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Short Communication

Time-lapse videography of human embryos: Using pronuclear fading rather than insemination in IVF and ICSI cycles removes inconsistencies in time to reach early cleavage milestones

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ABSTRACT

Time-lapse videography showed that human early cleavage embryos were quicker following intracytoplasmic sperm injection to reach developmental milestones compared to in vitro fertilization when using insemination as the timing start point (t0), due to differences in the time taken for embryos to reach pronuclear fading (PNF). These differences disappeared when PNF was used as t0. Using a biological rather than procedural t0 will allow a unified assessment strategy to be applied to all cycles irrespective of the insemination method.

1. Introduction

Time-lapse videography combined with uninterrupted incubation of human embryos has shown considerable promise in improving embryo assessment and clinical outcomes [1–3] although its efficacy has been questioned more recently [4,5]. In addition to using timing parameters to aid embryo selection prior to transfer [1–3,6], abnormal cleavage patterns of embryos identified via time-lapse imaging have been linked with compromised subsequent development [3,7] and reduced implantation potential [7,8] providing a basis for embryo de-selection. Notably, the vast majority of published studies are based on intracytoplasmic sperm injection (ICSI) treatment cycles [1] as ICSI oocytes have a more easily identified starting time point (t0) – sperm injection – allowing further developmental milestones to be timed. In practical terms though, the exact t0 for each individual oocyte from a cohort is sometimes

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difficult to define with certain existing time-lapse equipment [9], e.g. Embryoscope™ (Unisense Fertilitech, Aarhus, Denmark), where only one time point is possible to be defined as t0 for a cohort of injected oocytes contained on the same slide. So a recently published set of guidelines suggested that in such cases the mid-time point of the ICSI procedure of the patient is used as t0 for the entire cohort of injected oocytes [9]. This however is not ideal as the actual t0 for each oocyte varies dependent upon the time taken to identify, select and inject the sperm. Therefore, an alternative t0, with improved precision, may well lead to improved reliability of morphokinetic grading models for embryo selection.

Whilst current reported morphokinetic algorithms have been constructed using ICSI data [2], an unified algorithm for both ICSI and conventional *in vitro* fertilization (IVF) embryos would be useful. The chief obstacle when timing oocytes fertilized *via* conventional IVF insemination is the difficulty in defining the exact time of sperm entry. Indeed, the time of mixing the sperm and oocytes together in IVF cases has been suggested as a surrogate marker for t0 [9]. Earlier time-lapse studies showed significant differences in morphokinetic parameters according to the method of insemination because delayed pronuclear fading (PNF) was seen in IVF embryos compared to ICSI [10], resulting in significantly slower cleavage rates observed in IVF embryos compared to ICSI [11]. The timing differences however were only evident when relative to the insemination time, but not when relative to PNF [11]. The aim of the present study is to investigate the value of PNF as a biological t0 to time subsequent early developmental milestones of human embryos fertilized *via* either IVF or ICSI, with the purpose of enabling unified assessment of embryos originating from different insemination methods.

2. Materials and methods

A total of 193 women attending Fertility North and undertaking 223 consecutive IVF/ICSI treatment cycles between February 2013 and September 2014 were included for analysis, with 1400 (676 IVF and 724 ICSI) embryos cultured in the Embryoscope™ time-lapse incubator. Informed consent was obtained from both partners of participating couples and retrospective data analysis was approved by the Research and Ethics committees of both Joondalup Health Campus and Edith Cowan University.

Patient management and IVF/ICSI procedures were performed as previously described [7]. After fertilization was confirmed post IVF or ICSI, embryos were placed in the Embryoscope™ for 3 days of culture and monitoring, with images taken every 10 min per embryo at 7 planes of focus, followed by either replacement or storage.

Developmental events were manually annotated up to the 5-cell stage for each embryo by the same embryologist (YL) with more than 10 years of experience in clinical embryology, and recorded into the Embryoviewer® (Unisense Fertilitech, Aarhus, Denmark) software. Cleavage milestones of embryos were timed relative to either insemination (time of mixing sperm and oocytes for IVF, and mid-time point of the ICSI procedure for a cohort of oocytes that were cultured on the same slide) [9], or pronuclear fading when both nuclear envelopes had completely disappeared (see example photograph in Fig. 1). Timing parameters analyzed included time to reach 2- (t2), 3- (t3), 4- (t4) and 5-cell (t5) stages relative to either insemination (t0 = insemination) or PNF (t0 = PNF); and the duration of 2- (cc2) and 3-cell (s2) stages. Abnormal cleavage events, such as direct cleavage 2–3 cells (DC 2–3) [3,8] where the duration of 2-cell stage was less than 5 h (cc2 $<$ 5 h) and reverse cleavage (RC) as described previously [7], were also noted for embryos that had been affected. Annotation details and comparisons between two timing systems are shown in Fig. 1.

Conventional grading of embryos was performed by the same embryologist (YL) on day 3 based on the images captured by the Embryoscope™ at 68 h post insemination, using criteria described previously [7,12]. One or two embryos with the best grade were selected for transfer. All cycles with positive serum β-hCG test were followed until the confirmation of a fetal heart beat at 7 weeks of pregnancy.

A total of 196 treatment cycles resulted in a transfer; and in the remaining 27 cycles, all good quality embryos were frozen due to risk of ovarian hyperstimulation syndrome or elevated progesterone level on the ovulatory trigger day. Of the 196 transferred cycles, a total of 50 embryos (30 from IVF and 20 from ICSI) were identified with known implantation (KID+ve), where the number of fetal hearts matched the number of embryos transferred.

All timing parameters were compared using the Student *t*-test. Statistical analysis was performed using the Statistical Package for the Social Sciences 20.0 (SPSS) where $p < 0.05$ was considered statistically significant.

3. Results and discussion

In the 95 IVF and 128 ICSI cycles (female age, 34.96 ± 3.94 vs 35.17 ± 5.05 years, $p > 0.05$), IVF embryos ($n = 462$) had significantly delayed cleavage divisions up to the 5-cell stage compared to ICSI ($n = 509$) when using insemination as a reference starting time point (t0 = insemination), but the differences disappeared when PNF was used as the reference starting time point (t0 = PNF) (Table 1). These results are in line with previous publications [10,11].

Furthermore, in the present study, similar pattern was also observed in a subset of embryos with known implantation following uterine transfer (Table 1), thus indicating differences in cleavage division timings from the 2- to 5-cell stages were predominately related to variations in the time taken to reach PNF post insemination. Supporting this were the reduced standard deviations in the timings of early cleavage divisions relative to PNF rather than insemination (Table 1), possibly due to the increased uncertainty (IVF) or imprecision (ICSI) of actual sperm entry time point for each individual oocyte as illustrated in Fig. 1. These results are consistent with our previous findings [13] where a large variation in the timing of second polar body extrusion was evident in the metaphase II oocytes injected at ICSI, possibly due to varying degrees of cytoplasmic maturity. Second polar body extrusion is extremely difficult to see by time-lapse in oocytes inseminated via IVF due to the presence of cumulus and coronal cells. Thus, the results of the present study using PNF as t0 would provide

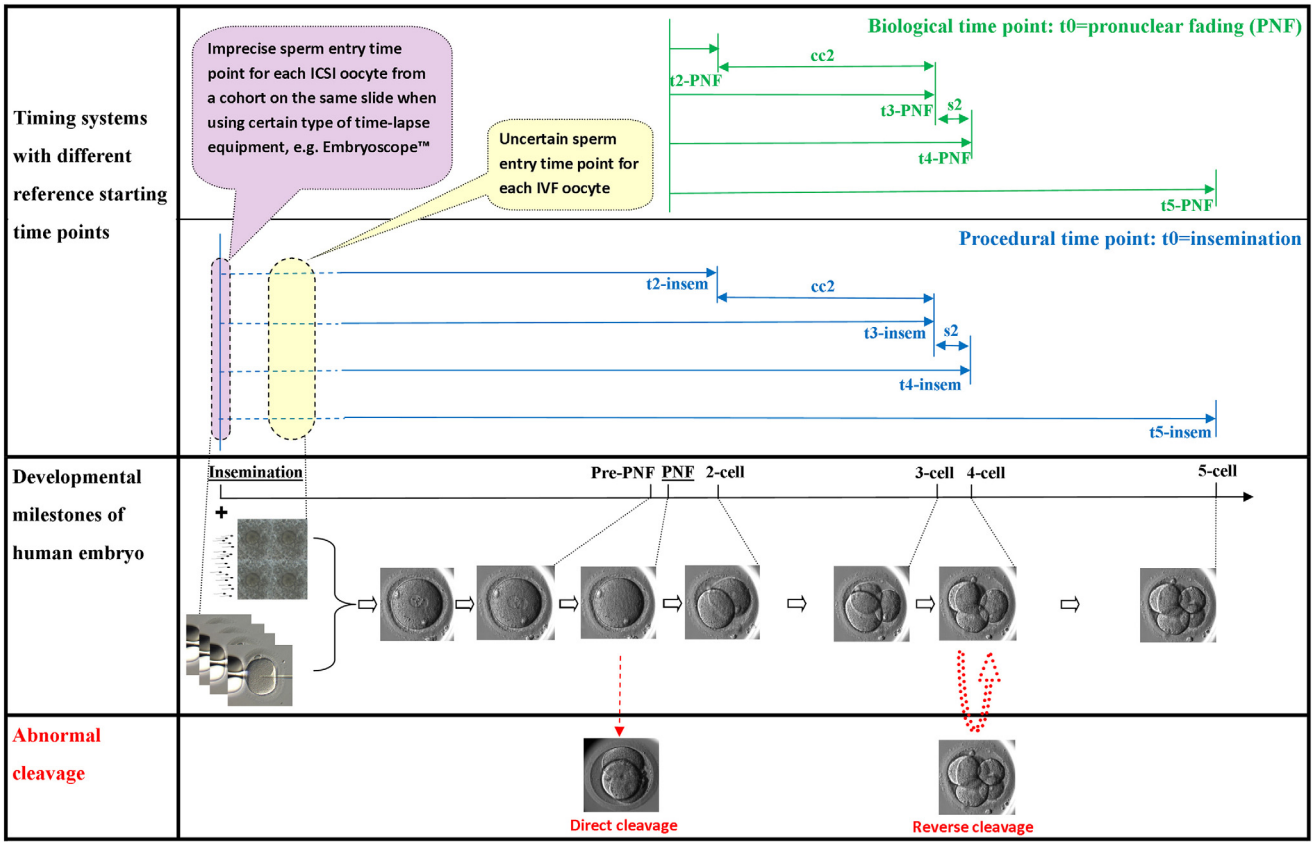

Fig. 1 – Annotations of early developmental milestones of human embryos observed via time-lapse incubation and a comparison of two morphokinetic timing systems with different reference starting time points.

a practical advantage when used as a reference starting time point to compare the morphokinetics between IVF and ICSI embryos.

In addition to the 2- to 5-cell stage timings from insemination, two relative timing parameters (cc2 and s2) have been shown to be predictive of implantation following transfer [2]. Furthermore the benefit of using relative rather than absolute timing expressions to predict blastulation in time-lapse monitored embryos was recently demonstrated [14]. In the present study, cc2 and s2 did not differ between IVF

Table 1 – Time (mean ± SD) to reach 2-, 3-, 4- and 5-cell stages of human embryos relative to either insemination (t0 = insemination) or pronuclear fading (t0 = PNF) and the duration (mean ± SD) of 2-cell (cc2) and 3-cell (s2) stages comparing IVF and ICSI cycles.

Time interval (h)	All embryos[a]		Implanting embryos[b]	
	IVF (n = 462)	ICSI (n = 509)	IVF (n = 30)	ICSI (n = 20)
t2-insem	27.26 ± 2.90	25.40 ± 2.76*	26.70 ± 2.07	24.54 ± 2.14*
t3-insem	38.20 ± 3.59	36.49 ± 3.43*	37.48 ± 2.43	35.27 ± 2.61*
t4-insem	39.52 ± 4.66	37.98 ± 4.90*	37.97 ± 2.50	35.58 ± 2.61*
t5-insem	50.81 ± 5.44	49.11 ± 5.56*	49.61 ± 3.40	47.61 ± 3.12*
t2-PNF	2.87 ± 0.69	2.78 ± 0.66	2.60 ± 0.52	2.42 ± 0.46
t3-PNF	13.84 ± 1.72	13.91 ± 1.56	13.40 ± 0.97	13.25 ± 0.91
t4-PNF	15.13 ± 3.37	15.38 ± 3.52	13.94 ± 1.03	13.56 ± 0.83
t5-PNF	26.42 ± 3.99	26.50 ± 4.39	25.53 ± 2.13	25.58 ± 1.46
cc2 (t3 – t2)	10.95 ± 1.46	11.11 ± 1.30	10.78 ± 0.72	10.78 ± 0.94
s2 (t4 – t3)	1.26 ± 2.85	1.46 ± 3.06	0.46 ± 0.68	0.31 ± 0.36

* $p < 0.05$ compared to corresponding IVF group.
[a] Only fully annotated embryos without direct cleavage 2–3 cells were included for comparisons.
[b] All implanting embryos were fully annotated and did not display direct cleavage 2–3 cells.
t2-insem, t3-insem, t4-insem and t5-insem are the times from insemination to 2-cell, 3-cell, 4-cell and 5-cell respectively.
t2-PNF, t3-PNF, t4-PNF and t5-PNF are the times from pronuclear fading to 2-cell, 3-cell, 4-cell and 5-cell respectively.

and ICSI in either the entire embryo cohorts or the subset of known implanted embryos (Table 1). These results further indicate that differences in timings are due to variations in the initial stages of fertilization rather than differences in cell cycle times, and confirm the practical value of using PNF as a reference starting point of subsequent cleavage divisions irrespective of the insemination method.

Increased frequency of observations via time-lapse incubation of human embryos enables embryologists to identify abnormal cleavage patterns leading to their reduced viability over a longer period [3,7,8]. In the present study, none of the 50 implanting embryos in the subset (Table 1) displayed DC 2–3 or RC. Embryos with DC 2–3 could result from a tripolar mitosis of the zygotes (cc2 = 0 h) [3] or an early division (cc2 < 5 h) in one blastomere of the 2-cell stage embryos [8], and embryos with either pattern were considered as confounding variables for their irregularity of cleavage timings and therefore excluded from comparisons in Table 1. Indeed, there were a few occasions where zygotes directly cleaved to 4 cells [15], but these were treated as "DC 2–3" in the present study since "cc2 = 0 h" applied in such embryos. Interestingly, in very few occasions, zygotes underwent immediate cell–cell fusion (resulting in 2 blastomeres) following tripolar cleavage as described in previous publications [3,15], resulting in a manual record of both DC 2–3 and RC.

In conclusion, preliminary results in the present study indicate that PNF could be used as an alternative reference starting time point for timing subsequent embryonic development. This may potentially: (a) result in improved accuracy of dynamic developmental timings of early cleavage embryos and (b) enable the development of a standardized universal algorithm for assessing both IVF and ICSI embryos based on morphokinetics. Further large scale studies exploring detailed equations in such algorithms are warranted.

Conflict of interest

None declared.

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