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Assessment of human embryos by time-lapse videography: A comparison of quantitative and qualitative measures between two independent laboratories

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ABSTRACT

A total of 488 Day 3 human embryos with known implantation data from two independent *in vitro* fertilization laboratories were included for analysis, with 270 from Fertility North (FN) and 218 from Canberra Fertility Centre (CFC). Implanting embryos grew at different rates between FN and CFC as indicated in hours of the time intervals between pronuclear fading and the 4- (13.9 ± 1.1 *vs.* 14.9 ± 1.8), 5- (25.7 ± 1.9 *vs.* 28.4 ± 3.7) and 8-cell stages (29.0 ± 3.2 *vs.* 32.2 ± 4.6), as well as the durations of 2- (10.8 ± 0.8 *vs.* 11.6 ± 1.1), 3- (0.4 ± 0.5 *vs.* 0.9 ± 1.2), and 4-cell stages (11.8 ± 1.4 *vs.* 13.6 ± 2.9), all $p < 0.05$. The application of a previously published time-lapse algorithm on ICSI embryos from the two participating laboratories failed to reproduce a predictive pattern of implantation outcomes (FN: AUC = 0.565, $p = 0.250$; CFC: AUC = 0.614, $p = 0.224$). However, for the qualitative measures including poor conventional morphology, direct cleavage, reverse cleavage and <6 intercellular contact points at the end of the 4-cell stage, there were similar proportions of embryos showing at least one of these biological events in either implanting (3.1% *vs.* 3.3%, $p > 0.05$) or non-implanting embryos (30.4% *vs.* 38.3%, $p > 0.05$) between FN and CFC. Furthermore, implanting embryos favored lower proportions of the above biological events compared to the non-implanting ones in both laboratories (both $p < 0.01$). To conclude, human embryo morphokinetics may vary between laboratories, therefore time-lapse algorithms emphasizing quantitative timing parameters may have reduced inter-laboratory transferability; qualitative measures are independent of cell division timings, with potentially improved inter-laboratory reproducibility.

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1. Introduction

Time-lapse videography permits continuous monitoring of human embryo growth over the entire culture period to assess both quantitative morphokinetic measures [1–7] and the occurrence of qualitative indicators of atypical cleavage [7–11]. The use of enclosed incubators equipped with built-in video cameras does not appear to be detrimental to embryo quality [12], possibly improving culture conditions [13], and may be advantageous in improving clinical pregnancy rates [4,14] although further high quality evidence is required before routine application [15,16]. Selection of the best embryo(s) for transfer using time-lapse videography requires a set of defined limits which embryos can be assessed against, and published algorithms that exist use predominantly morphokinetic parameters [6,17,18]. Unfortunately, such algorithms may lose discriminatory power for embryo selection when transferred for use in other laboratories [19–21]. This has led to the exploration of algorithms based upon other markers, such as qualitative measures of cleavage [8].

The reason behind the limited transferability of algorithms is unclear. However, if one applies the same criteria to the parameters measured by time-lapse videography as those applied to any other diagnostic test, then each parameter measured should have minimal technical and biological variability [22]. Previously, an assessment of technical precision in time-lapse observations found whilst measurements were more objective than conventional assessments, some parameters measured were more variable than others [23]. It must also be noted that the growth of embryos is not constant, and may be influenced by external factors. Such confounding factors have been associated with the patient (*e.g.*, the stimulation regimen used for ovarian stimulation [24,25], gonadotropin dose and hormonal levels [24,26], smoking [27], the presence of hyperandrogenic polycystic ovarian syndrome in the female [28], and sperm DNA fragmentation in the male [29]) and also the culture system (*e.g.*, oxygen concentration in the incubator [30] and the use of different culture media formulations [31]).

The successful application of a time-lapse algorithm to assess embryo implantation potential in different laboratories requires that the embryos must behave in a similar manner between laboratories despite differences in laboratory culture conditions and patient profiles. The aim of the present study was therefore to describe the culture system and cycle data for two independent laboratories, and then compare the time-lapse videography findings for (a) the quantitative morphokinetic parameters of embryos with known implantation data (KID), (b) the suitability of a published algorithm to assess the implantation potential of embryos transferred, and (c) the prevalence of qualitative biological events in embryos categorized according to their KID status.

2. Materials and methods

2.1. Laboratories

The two Australian laboratories (Fertility North and Canberra Fertility Centre) were both accredited by the National Association of Testing Authorities for their biochemistry and andrology services, and the Reproductive Technology Accreditation Committee for their *in vitro* fertilization (IVF) treatment services. In addition, both laboratories participate in the embryo grading and embryo time-lapse modules of the External Quality Assurance Schemes for Reproductive Medicine (Northlands, Western Australia, Australia).

Table 1 – Comparisons of cycle characteristics between Fertility North and Canberra Fertility Centre.

	Fertility North	Canberra Fertility Centre
No. cycles included	212	160
Age (y, mean ± SD)	34.5 ± 4.6	36.3 ± 5.0*
No. IVF:ICSI cycles	84:128	89:71*
No. Agonist:antagonist cycles	89:123	61:99
Peak E_2 (pmol/L, mean ± SD)	6612.2 ± 3405.1	7766.4 ± 3918.8*
Days of FSH (mean ± SD)	10.3 ± 1.9	11.5 ± 2.0*
No. oocytes collected (mean ± SD)	9.5 ± 4.6	8.2 ± 4.1*
No. oocytes fertilized (mean ± SD)	6.6 ± 3.4	5.7 ± 2.8*
No. embryos transferred (mean ± SD)	1.3 ± 0.5	1.5 ± 0.5*

E_2 – estradiol; FSH – follicle-stimulating hormone.
* $p < 0.05$ when compared with Fertility North.

2.2. Patient recruitment and management

The study included 212 IVF or intracytoplasmic sperm injection (ICSI) treatment cycles (aged at 34.5 ± 4.6 years) at Fertility North between February 2013 and December 2014, and 160 IVF/ICSI treatment cycles (aged at 36.3 ± 5.0 years, $p < 0.05$) at Canberra Fertility Centre between January and December 2014. All cycles included KID results for transferred embryos as previously defined [8], with KID+ referring to known implanting embryos whilst KID− as known non-implanting embryos. Within these treatment cycles, a total of 488 fully annotated embryos had reached at least the 5-cell stage by 68 h post-insemination; 270 from Fertility North and 218 from Canberra Fertility Centre were included for analysis after culture in the Embryoscope™ (Vitrolife, Göteborg, Sweden) time-lapse system. Patients were managed according to the clinic's own standard operating protocols by the two respective and separate teams of clinicians. Comparisons of cycle characteristics between the two clinics are shown in Table 1.

2.3. Embryo culture

A summary of the main laboratory conditions for the two laboratories for oocyte fertilization and embryo culture is shown in Table 2. After insemination *via* either conventional IVF or ICSI, fertilized oocytes were placed in the respective Embryoscope™ incubators and cultured until at least Day 3.

2.4. Embryo assessment

Images on the Embryoscope™ were taken every 10 min for each embryo over seven focal planes. All embryos included in

Table 2 – A summary of the main laboratory features in operation at Fertility North and Canberra Fertility Centre.

Procedure	Fertility North	Canberra Fertility Centre
Sperm density gradient	95:50% PureSperm (Nidacon)	80:40% PureCeption™ (Origio)
Suite of media/oil	Medicult (Origio)	Sage® (Origio)
IVF sperm insemination density	50,000/oocyte	20,000/oocyte
ICSI needles	The Pipette Company	The Pipette Company
ICSI oocyte denuding	Synvitro Hyadase (Origio)	Hyalase® (Sanofi-Aventis)
Culture incubator	Embryoscope™ (Vitrolife)	Embryoscope™ (Vitrolife)
Incubator temperature	37.0 °C	37.0 °C
Gas phase	6% CO_2, 5% O_2, 89% N_2	5.5% CO_2, 5% O_2, 89.5% N_2

Nidacon, Mölndal, Sweden.
Origio, Måløv, Denmark.
The Pipette Company (Thebarton, SA, Australia).
Vitrolife, Göteborg, Sweden.
Sanofi-Aventis, Macquarie Park, NSW, Australia.

the study from both clinics were retrospectively annotated by one scientist (YL) to minimize potential inter-operator variability [23]. Conventional embryo morphology scoring was performed as previously described [32], using images captured by the EmbryoScope™ at 68 h post-insemination (independent of the time-lapse information). Embryos were scored between 1 and 4, based on the cell count, symmetry and degree of fragmentation; 1 was the highest score and 4 the lowest.

Morphokinetic analysis of embryos cultured in the Embryoscope™ imaging system was performed using the EmbryoViewer® (Vitrolife) software. Each cleavage event was documented as the time interval between insemination and the first observed image of completion of the event, *e.g.*, T2 is the time taken from insemination until the complete separation of the two daughter blastomeres [33]. Cleavage timings were also analyzed relative to pronuclear fading (PNF) rather than using insemination as a reference starting point, this enabled both IVF and ICSI embryos to be included in the analysis [34,35]. Morphokinetic parameters included T4_PNF (time from PNF to the 4-cell stage), T5/T5_PNF (time from insemination/PNF to the 5-cell stage), CC2 (time from 2- to 3-cell), S2 (time from 3- to 4-cell), T5_T4 (time from 4- to 5-cell), T8_PNF (time from PNF to the 8-cell stage) and S3 (time from 5- to 8-cell). Embryos with 2- or 4-cell stages <5 h were classified as direct cleavage (DC) [8,10], and excluded from morphokinetic comparison. Reverse cleavage (RC), which was also excluded from morphokinetic comparison, was defined as either (a) two daughter blastomeres re-combining after complete separation following cleavage division, or (b) failed cytokinesis following the disappearance and reappearance of the nucleus [9]. The degree of cell contact observed between blastomeres was also assessed at the end of the 4-cell stage by recording the number of intercellular cell count points (ICCPs) [8].

In addition, all the ICSI embryos were re-assessed with annotations timed from insemination, and the embryos were graded according to the algorithm proposed by Meseguer et al. [18]. The evenness of blastomeres at the 2-cell stage and the presence of multinucleation in at least one blastomere at the 4-cell stage were included as per their protocol. Briefly, Day 3 embryos were initially assessed at 68 h post-insemination according to conventional grades [32], and the poor quality embryos were graded "F"; thereafter embryos displaying uneven blastomere at the 2-cell stage, and/or DC (CC2 < 5 h), and/or multinucleation at the 4-cell stage were graded "E"; the remaining embryos were evaluated against morphokinetic features and graded D− (T5 > 56.6 or <48.8 h, and S2 > 0.76 h, and CC2 > 11.9 h), D+ (T5 > 56.6 or <48.8 h, and S2 > 0.76 h, and CC2 ≤ 11.9 h), C− (T5 > 56.6 or <48.8 h, and S2 ≤ 0.76 h, and CC2 > 11.9 h), C+ (T5 > 56.6 or <48.8 h, and S2 ≤ 0.76 h,

Table 3 – Comparison of quantitative morphokinetic parameters (mean ± SD) of embryos with known implantation data (KID) in two independent IVF laboratories at Fertility North and Canberra Fertility Centre.

Timing parameters (T0 = PNF)	Fertility North		Canberra Fertility Centre	
	KID−	KID+	KID−	KID+
T4_PNF (*n*)	14.5 ± 1.5 (163)^{a}	13.9 ± 1.1 (63)^{b}	15.3 ± 2.4 (139)^{c}	14.9 ± 1.8 (29)^{ac}
T5_PNF (*n*)	26.8 ± 2.6 (163)^{a}	25.7 ± 1.9 (63)^{b}	29.6 ± 3.8 (139)^{c}	28.4 ± 3.7 (29)^{c}
CC2 (*n*)	11.1 ± 1.0 (163)^{ab}	10.8 ± 0.8 (63)^{ac}	12.1 ± 1.8 (139)^{d}	11.6 ± 1.1 (29)^{bd}
S2 (*n*)	0.7 ± 0.9 (163)^{ab}	0.4 ± 0.5 (63)^{a}	0.8 ± 1.0 (139)^{b}	0.9 ± 1.2 (29)^{b}
T5_T4 (*n*)	12.2 ± 1.9 (163)^{a}	11.8 ± 1.4 (63)^{a}	14.2 ± 2.7 (139)^{b}	13.6 ± 2.9 (29)^{b}
T8_PNF (*n*)	29.8 ± 3.8 (127)^{a}	29.0 ± 3.2 (58)^{a}	32.6 ± 4.4 (106)^{b}	32.2 ± 4.6 (25)^{b}
S3 (*n*)	3.6 ± 3.2 (127)^{a}	3.4 ± 3.3 (58)^{a}	4.1 ± 2.9 (106)^{a}	4.5 ± 4.1 (25)^{a}

The data excludes embryos that displayed direct cleavage and/or reverse cleavage.
PNF = pronuclear fading; CC2 = time from 2- to 3-cell; S2 = time from 3- to 4-cell; T5_T4 = time from 4- to 5-cell; S3 = time from 5- to 8-cell.
ANOVA analysis followed by LSD *post hoc* tests.
Different subscripts in the same row indicate statistical significance ($p < 0.05$).

Table 4 – Implantation rates of ICSI embryos with known implantation data (KID) categorized according to Meseguer et al's model [18] and the predictive power for implantation when applied in (a) Fertility North, (b) Canberra Fertility Centre, and (c) the original published algorithm by Meseguer et al. [18].

Grade	Fertility North		Canberra Fertility Centre		Meseguer et al. [18]*	
	No. embryos	No. KID+ embryos (%)	No. embryos	No. KID+ embryos (%)	No. embryos	No. KID+ embryos (%)
A+	26	9 (34.6)	15	3 (20.0)	29	19 (65.5)
A−	15	2 (13.3)	10	1 (10.0)	25	9 (36.0)
B+	11	0	10	1 (10.0)	24	7 (29.2)
B−	11	0	7	2 (28.6)	25	6 (24.0)
C+	58	19 (32.8)	13	2 (15.4)	32	8 (25.0)
C−	3	0	9	1 (11.1)	21	2 (9.5)
D+	19	2 (10.5)	4	0	10	1 (10)
D−	5	0	10	1 (10.0)	33	5 (15.2)
E	11	1 (9.1)	8	0	48	4 (8.3)
Total	159	33 (20.8)	86	11 (12.8)	247	61 (24.7)
AUC (95%CI)	0.565 (0.458–0.672)		0.614 (0.452–0.776)		0.735 (0.661–0.809)	
p-Value	0.250		0.224		0.000	

Receiver operating characteristic (ROC) analysis was performed after converting embryo grades (E to A+) to numeric ranking (1–9, respectively).
AUC (95%CI) = area under the ROC curve (95% confidence interval).
* Permission granted to the Authors by Oxford University Press to use data provided in this table.

and CC2 ≤ 11.9 h), B− (48.8 ≤ T5 ≤ 56.6 h, and S2 > 0.76 h, and CC2 > 11.9 h), B+ (48.8 ≤ T5 ≤ 56.6 h, and S2 > 0.76 h, and CC2 ≤ 11.9 h), A− (48.8 ≤ T5 ≤ 56.6 h, and S2 ≤ 0.76 h, and CC2 > 11.9 h), A+ (48.8 ≤ T5 ≤ 56.6 h, and S2 ≤ 0.76 h, and CC2 ≤ 11.9 h), respectively.

2.5. *Statistical analysis*

Proportions were compared using the chi-squared test, and parametric data analyzed using Student *t*-test or analysis of variance (ANOVA) with *post hoc* analysis using Fisher's least significant difference (LSD) test. Predictive power of embryo classification in different laboratories was tested using receiver operating characteristic (ROC) with area under the ROC curve (AUC) analysis. Differences were considered significant if $p < 0.05$.

3. Results

3.1. *Patient and laboratory differences*

Significant differences in the two patient populations can be seen in Table 1, with the patients at Fertility North being younger, more likely to have ICSI than IVF, lower peak serum estradiol concentrations, shorter stimulation times, more oocytes collected and fewer embryos transferred. Table 2 shows that the two laboratories also varied considerably in the details of their culture systems, with differences seen in the materials used in sperm density gradients, culture media, IVF sperm insemination density, enzyme preparation for denuding ICSI oocytes and the gas phase used for embryo culture.

3.2. *Quantitative parameters*

A summary of the quantitative morphokinetic parameters of both KID+ and KID− embryos from both laboratories is shown in Table 3. At Fertility North, KID+ embryos were significantly faster than KID− embryos for T4_PNF (13.9 ± 1.1 *vs.* 14.5 ± 1.5 h, $p < 0.05$) and T5_PNF (25.7 ± 1.9 *vs.* 26.8 ± 2.6 h, $p < 0.05$). However, no differences were seen between the KID+ and KID− embryos at Canberra Fertility Centre. Considering only KID+ embryos, significant differences were seen between the two laboratories for all parameters except S3, with the Fertility North KID+ embryos progressing faster. When the ICSI embryos for both laboratories were categorized as per a published algorithm [18], the implantation rates in groups A+ to E showed no trend (see Table 4). Furthermore, ROC analysis of the predictive power of the algorithm showed no statistical significance when applied to either participating laboratory (Fertility North: AUC = 0.565, 95%CI 0.458–0.672, $p = 0.250$; Canberra Fertility Centre: AUC = 0.614, 95%CI 0.452–0.776, $p = 0.224$), despite the significant prediction (AUC = 0.735, 95%CI 0.661–0.809, $p = 0.000$) calculated *via* the same method using the original published dataset [18].

3.3. *Qualitative parameters*

At Fertility North, the implantation rate for embryos with ≥1 atypical biological events (2/72, 2.8%) was significantly lower than those embryos with typical patterns of cleavage (62/198, 31.3%; $p < 0.0001$). Embryos with ≥1 atypical biological events at Canberra Fertility Centre were also seen to implant less frequently (1/73, 1.4%) than embryos with typical patterns of cleavage (29/145, 20.0%; $p < 0.0005$). Viewing it from a different perspective, the incidence of atypical biological events in KID embryos for both laboratories is shown in Table 5. The proportion of embryos with at least one atypical biological event was significantly higher in KID− embryos than KID+ embryos for both Fertility North (30.4% *vs.* 3.1%, $p < 0.05$) and Canberra Fertility Centre (38.3% *vs.* 3.3%, $p < 0.05$). However, both laboratories had similar proportions showing at least one atypical biological event in either KID+ ($p > 0.05$) or KID− embryos ($p > 0.05$).

Table 5 – Incidence of qualitative atypical biological events in embryos with known implantation data (KID) which were grown and transferred in two different laboratories at Fertility North and Canberra Fertility Centre.

Qualitative atypical biological events	Fertility North		Canberra Fertility Centre	
	KID− embryos (n = 206)	KID+ embryos (n = 64)	KID− embryos (n = 188)	KID+ embryos (n = 30)
Poor conventional morphology	7 (3.4%)	0	11 (5.9%)	0
Direct cleavage	16 (7.8%)	1 (1.6%)	33 (17.6%)	1 (3.3%)*
Reverse cleavage	36 (17.5%)	0*	25 (13.3%)	0*
<6 intercellular contact points at the end of 4-cell stage	31 (15.0%)	1 (1.6%)*	21 (11.2%)	0
Embryos with ≥1 of the above	70 (30.4%)	2 (3.1%)*	72 (38.3%)	1 (3.3%)*

* Statistical significance ($p < 0.05$) between KID+ and KID− embryos within the same laboratory.

4. Discussion

Despite a number of studies showing the predictive value of morphokinetic analysis on embryo implantation outcomes in a single laboratory or multiple laboratories [2,4,5,17,18,36], there is limited evidence available that directly compares the embryo morphokinetics between independent laboratories, particularly for implanting embryos that progress to form viable fetuses. However, evidence does exist to show external factors may alter embryo morphokinetics, and such factors may originate from either the patient population [27–29], ovarian stimulation [24–26], or laboratory culture conditions [30,31]. In the present study, the two participating laboratories' datasets had significantly different cycle characteristics (Table 1) and embryo culture conditions (Table 2), which may have contributed to the significant differences in morphokinetics that were found (Table 3). It should also be noted that, however, KID+ embryos in the two laboratories had significantly different morphokinetics in addition to the KID− embryos (Table 3); thus suggesting caution is required when applying a morphokinetic algorithm to predict implantation based on KID embryos from a different laboratory. Although a previous study [25] concluded there was no difference between the embryo morphokinetic categories (A+ to E) comparing two different ovarian stimulating regimens, there were actual differences in the timings of early developmental milestones. This study failed to reproduce the pattern of the original publication [18], and similarly, other recent papers reported inconsistent implantation outcomes after applying the same algorithm [19–21]. Rather like reference ranges associated with diagnostic pathology tests, time-lapse algorithms require limits of normality against which to compare the growth of an embryo for selection purposes. However, it is clearly seen in the current study that embryos from the two different clinics do grow at different rates, probably due to differences in culture systems and patient profiles. Accordingly, the limits of normality would either not be applicable to both populations of embryos, or would need to be extended to cover the full range for the two embryo populations thereby inevitably reducing the discriminatory power of the algorithm.

In addition to establishing positive embryo morphokinetic data, embryo deselection may be another benefit of time-lapse monitoring [33] as mentioned above. Recently several atypical cleavage patterns have been identified using time-lapse observations *via* the Embryoscope™, including DC [7,8,10], RC [9], and <6 intercellular contact points [8]. A qualitative time-lapse deselection algorithm was also proposed incorporating the above parameters to potentially improve the selection of human embryos with better implantation potential [8]. The transferability of such an algorithm, though, has not yet been investigated. To begin to address this issue the present study compared the incidences of atypical biological events in embryos annotated by the same operator between the two laboratories. In contrast to the differences observed in quantitative timing parameters, the study found similar incidences of the qualitative parameters in embryos between the two laboratories (Table 5). The similarities in qualitative measures may be attributed to their very nature (measured as either a positive or negative event), and also being independent of absolute cell division timings of embryos. The qualitative parameters alone could potentially be a practical starting point for new time-lapse users who wish to maximize the benefits of time-lapse culture but are concerned about transferability of existing quantitative selection models. In addition, the Embryoscope™ and other similar instruments appear to be superior platforms for embryo monitoring within an uninterrupted culture condition, due to their increased frequencies of observations [1,4], although further prospective studies are required to validate the practical usefulness.

In the present study, all embryo annotations in the two laboratories were performed by one embryologist to minimize potential inter-observer variability. A previous study [23] showed close inter- and intra-observer agreement (intra-class correlation coefficients >0.8) could be achieved in annotations of pronuclear fading and cleavage divisions from the 2- to 8-cell stages. However, such an assessment of reproducibility on the qualitative time-lapse parameters of DC, RC, and <6 ICCP at the end of the 4-cell stage has not yet been performed; this requires further investigation. It is anticipated that training embryologists and participation in annotation comparisons may improve the consistency of time-lapse results, in line with previous studies on the conventional embryo grading system [37,38].

The present study included only KID embryos [8] to minimize the confounding factors in treatment cycles where a single fetal heart was detected following a double embryo transfer. As a result, KID+ embryos only included embryos transferred in the cycles with 100% implantation, where either a single fetal heart from a single embryo transfer or double fetal hearts from a double embryo transfer were detected. While KID− embryos were those transferred in all treatment

cycles with a negative outcome regardless of the number of embryos transferred. Therefore, KID embryo implantation rates do not reflect pregnancy rates, as negative pregnancy tests are over represented due to the exclusion of double embryo transfers that resulted in a single fetal heart. For example, Canberra Fertility Centre cannot be said to be different to Fertility North (Table 4) due to higher proportion of double embryo transfers (1.5 ± 0.5 *vs.* 1.3 ± 0.5, $p < 0.05$), and the older age of the patient population (36.3 ± 5.0 years *vs.* 34.5 ± 4.6 years, $p < 0.05$).

In conclusion, embryo morphokinetics may vary in both KID+ and KID− embryos cultured in different IVF laboratories probably due to differences in embryo culture conditions and patient profiles. The present study applied a previously published algorithm mainly based on quantitative measures which was of only limited value in assessing embryo implantation potential, confirming that one should be cautious about utilizing such an algorithm developed outside of one's own laboratory. Conversely, the occurrence of qualitative atypical biological events was similar between the two laboratories in both KID+ and KID− embryos, suggesting an increased reproducibility between laboratories of algorithms based upon qualitative measures.

Conflict of interests

All authors declare that they have no conflict of interests.

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