar body II (PBII) emission, pronuclear appearance and fading, and first cleavage. This is not consistent with the complexity and importance of this process, calling for further TLM studies aimed at describing previously unrecognized or undetected morphokinetic events and identifying novel developmental biomarkers.

STUDY DESIGN, SIZE, DURATION: The study involved a retrospective observation by TLM of the fertilization process in 500 oocytes utilized in consecutive ICSI cycles carried out in 2016. A maximum of five fertilized oocytes per patients were included in the analysis to reduce possible patient-specific biases. Oocytes of patients with different diagnoses of infertility where included in the analysis, while cases involving cryopreserved gametes or surgically retrieved sperm were excluded.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: Microinjected oocytes where assessed by a combined TLM-culture system (Embryoscope). Oocytes that were not amenable to TLM assessment, due to excess of residual corona cells or inadequate orientation for the observation of PBII emission, were not analysed. We identified and monitored 28 parameters relevant to meiotic resumption, pronuclear dynamics, chromatin organization, and cytoplasmic/cortical modifications. Times (T) were expressed as mean ± SD hours post-insemination (p.i.) and analysed, where appropriate, by Paired T Student or Fisher's exact tests.

MAIN RESULTS AND ROLE OF CHANCE: PBII emission was occasionally followed (4.3% of cases) by the transient appearance of a protrusion of the cell surface, the fertilization cone (FC), probably resulting from interaction of the male chromatin with the oocyte cortex.

Pronuclear formation was always preceded by a radial CW originating from the initial position of the male pronucleus (PN) and extending towards the oocyte periphery. The appearance of the CW followed a precise sequence, occurring always 2–3 h after PBII emission and shortly before PN appearance.

Male and female PN appeared virtually simultaneously at approximately 6.2 h p.i. However, while the female PN always formed cortically and near the site of emission of the PBII, the initial position of the male PN was cortical, intermediate, or central (15.2%, 31.2% and 53.6%, respectively). PN juxtaposition involved rapid and straight movement of the female PN towards the male PN. In addition, the initial position

of male PN formation was predictive of the position of PN juxtaposition. It was also observed that nucleolar precursor bodies (NPBs) aligned along the juxtaposition area and this happened considerably earlier for the female PN (8.2 ± 2.6 vs.11.2 ± 4.1 h, $P = 0.0001$).

Although it occurred rarely, displacement of juxtaposed PN to the cortex was strongly associated ($P < 0.0001$) with direct cleavage into three blastomeres at the first cell division. The times of PN breakdown and first cleavage showed a very consistent trend, occurring earlier or progressively later depending on whether initial male PN positioning was central, intermediate or cortical, respectively.

Finally, time intervals between discrete fertilization events were strongly associated with embryo quality on Day 3. For example, longer intervals between disappearance of the cytoplasmic halo and PN breakdown were highly predictive of reduced blastomere number and increased fragmentation ($P = 0.0001$).

LARGE SCALE DATA: N/A

LIMITATIONS, REASON FOR CAUTION: Some of the morphokinetic parameters assessed in this study may require better definition to reduce inter-operator annotation variability.

WIDER IMPLICATIONS OF THE FINDINGS: To our knowledge, overall, these data represent the most detailed morphokinetic description of human fertilization. Many of the illustrated parameters are novel and may be amenable to further elaboration into algorithms able to predict embryo quality, as suggested by the findings presented in this study.

STUDY FUNDING/COMPETING INTERESTS: None.

Key words: fertilization / ICSI / time-lapse microscopy / pronuclei / morphokinetics / embryo quality

Introduction

Over many decades of observations and experimentations, classical embryology and recent cellular and molecular research have moulded the concept of a continuum between oogenesis and embryogenesis. Indeed, competencies that are key to successful development of the preimplantation embryo are established during oocyte growth and maturation (Li *et al.*, 2008; Bebbere *et al.*, 2016), often with the support of the somatic compartment of the ovarian follicle.

In such a continuity between the life of the gamete and that of the embryo, fertilization plays a unique and central role. In fact, it represents a bridge between gamete differentiation and totipotency, between meiosis and mitosis and, ultimately, between generations. It should not surprise, therefore, that an important part of the developmental fate of the embryo depends on how fertilization unfolds. For such reasons, assessment of fertilization is an essential part of IVF treatment. However, until recently, clinical embryologists have looked at this developmental stage in a rather static modality that has involved the mere search for signs of normal fertilization, i.e. the presence of two pronuclei (2PN) at 16–18 h post-insemination (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). This approach has been imposed by inevitable practical constraints derived from the time schedule of human IVF treatment, as well as concerns of detrimental effects on embryo health caused by exposure to non-controlled atmospheric conditions during microscopic observation. Notably, static assessment of fertilization at a single time point may have frustrated attempts to qualify, as biomarkers for embryo quality, highly dynamic cellular manifestations, such as patterns of nucleolar precursor bodies (NPBs) that are believed to change at various phases of the pronuclear stage (Tesarik and Greco, 1999; Scott, 2003).

The introduction of time-lapse microscopy (TLM) in clinical embryology has changed the rules of the game of embryo morphological assessment, by allowing uninterrupted observation of a process, preimplantation development, that is in fact a continuum. With the aid of TLM, a plethora of studies have been carried out with the aim of identifying morphokinetic markers of embryo implantation potential or to reveal unsuspected aspects of the biology of preimplantation development. However, while the overwhelming majority of such studies have focused on morphokinetics of developmental events starting from the first cleavage (Meseguer *et al.*, 2011; Dal Canto *et al.*, 2012b; Armstrong *et al.*, 2014; Petersen *et al.*, 2016), so far fertilization has been described rather poorly, merely in terms of times of polar body II (PBII) emission and pronuclear appearance, juxtaposition and disappearance (Aguilar *et al.*, 2014; Chamayou *et al.*, 2015). This is rather unexpected and disappointing for at least two important reasons. Firstly, many essential fertilization events cannot be assessed in clinical IVF, considered that their detection would require invasive analytical techniques; nevertheless some crucial steps of fertilization are reflected in morphological epiphenomena that can be captured by conventional transmitted light microscopy. Secondly, unlike several other mammalian species, the cytoplasm of the human oocyte is translucent, making human fertilization amenable to morphological observation of subcellular events.

Providing scope for this study, we addressed the question of whether the morphokinetic map of human fertilization could be redrawn in much finer detail by better harnessing the potential of TLM technology currently used in ART laboratories, thereby proposing novel approaches and concepts for embryo evaluation.

To our knowledge, collectively our data represent the most detailed spatial-temporal view of human fertilization, revealing phenomena that were previously unrecognized or poorly characterised. They also suggest that the morphokinetics of such phenomena have the potential to predict embryo development and therefore, with further elaboration, to be used as non-invasive biomarkers of embryo implantation.

Materials and Methods

Patients

Data presented in this study were obtained from consecutive treatment cycles of infertile couples requiring ART therapy and were performed in

2016 at Biogenesi Reproductive Medicine Centre, Monza, Italy. Diagnosis of infertility included various causes, including male factor, tubal factor and polycystic ovary (PCO, but not PCOS) with or without chronic anovulation. The study was approved by the competent ethical committee (ASST, Monza). Written informed consent was obtained from all couples before starting treatment.

Semen preparation

Semen samples were prepared by discontinuous gradients (47.5% and 90%) of Sil-Select (Ferti-Pro, Belgium). Spermatozoa were washed and re-suspended in HEPES-buffered Sperm Preparation Medium (Origio, Måløv, Denmark) and stored in an incubator at room temperature in an unmodified atmosphere until use (Dal Canto *et al.*, 2012a).

Oocytes

Pituitary down-regulation was achieved by GnRH antagonist (Ganirelix, Merck Sharp & Dohme, Rome, Italy). Ovarian stimulation was carried out with rFSH (Puregon, Merck Sharp & Dohme, Rome, Italy or Gonal-F, Merck, Rome, Italy) (Fadini *et al.*, 2015). A dose of 10 000 IU hCG was administered 36 h prior to oocyte collection (Ovitrelle Merck, Rome, Italy). After retrieval, cumulus cell-oocyte complexes were cultured in fertilization medium (Sequential Fert, Origio, Måløv, Denmark). Within 3 h from collection, cumulus cells were removed by brief exposure to culture medium containing cumulase (80 U/ml; ICSI Cumulase, Origio, Måløv, Denmark), followed by mechanical action. Oocytes displaying the polar body 1 (PBI) were moved to microdrops of cleavage medium (Sequential Cleav, Origio, Måløv, Denmark), selected for treatment and fertilized by ICSI starting precisely at 4 h from collection.

ICSI and embryo culture

ICSI was carried out according to conventional methodology. Oocytes were positioned in order to place the first polar body at the 12 o'clock position, while the injecting needle was inserted from the 3 o'clock position. Microinjected oocytes were moved to Embryoslide™ Culture slides (Vitrolife, Göteborg, Sweden), a specific 12-well plate prepared with a 30-µl microdrop of cleavage medium placed in each well and covered in paraffin oil (Origio, Måløv, Denmark). The plates were equilibrated overnight before use. Prezygotes and embryos were cultured up to 3 days in cleavage medium. A $N_2/CO_2/O_2$ (89:6:5, v/v) atmosphere without humidity control and a 37°C temperature were adopted as culture conditions. Embryos were cultured and analysed for morphokinetic parameters in an integrated embryo culture TLM system, i.e. the EmbryoScope™ Time-lapse System (Vitrolife, Göteborg, Sweden) (Dal Canto *et al.*, 2012b). Image acquisition was set every 10 min at seven different focal planes for each embryo. Images (1280 × 1024 pixels) were acquired by a Leica 20 × 0.40 LWD Hoffman Modulation contrast objective specialized for 635-nm illumination. Illumination for image acquisition was <0.5 s per image, using single 1-W red LED. Only one embryologist was in charge of annotation of fertilization and embryo parameters, in order to reduce bias due to inter-operator differences. Embryos were classified according to morphological parameters, such as fragmentation and blastomere shape and number (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

Annotation of morphokinetic parameters of fertilization

Following the sequential order of oocyte injection, one to five fertilized oocytes per patient were included in the analysis to reduce possible patient-specific biases. Oocytes that were not amenable to TLM assessment, due to excess of residual corona cells or inadequate orientation for the observation PBII emission, were not included in the study. A total of 28 morphokinetic parameters of fertilization were identified and monitored. Times were expressed in terms of hours post-insemination (p.i.). Detected parameters were relevant to meiotic resumption, pronuclear dynamics, chromatin organization, and cytoplasmic/cortical modifications. Most of the paramenters (13 time-based and 10 positional or morphological) were considered relevant to the study object and further analysed (Tables II and III). Annotation options for such parameters are not included in the design of the Embryoscope software. Therefore, an 'ad hoc' independent database was created and used for elaboration.

Statistics

Data were collected in a dedicated and anonymized database and summarized as mean ± standard deviation (SD) for time parameters and as percentages and absolute frequencies for categorical data. A box plot was used to summarize data distribution. Kruskall–Wallis' sum rank test was performed to compare times for female and male PN breakdown and for the first cleavage, stratified according to the position of male PN appearance. Time intervals between temporal parameters were also calculated and their association with embryo cell number and percentage of fragmentation on Day 3 was investigated. A level of $P < 0.05$ was adopted for significance. Statistical analysis was performed by Stata software 9.0 (Stata Corporation, College Station, TX).

Results

Data elaborated in this study were derived from ICSI cycles of 168 women. Female age, BMI, ovarian reserve and cause of infertility are reported in Table I.

In 500 fertilized oocytes displaying 2PN, 28 parameters were identified and monitored, relevant to meiotic resumption, pronuclear dynamics, chromatin organization, and cytoplasmic/cortical modifications. Most of the parameters (13 time-based and 10 positional or

Table I Female characteristics and diagnosis of infertility of patients whose fertilized oocytes were subject to morphokinetic analysis. A maximum of five fertilized oocytes from each treatment were analysed.

Patient characteristics and causes of infertility	
Female characteristic (median and range)	
Age (years)	36.5 (25.5–45.9)
BMI	21.9 (21.3–22.7)
AMH (ng/ml)	2.2 (0.2–11.6)
Cause of infertility (%)	
Unexplained	36.3
Male factor	35.7
Endometriosis	3.6
PCO	4.0
Tubal	7.7
PCO and tubal	0.6
PCO and male factor	2.4
Reduced ovarian reserve	5.4
Reduced ovarian reserve, endometriosis and tubal	0.6
Reduced ovarian reserve and male factor	3.6

morphological) were considered relevant to this study and reported in this manuscript. Mean times (± SD) of major temporal parameters are described in Table II, while temporal distributions of the same parameters are illustrated in Fig. 1. Positional parameters are reported in Table III.

Fertilization cone

Table II does not include a temporal event, the fertilization cone (FC), which was exhibited only sporadically by fertilized oocytes. This phenomenon was previously described as a transient cortical protrusion, similar to a polar body, occurring in human oocytes fertilized by standard IVF (Mio, 2013), but it went totally neglected in hundreds of TLM studies involving oocytes fertilized by ICSI. We observed the FC only in a minute minority (3.6%) of fertilized oocytes (Movie 1). Interestingly, appearance of the FC (4.1 ± 1.3 h) was always successive to PBII emission (3.3 ± 1.1 h), while its position was always in close proximity to the initial position of the male PN. The hypothesis that in ICSI fertilization the FC represents a casual interaction between male DNA and oocyte cortex is illustrated in the discussion.

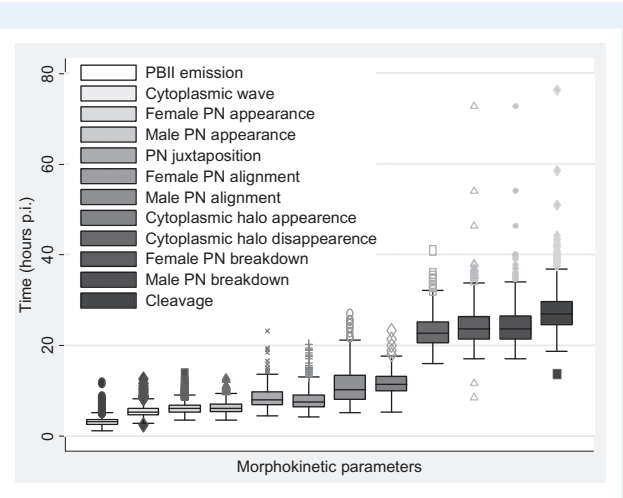

Figure 1 Temporal distributions of morphokinetics events occurring during fertilization and relevant to meiotic resumption, pronuclear dynamics, chromatin organization and cytoplasmic/cortical modifications.

Table II Mean times of morphokinetics events occurring during fertilization and pertaining to meiotic resumption, pronuclear dynamics, chromatin organization and cytoplasmic/cortical modifications.

Parameter	Hours from ICSI (mean ± SD)
PBII emission	3.3 ± 1.1
Cytoplasmic wave	5.5 ± 1.3
Female PN appearance	6.2 ± 1.4
Male PN appearance	6.3 ± 1.4
PN juxtaposition	8.5 ± 2.3
Female NPB alignment	8.2 ± 2.6
Male NPB alignment	11.2 ± 4.1
Cytoplasmic halo appearance	11.7 ± 2.5
Cytoplasmic halo disappearance	23.1 ± 3.5
Female PN breakdown	24.4 ± 4.7
Male PN breakdown	24.5 ± 4.7
Cleavage	27.7 ± 5.0

Cytoplasmic wave

During fertilization, the cytoplasm is subject to major rearrangements and movements. In all 500 fertilized oocytes subject to analysis, we observed one very consistent example of these manifestations. It appeared as a well coordinated radial cytoplasmic wave (CW) originating from the initial position of the male PN and extending towards the oocyte periphery (Movie 2). The appearance of the CW followed a precise sequence in the timeline of fertilization occurring on average 2–3 h after PBII emission and always shortly before PN formation (3.3 ± 1.1, 5.5 ± 1.3 and 6.2 ± 1.4 h, respectively). We also assessed the relationships between PN appearance and PBII

Table III Positional parameters of morphokinetics events occurring during fertilization and relevant to meiotic resumption, pronuclear dynamics, chromatin organization and cytoplasmic/cortical modifications.

Positional parameter	Percent (no.)
Position of female PN appearance	
Cortical	100 (500)
Intermediate	0 (0)
Central	0 (0)
Position of male PN appearance	
Cortical	15.2 (76)
Intermediate	31.2 (156)
Central	53.6 (268)
Relative PN movement	
Female-towards-male	92.4 (462)
Mutual	7.6 (38)
Male-towards-female	0 (0)
Shape of female PN trajectory	
Rectilinear	95.6 (478)
Curved	4.4 (22)
PN juxtaposition	
Cortical	12.4 (62)
Intermediate	33.0 (165)
Central	54.6 (273)
Displacement of juxtaposed PN	5.4 (27)
PN separation following juxtaposition	0 (0)
PN breakdown and reappearance	0.2 (1)
Shape of cytoplasmic halo	
Symmetric	28.4 (142)
Asymmetric	63.0 (315)
Absent	8.6 (43)
Direct cleavage into three or more blastomeres	15.8 (79)

emission (Fig. 2A) or occurrence of the CW (Fig. 2B), observing that the CW is a much better predictor of the time of PN formation. Timing and geometry of the CW are suggestive of the sperm aster, as reported in the discussion.

PN formation and movement

Male and female PN appeared virtually simultaneously at around 6.2 h p.i. (Table II). However, while the female PN formed initially always cortically and near the site of emission of the PBII (Table III), the initial position of the male PN was cortical, intermediate, or central (15.2%, 31.2% and 53.6%, respectively). In 92% (n = 462) of samples, the female PN moved towards the male counterpart, describing a rectilinear path (96%, n = 478) (Table III), in some cases approaching a linear speed of 50 μm/h (data not shown) (Movie 3). On average, the time of juxtaposition occurred 2.3 h after the time of PN formation (Table II). Notably, a close association (78.2%, $P < 0.0001$) was observed between the initial position of male PN formation (Table III) and the position of PN juxtaposition (cortical: 12.4%; intermediate: 33.0%; central: 54.6%).

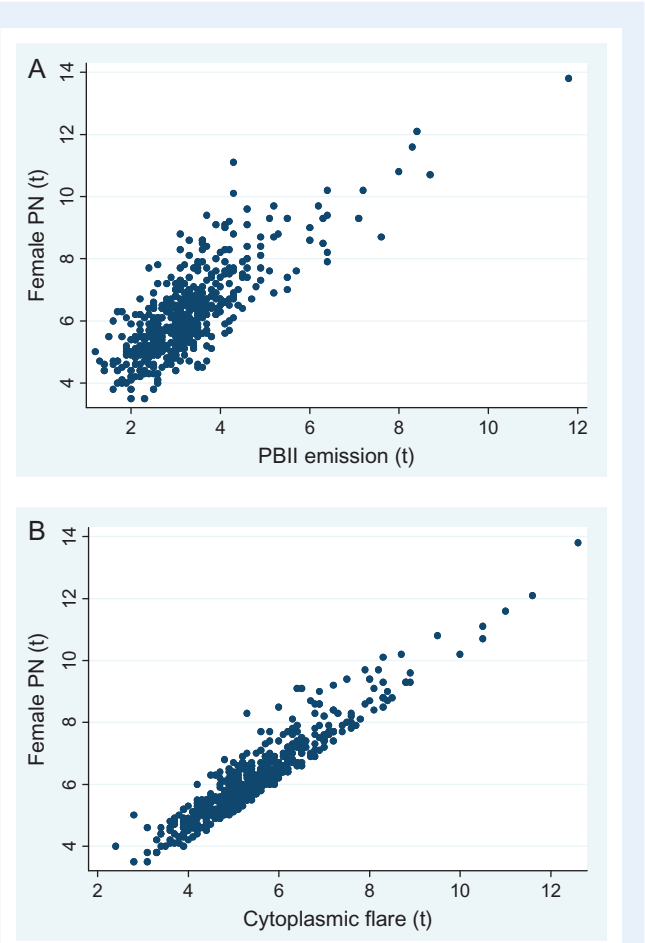

Figure 2 Temporal relationship between times of female pronucleus (PN) appearance and times of polar body II (PBII) emission (**A**) or occurrence of the cytoplasmic wave (**B**). Values are expressed in hours post-insemination (p.i.).

NPB dynamics

We observed that NPB polarized (aligned) along the juxtaposition area earlier for the female PN than for the male PN (8.2 ± 2.6 vs.11.2 ± 4.1 h, $P = 0.0001$) (Table II). Also, by the time of PN juxtaposition, most (67.3%) female PN had their NPB already polarized. On the contrary, in the male PN chromatin polarization occurred after juxtaposition in the large majority of cases (81.1%, $P < 0.0001$). Finally, it was found that failure in NPB alignment was four times more likely to occur in the male PN (2.4% vs. 8.8%, $P < 0.0001$).

Cytoplasmic halo

Formation of the cytoplasmic halo is a known phenomenon of fertilization, but its morphokinetic characteristics are at present undetermined. On average, its formation occurred at approximately 11 h p.i. (Table II). Its position relative to the centre of the oocyte was in most cases (63%) asymmetrical. Absence of a halo was observed in a small minority (8.6%) of fertilized oocytes (Table III). Halo dissolution involved a radial redistribution of cytoplasmic material towards the cortex and strictly preceded PN breakdown by less than 1.5 h.

Space–time of pronuclear development and first cleavage

The times of male and female PN breakdown and first cleavage were analysed in relation to the initial position of appearance of the male PN (Table IV). Indeed, times of PN breakdown and first cleavage showed a very consistent trend, occurring progressively later depending on whether initial male PN positioning was central, intermediate or cortical ($P = 0.0044$, 0.0016 and 0.0087, respectively).

Pronuclear displacement and breakdown

Following juxtaposition, if not already localized centrally, male and female PN were repositioned in a central region of the oocyte. Occasionally (5%, n = 25), following juxtaposition and repositioning, both male and female PN were displaced from a central position to the oocyte cortex (Table III), in concomitance with a wave of cortical deformation (Movie 4). Although rare, this event was strongly associated ($P < 0.0001$) with direct cleavage into three blastomeres at the first cell division. PN disjunction after juxtaposition was never observed, while in a single case PN disappearance was followed by reappearance. Female and male PN disappeared very synchronously (24.4 ± 4.7 and 24.5 ± 4.7 h,

Table IV Times (hours post-insemination) of male and female pronucleus (PN) breakdown (PNBD) and first cleavage analysed in relation to the initial position of appearance of the male PN.

	Position of male PN appearance			*P*
	Central	Intermediate	Cortical	
Time of female PNBD	23.8 ± 4.3	24.7 ± 3.7	25.5 ± 6.8	0.0044
Time of male PNBD	23.9 ± 4.3	25.0 ± 4.2	25.6 ± 6.8	0.0016
Time of first cleavage	27.1 ± 4.5	28.2 ± 4.6	28.8 ± 7.0	0.0087

respectively) (Table II). Time differences in the breakdown of the two PN larger than 1 h were rare (2.0%), but if this occurred, it was frequently associated with developmental failure due to direct cleavage into thee blastomeres at the first cell division (6/10, 60%) (Movie 5) and/or times of first cleavage longer than 40 h (4/10, 40%).

Time intervals and embryo quality

To explore the hypothesis that the morphokinetics of fertilization can predict embryo quality, different time intervals between discrete fertilization events were assessed in relation to the number of cells and degree of fragmentation of resulting embryos on Day 3 of development (68 h p.i.). Nine different intervals were assessed, setting as extremes of such intervals the times of PBII emission, CW, PN appearance, PN juxtaposition, male and female chromatin polarization, halo appearance and disappearance, PN breakdown and first cleavage (Table V). Four of such intervals were strongly associated with embryo quality on Day 3. For example, longer intervals between halo disappearance and PN breakdown were highly predictive of reduced blastomere number and increased fragmentation ($P = 0.0001$). Likewise, an increased interval between PN breakdown and first cleavage was positively associated with poor embryo quality ($P = 0.0001$).

Discussion

Fertilization plays a fundamental role in biology assuring continuity between generations by providing the link between gametes and embryo. This astonishing task requires a regulation of biochemical and cellular events that, for its magnitude on a cellular scale and complexity, was quite correctly described as a 'choreography' (Ramalho-Santos *et al.*, 2000; Coticchio and Brambillasca, 2013).

By better harnessing the potential of current TLM technology applied to ART, in the present study we reveal the morphokinetics of fertilization with unprecedented details, offering the clinical embryologist new insights on the subcellular dynamics of this process and expanding the range of possible morphokinetic parameters that may be developed as biomarkers of embryo quality and, ultimately, implantation ability.

Despite its founding role in development, relatively little is known about human fertilization. Affected by the constraints imposed by observation at a single time point, until a few years ago clinical embryologists had a minimalist approach to the assessment of fertilization, (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011), relying only on the presence of the PBII and the number of pronuclei. So far, the advent of TLM in routine ART has not changed this scenario significantly. In 2014, Aguilar and colleagues (2014) investigated possible associations between fertilization events and embryo implantation, however, limiting their observation to the times PBII extrusion and PN appearance, juxtaposition and breakdown, without discriminating between male and female PN. A year later, Chamayou *et al.* (2015) described the morphokinetic behaviour of embryos developed from fresh or vitrified oocytes, but likewise without extending the range of their observations to novel fertilization parameters. Of note, exactly 20 years ago, Payne *et al.* (1997) reported an inspiring study on the morphokinetics of fertilization, but their preliminary inferences were limited to only 38 normally fertilized oocytes.

For such reasons, we undertook a systematic analysis of the morphokinetics of fertilization, including initially 28 parameters, many of which were considered relevant and illustrated in the present manuscript.

Fertilization cone

In a very small proportion of fertilized oocytes, we detected the FC, observed as a transient protrusion of the oocyte cortex, appearing shortly after PBII emission and disappearing after a couple of hours. This phenomenon was described repeatedly in the mouse (Davies and Gardner, 2002) and sporadically in the human (Mio, 2013). With reference to mammalian fertilization, 'fertilization cone' is probably a misnomer. In fact, this term has been used for decades to describe a rather different phenomenon occurring in echinoderm fertilization (Tilney and Jaffe, 1980). In such species, the oocyte responds with a large conical projection of the cortex to contact with the sperm acrosomal process (a thin actin-rich extension of the sperm head). This occurs while the sperm is still entirely external to the ooplasm. On the

Table V Time intervals (±SD) between discrete fertilization events assessed in relation to the number of cells and degree of fragmentation of resulting embryos on Day 3 of development (68 h post-insemination).

Interval	Embryo cell no. and percent of fragmentation on Day 3				*P*
	<8 cell >20% frag (*n* = 73)	<8 cell <20% frag (*n*° = 84)	≥8 cell >20% frag (*n*° = 13)	≥8 cell <20% frag (*n*° = 281)	
PBII emission—Cytoplasmic wave	2.3 ± 0.8	2.2 ± 0.8	2.0 ± 0.5	2.2 ± 0.8	>0.05
Cytoplasmic wave—Female PN appearance	0.8 ± 0.4	0.8 ± 0.5	0.8 ± 0.3	0.7 ± 0.4	>0.05
Cytoplasmic wave—Male PN appearance	0.9 ± 0.5	0.9 ± 0.6	0.8 ± 0.4	0.8 ± 0.5	>0.05
Male PN appearance—Juxtaposition	2.3 ± 1.9	2.2 ± 1.6	1.7 ± 1.0	2.2 ± 2.1	>0.05
Female NPB polarization—Juxtaposition	0.3 ± 2.3	0.4 ± 2.1	0.5 ± 2.0	0.4 ± 2.8	>0.05
Male NPB polarization—Juxtaposition	2.9 ± 4.2	2.7 ± 4.5	2.9 ± 2.8	2.6 ± 4.1	>0.05
Halo appearance—Halo disappearance	12.8 ± 4.8	12.7 ± 4.1	14.0 ± 3.3	10.7 ± 3.1	0.0001
Halo disappearance—PN breakdown	1.9 ± 3.4	1.1 ± 1.4	1.0 ± 0.5	0.9 ± 0.6	0.0001
PN breakdown—Cleavage	4.7 ± 4.6	3.3 ± 1.4	3.4 ± 1.1	3.1 ± 1.0	0.0001
Male PN appearance—Male PN breakdown	20.5 ± 6.2	20.5 ± 6.3	18.9 ± 3.1	17.1 ± 2.7	0.0001

contrary, we observed the FC in oocytes fertilized by ICSI and after PBII emission, therefore in a situation in which the sperm was definitely inside the oocyte. We would tend to interpret the FC as a reaction of the oocyte cytoskeleton to the casual capacity of the male chromatin to organize, once released from the sperm nuclear vestments, a cortical acto-myosin cap (Van Blerkom *et al.*, 2004; Deng *et al.*, 2007; Coticchio, 2013). In addition, we think, due to the fact that the FC was observed in less than 4% of fertilized oocytes, it depended on the position of sperm deposition during microinjection. In fact, in the mouse model the above described capacity of the chromatin, indeed DNA of any source, to elicit a response of the oocyte cortex is known to occur within a range of 20 µm (Deng *et al.*, 2007). Therefore, only sperm deposited by chance very near to the oocyte cortex may be able to induce formation of the FC.

Cytoplasmic wave

A striking phenomenon systematically observed during fertilization was a movement of cytoplasmic material having the form of a radial wave and originating from the position of initial appearance of the male PN. In all oocytes, appearance of the CW followed a precise sequence, occurring after the emission of the PBII and preceding PN appearance by less than 1 h. In addition, compared to the time of PBII extrusion, its timing had a much higher ability to predict appearance of the PN. The shape, positional relationship with the male PN and timing are consistent with the hypothesis that the CW is the morphokinetic manifestation of the formation of the microtubule sperm aster. At fertilization, a new centrosome is formed from the assembly of the sperm proximal centriole with pericentriolar material of maternal origin. At this stage, the newly formed centrosome is crucial for the nucleation of microtubules that, extending radially throughout the cytoplasm, form the cytoskeletal infrastructure through which PN are repositioned becoming juxtaposed. Interestingly, we observed the CW in 3PN, but not failed fertilized oocytes (data not shown), consistent with the supposition that this phenomenon is specific to and invariably associated with fertilization. This suggestion offers scope for future observations aimed at, for example, assessing the possibility of using the CW as a biomarker of sperm function. The CW is also amenable to particle image velocimetry analysis as suggested by studies in progress in our laboratory (data not shown).

PN formation and movement

Previous studies, carried out with the same TLM apparatus used to produce the present data, reported times of PN appearance and breakdown (Aguilar *et al.*, 2014; Chamayou *et al.*, 2015). However, our analysis offers a much wider information on PN dynamics. (i) On average, PN appearance was observed at least 1 h earlier than previously reported, probably also because of a more careful selection of oocytes amenable to analysis (see Materials and Methods). (ii) Unlike the study of Aguilar *et al.* (2014), our data suggest a virtually simultaneous PN appearance, discriminating also between male and female PN. (iii) Initial female PN formation was always observed in close vicinity with the site of PBII emission, suggesting that naked female chromatin is not displaced in the cytoplasm before organization of the pronuclear envelope. (iv) Male PN formation was observed centrally, cortically or in an intermediate position, predictably as an uncontrolled effect of sperm deposition at ICSI. Different localization of the appearance of the male PN might have a role in the position of the cytoplasmic halo (symmetric or asymmetric), although the present data are not sufficient to confirm this hypothesis. (v) In almost all cases, the female PN approached the male PN rectilinearly, confirming that this step is highly regulated and suggesting for the first time that the position in which the male PN appears initially influences the position of PN juxtaposition and, in the final analysis, the kinetics of fertilization (see below).

NPB dynamics

Pronuclear DNA organizes in NPB, small chromatin aggregates of a few µm in diameter that ultimately tend to polarize along the region of contact between PN. Although described in several previous studies in the era preceding the introduction of TLM in routine IVF, such suborganellar structures have never been studied in a dynamic fashion. By reporting for the first time that female NPB polarization occurs earlier, our study indicates that an asymmetry in male and female chromatin distribution may be a physiological manifestation, at least during a certain interval of fertilization, suggesting also an intrinsic difference in the constitution of maternal and paternal chromatin. Alternatively, asynchrony in NPB polarization could be a consequence of ICSI. However, this latter hypothesis is destined to remain unverified as long as morphokinetic data on standard fertilization will not be generated. For such reasons, our data suggests caution in the interpretation of previous studies that attempted to attribute a prognostic value to NPB configuration (Tesarik and Greco, 1999).

Space–time of pronuclear development and first cleavage

Perhaps one of the most novel and significant observations of the present study is the association that was observed between position of PN juxtaposition and successive fertilization events. Essentially, we ascertained that the more peripherally PN juxtaposition occurred, the later were the times of PN breakdown and first cleavage. This relationship can be explained by the fact that almost invariably is the female PN to approach the male PN. Therefore, considering that PN repositioning in the cell centre occurs only after juxtaposition, it is plausible to imagine that if the position of male PN formation is peripheral, once juxtaposed the two PN require an extra time to achieve central repositioning. On the contrary, if male PN formation occurs centrally, central repositioning of PN after juxtaposition is not required. This is not without implications, in consideration that the time of first cleavage is a recognized marker of embryo quality (Lundin *et al.*, 2001). If correct, this hypothesis would introduce the concept that positional cues generated at initial phases of fertilization influence the timing of later events, i.e. that to some extent space and time are integrated in a same mechanism.

PN displacement and breakdown

We observed that perturbations in the timing (asynchrony between the two PN) or localization (displacement to the cortex from a central position) of PN breakdown were highly disruptive of the developmental process, being strongly associated with direct division of the fertilized oocyte into three or more blastomeres or to highly delayed cleavage. These findings identify PN breakdown as an extremely delicate and important step of fertilization that remained totally unexplored until the introduction of TLM. They also highlight the importance of the acquisition or maintenance

of a specific cell design (symmetric vs. asymmetric, or vice versa) at definite fertilization stages.

Time intervals and embryo quality

The importance of PN breakdown is demonstrated also by a further analysis in which we tested the hypothesis that at least part of the observed morphokinetic parameters, elaborated as time intervals, could be predictive of later stages of development. Indeed, we observed that several time intervals were indicative of embryo quality on Day 3 of development. In particular, we found that longer intervals between disappearance of the cytoplasmic halo and PN breakdown were strongly associated with reduced number of blastomeres and high fragmentation. Disappearance of the cytoplasmic halo is probably the morphological manifestation of redistribution of organelles, previously clustered around the PN, in preparation for their equal segregation in the two daughter blastomeres after the first cleavage. A long interval between halo disappearance and PN breakdown might reflect a defect in this mechanisms of redistribution and segregation, with predictable implications for embryo development. The relationship between the observed time intervals and embryo morphology prompts interesting goals for translational research. In particular, identification within the time window of fertilization of reliable morphokinetic markers of embryo developmental competence would offer an alternative to embryo selection achieved by extended culture. In fact, while the advantages or embryo self-selection during development to the blastocyst stage are indisputable, on the other hand embryo selection at much earlier stages would solve several problems and concerns associated with extended culture, such as higher costs, longer working times, and possible (although so far undemonstrated) effects on the health of the foetus and the newborn.

Conclusions

We trust this manuscript offers the most detailed and comprehensive description of human fertilization. This was achieved using TLM equipment nowadays normally present in IVF laboratories, suggesting that this technology has not been fully harnessed to the best of its capacity. We redefined with higher precision the timing of previously described parameters (e.g. PN appearance and juxtaposition). More importantly, we illustrated previously unrecognized or neglected aspects of fertilization, such as the spatial dynamics of PN interaction and the occurrence of the FC and the CW. Also, our data suggests novel concepts, such as the relationship between positional cues and timing of fertilization. This evidence sets the stage for future research scenarios. For example, it will be important to establish if and to what extent ICSI fertilization differs from standard fertilization. Finally, and importantly, while having significant relevance to understand the biology of early human development, potentially our data have practical implications, as suggested by the finding that combined elaboration of several of the observed parameters can predict embryo quality at later stages of development. Studies, for example particle image velocimetry analysis of the CW, are currently ongoing in our laboratory to explore this hypothesis.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

G.C.: study design, data entry and collection, data analysis and interpretation, manuscript writing; M.M.R.: clinical intervention, critical reading of manuscript; P.V.N.: embryology, data collection, critical reading of manuscript; M.L.: embryology, critical reading of manuscript; E.D.P.: study design, analysis and interpretation, critical reading of manuscript; D.T.: embryology, critical reading of manuscript; R.F.: clinical intervention, critical reading of manuscript; M.D.C.: embryology, data collection, data analysis and interpretation, critical reading of manuscript.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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