ORIGINAL ARTICLE: ASSISTED REPRODUCTION

An investigation into the effect of potential confounding patient and treatment parameters on human embryo morphokinetics

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Objective: To determine the effect of patient and treatment parameters on 19 embryo morphokinetic parameters using pronuclear fading as time zero.
Design: Single-site, retrospective cohort analysis.
Setting: Fertility treatment center.
Patients(s): Patients undergoing treatment between September 2014 and January 2016 (n = 639) whose embryos were cultured in the EmbryoScope for 6 days (n = 2,376).
Intervention(s): None.
Main Outcome Measure(s): Multiple regression analysis of body mass index; maternal age; infertility diagnosis; treatment type; suppression protocol on time to each cellular division (tn): t2, t3, t4, t5, t6, t7, t8, t9, time to start of compaction (tM), start of blastulation (tSB), full blastocyst (tB); and interval measurements: s2, s3, cc2, cc3, cc4, t9-tM, tM-tSB, and tSB-tB. Beta coefficients were analyzed to quantify any significant effects.
Result(s): Embryos appeared to be subtly affected by patient and treatment parameters, exhibiting complex relationships between various morphokinetic parameters and specific patient and treatment factors, rather than a systemic effect.
Conclusion(s): These findings outline the need for the consideration of confounding factors when assessing an embryo's ability to achieve implantation. Although morphokinetic parameters have been related to embryo viability, it is likely that this will vary depending on the embryo's origin. (Fertil Steril® 2020;■:■–■. ©2020 by American Society for Reproductive Medicine.)
Key Words: Embryo culture, embryo viability, reproductive technology

Time-lapse imaging is no longer a novel technique for the culturing of human embryos. Time-lapse imaging is employed by many internationally and has gained a high degree of attention based on little scientific evidence (1). In theory, time-lapse systems (TLS) offer two potential benefits: a highly controlled, undisturbed culture environment and an increased level of detail when analyzing the embryos contained within the system. However, a recent Cochrane review concluded that "there is insufficient evidence of differences in live birth, miscarriage, still birth or clinical pregnancy to choose between TLS [time lapse systems] and conventional incubation" (1). It is notoriously difficult for clinics to perform the much-needed randomized controlled trials (RCTs) for a multitude of reasons: funding availability, lack of patient interest, and difficulty in the approval process. As a result, many researchers turn to retrospective, observational investigations to determine the relevance and significance of the environment and the information that TLS can provide, the pitfalls of which have been highlighted (2).

What does remain novel about TLS is not their use per se, in the simplest form, but how the information gleaned from them is put to use. Time-lapse systems can capture images of embryos every five minutes over a period of six days, generating thousands of images per embryo. The wealth of information available to the user regarding one embryo is, undeniably, substantial, but

Received August 4, 2020; revised September 21, 2020; accepted October 7, 2020.
A.B. has nothing to disclose. G.M. has nothing to disclose. S.T. has nothing to disclose.
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Fertility and Sterility® Vol. ■, No. ■, ■ 2020 0015-0282/$36.00

https://doi.org/10.1016/j.fertnstert.2020.10.037

exactly how to use this information is an issue and the reason this feature of TLS remains novel.

The correlation of morphokinetic data (the times at which an embryo reaches a developmental milestone) provided by TLS with the embryo's ability to create a pregnancy both in humans and animals has been identified: the appearance and disappearance of pronuclei and nuclei at each cell stage (3–6), the length of time between early cytokineses (7–14), direct one to three cell divisions (15), and start times of blastulation (16), among others. With this information in tow, many researchers pursued the development of embryo selection algorithms (ESAs), which incorporate a set of instructions for the user, where, depending on the answers to the questions asked, a score is given that will aid in the selection, or deselection, of embryos in any given cohort. Many ESAs have now been developed and published, each using differing outcome parameters, exclusion and inclusion criteria, and morphokinetic parameters to define the selection of an embryo (6, 12, 14, 16–22). Crucially, a number of these ESAs have been validated externally with varying degrees of success (23, 24).

Unfortunately, the lack of control for confounding variables in time-lapse investigations, especially those involving the derivation of ESAs, reduces their transferability, meaning they are likely to be applicable only to the patients on which, and environment in which, they were derived. The effect of confounders on seven embryo morphokinetics has previously been assessed to determine the effect of maternal age, treatment type, body mass index (BMI), cumulative gonadotropin dose, and the number of previous attempts (2). The investigators concluded that patient demographics rather than an embryo's viability, when considered as part of a large cohort of embryos, can explain a high degree of embryo timing variability. More recently, a new line of research has become apparent using machine learning to aid in the development of effective embryo selection methods (25–27). Still in its infancy, this method has significant promise to remove confounders and make embryo selection a relative and objective process.

The investigation presented here sought to determine the effects of a number of patient and treatment parameters on 19 morphokinetic parameters using a multiple regression analysis methodology. The purpose of this investigation was to echo previous findings; to inform future directions of research, specifically the consideration of embryo origin during the derivation of embryo selection methodologies; and to highlight that the power of TLS lies beyond the human eye.

MATERIALS AND METHODS

This investigation was a single-site, retrospective observational design approved by the North West Research Ethics Committee (ref. no. 14/NW/1043) as well as the Institutional Review Board. All procedures and protocols complied with UK regulations (Human Fertilisation and Embryology Act, 1990, 2008). Data were obtained from 639 treatment cycles including 2,376 embryos cultured in EmbryoScope incubators between September 2014 to January 2016.

Ovarian Stimulation

Pituitary down-regulation was achieved using either a gonadotropin-releasing hormone agonist (buserelin, Suprecur, Sanofi Aventis) or antagonist (cetrorelix acetate, Cetrotide, Merck Serono). Ovarian stimulation was performed using urine-derived or recombinant follicle-stimulating hormone (Progynova, Bayer; Fostimon, Merional, IBSA Pharmaceuticals; Menopur, Ferring Fertility;, or Gonal f, Merck Serono). Doses were adjusted based on patient demographics and response. On identification of a lead follicle of 17 mm and at least two more of 16 mm, patients were given 5,000 IU of subcutaneous human chorionic gonadotropin (Gonasi HP, IBSA Pharmaceuticals) 36 hours before oocyte collection. Luteal support was provided using 400 mg of progesterone pessaries twice daily (Cyclogest, Actavis) until pregnancy test was performed.

Oocyte Retrieval and Embryology

Ultrasound-guided oocyte collection was performed transvaginally under sedation (Diprivan, Fresenius Kabi). Collected oocyte cumulus complexes were cultured in four-well dishes (Nunc, Thermo Scientific), each well containing 0.65 mL GIVF (Vitrolife) covered with 0.35 mL OVOIL (Vitrolife) in a standard incubator (Sanyo Multigas MCO 18M). Sperm preparation was performed using a standard gradient separation (ISolate, Irvine Scientific) at 0.3 relative centrifugal force for ten minutes followed by two washes at 0.6 relative centrifugal force for ten minutes using GIVF. Those oocytes destined for intracytoplasmic sperm injection (ICSI) were prepared using enzymatic (HYASE 10X, Vitrolife) and mechanical digestion. Intracytoplasmic sperm injection was performed on all metaphase II oocytes approximately four hours after collection, after which time all injected oocytes were placed in individual culture drops of GTL (Vitrolife) and cultured in the EmbryoScope (Vitrolife). Those oocytes destined for standard insemination (in vitro fertilization [IVF]) had this performed approximately four hours after collection and were replaced into a standard incubator until fertilization check the following day. Oocytes were then checked for fertilization approximately 16–18 hours post insemination (hpi), and all fertilized oocytes along with all unfertilized metaphase II oocytes were placed in individual culture drops, as with ICSI-derived embryos, and cultured in the EmbryoScope. Embryo selection was performed using the national grading scheme (Association of Clinical Embryologists/British Fertility Society guidelines (28)) along with an internally derived ESA. The ESA employed was used as an additive to morphology, with the latter remaining the gold standard. Embryo transfer was performed using the highest-grade embryo(s) 5 days post collection. Selected embryos were cultured in EmbryoGlue (Vitrolife) for 10–30 minutes in a standard incubator before embryo transfer. Embryos were cultured at 37°C, 6% CO_2, 5% O_2, 89% N_2 throughout. Where applicable, supernumerary embryos were cultured until day 6 for a decision regarding cryopreservation.

Analysis of Time-Lapse Information

The image interval on the EmbryoScope was set to ten minutes with seven focal planes. Images were collected for the

duration of culture immediately after ICSI or fertilization check (for IVF-derived embryos) until use. Annotation was performed manually as part of the clinical workload in the embryology laboratory using definitions previously described (29). Consistency of annotations was confirmed by participation of each embryologist in an internal quality assurance scheme. Time zero (t0) was assigned as pronuclei fading to eliminate the ambiguity regarding using time of insemination or injection. The absolute morphokinetic parameters assessed included time to two-cell (t2), three-cell (t3), four-cell (t4), five-cell (t5), six-cell (t6), seven-cell (t7), eight-cell (t8), nine-cell (t9), time to start of compaction (tM), start of blastulation (tSB), and full blastocyst (tB). The interval morphokinetic parameters assessed included the time between t2 and t3 (cc2), t4 and t5 (cc3), t8 and t9 (cc4), t3 and t4 (s2), t5 to t8 (s3), t9 and tM, tM and tSB, and tSB and tB.

Outcome Measures and Statistical Analysis

A multiple regression was performed on 2,376 embryos to determine the effect on t2, t3, t4, t5, t6, t7, t8, t9, tM, tSB, tB, cc2, cc3, cc4, s2, s3, t9-tM, tM-tSB, and tSB-tB of maternal age; maternal BMI; suppression protocol; and primary infertility diagnosis. All morphokinetic parameters were classed as continuous, dependent variables. Maternal age and BMI were classed as continuous, independent variables. Treatment type, primary diagnosis, and suppression protocol were categorical independent variables. However, because treatment type and infertility diagnosis were polytomous, they required the use of a reference category for statistical analysis. The reference category for treatment type was IVF, and the reference category for infertility diagnosis was male factor. As was the case for all morphokinetic parameters, linearity was assessed by partial regression plots and a plot of studentized residuals against the predicted values. There was independence of residuals, as assessed by a Durbin-Watson statistic (1.00–2.00). There was homogeneity of variance, as assessed by visual inspection of a plot of studentized residuals versus unstandardized predicted values. There was no evidence of multicollinearity (where one variable can be linearly predicted from others), as assessed by tolerance values greater than 0.1. No studentized deleted residuals were excluded from the analysis as they did not have advantage values greater than 0.2 and values for Cook's distance above one. The assumption of normality was met, as assessed by Q-Q plot. Results were considered significant at $P < .05$. Statistical analysis was performed using the statistical package SPSS (IBM, 2015).

RESULTS

In total, 2,376 embryos from 639 patients were included in this analysis. None of the patient or treatment parameters affected the morphokinetics of embryo development as a whole (Tables 1 and 2). Instead, complex relationships appeared to exist between specific morphokinetic parameters and patient and treatment parameters. Of all assessed parameters, suppression protocol (agonist or antagonist) had no significant effect on any morphokinetic parameter. Maternal age significantly affected t2, t4, tB, and tM-tSB. Female patient BMI affected t2 alone. In addition, those embryos created using ICSI (excluding those using donor sperm) had significantly different t2, tSB, tB, cc2, and tM-tSB measurements compared with those created using IVF.

Beta coefficients, indicating the amount of change elicited by the dependent variable (morphokinetic parameter) when a one-unit change in the independent variable is made (patient or treatment characteristics), were also assessed. Concerning maternal age, an increase of 1 year results in a decrease in t2 by 0.006 hours (h; equivalent to 21.6 seconds [s]), t4 by 0.029h (equivalent to 1.74 minutes [m]), an increase in tB by 0.78h and an increase in tM-tSB by 0.92h. This result indicates that embryos from younger patients undergo t2 and t4 slower than those from older patients; however, they are overall faster than older counterparts. Furthermore, where ICSI treatment has been performed (excluding donor sperm), embryos undergo t2 0.098h (equivalent to 5.88m) earlier, tSB 1.157h later, and tB 1.510h later than those undergoing IVF. Embryos derived from ICSI also have significantly longer cc2 (by 0.185h) and tM-tSB (by 0.637h). This result indicates that embryos derived from ICSI undergo the first cleavage of preimplantation embryo development earlier than those undergoing IVF; however, by the blastocyst stage of development ICSI embryos are overall slower than those derived from IVF.

When assessing causes of infertility and treatment type, seven categories were identified that had fewer than 20 patients in each; use of donor sperm, endocrine cause of infertility, secondary cause of infertility, intracytoplasmic morphologically selected sperm injection, testicular sperm extraction-ICSI, donor IVF, and donor ICSI. Statistical significance was found when analyzing the data; however, these will not be discussed at length owing to the sample size and likelihood of statistical insignificance should the sample size be increased. These results do allude to a need for further investigation with larger sample sizes and so remain in the overall data set for observation. Baseline characteristics of the patient cohort are shown in Table 3.

DISCUSSION

The effect of patient and treatment parameters on 19 morphokinetic parameters was assessed using a large group of embryos from an unselected cohort of patients. Using a defined time point as t0, a number of complex relationships between specific patient and treatment parameters and certain morphokinetic parameters were revealed. The analysis presented adds to the results seen by other invesetigators (2), and together with these highlight the presence of confounders when considering morphokinetics, confirming that machine learning should be the focus of future research when considering TLS for embryo selection.

Maternal age significantly affected four morphokinetic parameters, t2, t4, tB, and tM-tSB, demonstrating that embryos from younger patients undergo t2 slower than those from older patients; however, they are overall faster. Although the evidence is lacking in the literature regarding the specific relationship between morphokinetic parameters and maternal age, aneuploidy could be used as a proxy. It is

TABLE 1

Multiple regression analysis results for the effect of maternal age, maternal body mass index (BMI), suppression protocol, infertility diagnosis, and treatment type on absolute morphokinetic parameters.

	t2		t3		t4		t5		t6		t7		t8		t9		tM		tSB		tB	
	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B
Maternal age	.007[a]	−.006	.050	−.013	.007[a]	−.029	.791	−.004	.809	.004	.464	−.020	.152	−.052	.964	.001	.404	−.029	.058	.063	.043[a]	.078
Maternal BMI	.001[a]	−.009	.295	−.008	.362	−.012	.622	−.010	.093	−.037	.302	−.033	.267	−0.49	.330	−.036	.305	−.043	.133	−.060	.272	−.052
Suppression	.573	−.012	.613	−.030	.251	−.113	.754	0.47	.971	−.006	.558	−.144	.552	−.199	.625	.136	.577	.179	.843	.060	.229	.429
Infertility diagnosis																						
Ovarian	.913	−.004	.261	−.111	.866	−.028	.877	−.038	.326	−.269	.352	−.378	.928	.050	.472	.331	.863	−.091	.437	.390	.977	−.017
Uterine	.223	.045	.262	−.119	.958	−.009	.958	.014	.716	−.108	.662	.192	.173	.809	.593	.266	.494	.391	.156	.768	.204	.806
Donor	.027[a]	−.310	.019[a]	−.945	.044[a]	−1.340	.168	−1.388	.161	−1.572	.036[a]	−3.478	.230	−2.698	.021[a]	−4.343	.327	−2.121	.238	−2.419	.014[a]	−5.894
Unexplained	.571	−.019	.968	.004	.432	.123	.230	.285	.558	.155	.705	.148	.564	.306	.485	.310	.375	.454	.157	.685	.254	.647
Endocrine	.103	−.178	.802	.078	.403	.432	.385	.678	.216	1.077	.315	1.293	.220	2.140	.713	.536	.568	.960	.108	2.559	.404	1.557
Secondary	.002[a]	−.329	.418	−.250	.263	−.572	.746	−.250	.184	−1.143	.156	−1.806	.313	−1.741	.668	−.619	.013[a]	−4.137	.051	−3.069	.021[a]	−4.256
Treatment type																						
ICSI	.001[a]	−.098	.281	.087	.114	.211	.539	.124	.245	.262	.516	.216	.618	.255	.990	.005	.232	.520	.005[a]	1.157	.002[a]	1.510
IMSI	.306	−.074	.377	.184	.421	.277	.830	−.112	.512	−.381	.427	−.682	.501	−.783	.683	.397	.009[a]	2.938	.073	1.905	.210	1.560
TESE ICSI	.435	−.076	.462	.203	.811	.110	.337	.664	.275	.841	.455	.851	.373	1.378	.726	.453	.576	.831	.050	2.769	.272	1.817
D-IVF	.084	.183	.164	.422	.245	.583	.107	1.222	.514	.552	.090	2.121	.152	2.424	.024[a]	3.199	.407	1.353	.101	2.535	.007[a]	4.882
D-ICSI	.014[a]	.341	.001[a]	1.304	.084	1.137	.008[a]	2.650	.033[a]	2.367	.107	2.642	.248	2.571	.030[a]	4.036	.941	.160	.206	2.568	.099	3.930

Note: Time to two-cell (t2), three-cell (t3), four-cell (t4), five-cell (t5), six-cell (t6), seven-cell (t7), eight-cell (t8), nine-cell (t9), start of compaction (tM), blastulation (tSB), and time to full blastocyst (tB) are included. *P* values and beta coefficients (*B*) are shown for each parameter. A negative *B* indicates a decrease in the parameter in hours for every unit increase in the independent variable. ICSI = intracytoplasmic sperm injection; IMSI = intracytoplasmic morphologically selected sperm injection; TESE = testicular sperm extraction; D-IVF = donor in vitro fertilization; D-ICSI = donor ICSI.

[a] Statistically significant.

Barrie. Confounders of an embryo's morphokinetic profile. Fertil Steril 2020.

TABLE 2

Multiple regression analysis results for the effect of maternal age, maternal body mass index (BMI), suppression protocol, infertility diagnosis, and treatment type on interval morphokinetic parameters.

	cc2		cc3		cc4		s2		s3		t9-tM		tM-tSB		tSB-tB	
	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B
Patient age	.285	−.007	.094	.025	.082	.053	.081	−.016	.141	−.048	.348	−.031	<.001[a]	.092	.454	.016
BMI	.940	.001	.904	.002	.726	.013	.759	−.003	.319	−.039	.847	−.008	.584	−.017	.736	.009
Suppression	.749	−.018	.240	.160	.236	.335	.331	−.083	.410	−.245	.886	.043	.610	−.119	.055	.369
Infertility diagnosis																
Ovarian	.260	−.107	.962	−.011	.546	.381	.556	.083	.858	.088	.392	−.422	.211	.481	.200	−.407
Uterine	.108	−.164	.924	.023	.279	−.543	.470	.110	.134	.795	.814	.125	.363	.377	.912	.038
Unexplained	.805	.023	.456	.162	.993	.004	.380	.120	.964	.021	.763	.143	.534	.231	.902	−.038
Donor[a]	.102	−.635	.958	−.048	.387	−1.645	.494	−.394	.514	−1.309	.271	2.222	.850	−.298	.007[a]	−3.475
Endocrine[a]	.394	.256	.730	.246	.277	−1.604	.430	.354	.348	1.462	.787	.424	.190	1.599	.319	−1.002
Secondary[a]	.052	−.579	.648	.322	.441	1.122	.467	−.322	.333	−1.491	.023[a]	−3.518	.375	1.068	.232	−1.188
Treatment type																
ICSI	.018[a]	.185	.636	−.087	.564	−.220	.283	.124	.802	.101	.203	.515	.044[a]	.637	.175	.353
IMSI[b]	.198	.258	.413	−.389	.230	1.180	.755	.093	.518	−.671	.015[a]	2.541	.204	−1.033	.606	−.346
TESE ICSI[b]	.296	.279	.380	.555	.479	−.924	.813	−.094	.605	.714	.785	.377	.073	1.938	.285	−.952
D-IVF[b]	.414	.239	.355	.639	.588	.776	.711	.161	.427	1.201	.224	−1.846	.318	1.182	.016[a]	2.348
D-ICSI[b]	.012[a]	.963	.096	1.513	.436	1.465	.770	−.167	.968	−.079	.052	−3.876	.121	2.408	.288	1.362

Note: Duration of second cell cycle (cc2; t3-t2), third cell cycle (cc3; t5-t4), fourth cell cycle (cc4; t9-t8), synchrony of the second cell cycle (s2; t3-t4), synchrony of the third cell cycle (s3; t8-t5), time between t9 and tM, time between tM and tSB, and time between tSB and tB are included. *P* values and beta coefficients (*B*) are shown for each parameter. A negative *B* indicates a decrease in the parameter in hours for every unit increase in the independent variable. ICSI = intracytoplasmic sperm injection; IMSI = intracytoplasmic morphologically selected sperm injection; TESE = testicular sperm extraction; D-IVF = donor in vitro fertilization; D-ICSI = donor ICSI.

[a] Statistically significant.

[b] Reduced sample size (<20 patients included).

Barrie. Confounders of an embryo's morphokinetic profile. Fertil Steril 2020.

well accepted that the rate of aneuploidy increases with maternal age (30), and one investigation observed significant differences in blastulation morphokinetic parameters and risk of aneuploidy as determined through trophectoderm biopsy (16). From this investigation, a risk classification model was developed and, although when externally applied lost efficacy (23, 24), supports the notion that patient age, perhaps more specifically embryo ploidy, affects morphokinetic parameters. Of particular interest is the use of both tSB and tB in the classification model outlined by Campbell et al. (16). In the results presented here, tM-tSB and tB were both delayed in patients of increased maternal age. This is mirrored in the timings proposed by Campbell et al., where those embryos carrying a medium risk of aneuploidy were more likely to reach the start of blastulation later (>96.2 hpi) and those with a high risk were likely to reach tB later (>122.9 hpi). Other researchers have investigated the effect of maternal age with similar findings. In 2014, the correlation between maternal age and a number of morphokinetic timings, t5, cc2, cc3, s2, and t5-t2, was assessed (31). Although there was no statistically significant difference in these parameters in embryos from younger and older patients, there was a trend toward those from older patients being delayed in development. In 2016, time-lapse images of 1,730 biopsied embryos were analyzed, and a correlation between ploidy and blastulation parameters was detected where aneuploid embryos were delayed in development compared with euploid counterparts (32). In addition, the effect of maternal age on morphokinetic parameters was investigated using a similar study design to that presented here (2). In this investigation, embryos were seen to reach tSB 0.29h later with each one-unit increase in maternal age. The size of the time differences presented here may indicate a cumulative delay that becomes apparent by the time the blastulation stage of embryo development is reached. There is credence in considering that this may be due to the need for DNA repair in oocytes originating from patients with increased maternal age causing a prolonged cell cycle. Alternatively, there may be a relationship between the mechanism for blastocyst formation, perhaps linked to compaction, blastocoel formation, or cell differentiation that is directly affected by oocyte age. The differences of up to a 0.1h increase in blastocyst parameters observed in embryos from older patients here, and supported by other investigators, provide valuable information that could be useful when selecting embryos using ESAs, despite the scientific basis for this delay remaining unknown.

The underlying relationship between maternal BMI and embryo quality is yet to be determined; however, there are interesting investigations emerging that assess the composition of follicular fluid from patients with varying BMIs. The effect of BMI that is seen in this analysis could be due to the requirement for a higher dose of gonadotropins (33), which has been demonstrated to affect an embryo's morphokinetic profile (2). The influence of maternal BMI was evident only on t2, a gold standard for embryo viability dating back 20 years (34). Although this effect is not sustained throughout embryo development, the association could be a reflection of embryo viability. For every one-unit increase in BMI, t2 occurs 0.009h (equivalent to 32.4s) earlier. It has been demonstrated that patients with increased BMI have reduced pregnancy rates compared with normal BMI patients (33); thus a clinically relevant effect on a morphokinetic parameter, such as t2, is possible. A recent analysis aimed to determine the effect of BMI on the morphokinetics of 5,248

TABLE 3

Baseline patient information for the analysed embryo cohort.

Parameter	Value
No. of embryos	2,376
No. of patients	639
No. of cycles	639
Maternal age (mean ± SD)	32.9 ± 4.4
Maternal BMI (mean ± SD)	24.3 ± 3.7
Suppression protocol, n/%	
Agonist	275/41
Antagonist	364/59
Cause of infertility, n/%	
Male factor	225/35.2
Ovarian	114/17.8
Uterine	88/13.8
Unexplained	193/30.2
Donor[a]	4/0.6
Endocrine[a]	8/1.3
Secondary[a]	7/1.1
Treatment type (n/%)	
IVF	343/53.7
ICSI	266/41.6
IMSI[a]	17/2.7
TESE ICSI[a]	7/1.1
D-IVF[a]	4/0.6
D-ICSI[a]	2/0.3
No. of eggs collected, mean ± SD	14.7 ± 7.3
No. of embryo transfers	503
No. of embryos transferred	550
No. of positive pregnancy tests, n/BPR	213/42.3
No. of fetal hearts, n/IR	219/39.8

Note: BMI = body mass index; BPR = biochemical pregnancy rate (number of positive human chorionic gonadotropin tests/number of embryo transfers); D-ICSI = donor ICSI; D-IVF = donor IVF; ICSI = intracytoplasmic sperm injection; IMSI = intracytoplasmic morphologically selected sperm injection; IR = implantation rate (number of fetal hearts/number of embryos transferred); IVF = in vitro fertilization; SD = standard deviation; TESE = testicular sperm extraction.

[a] Reduced sample size (<20 patients included).

Barrie. Confounders of an embryo's morphokinetic profile. Fertil Steril 2020.

embryos. The investigators observed prolonged embryo development to t5 and t8 in obese women when compared with those of normal weight. Embryos from obese women were, on average, 1.60h slower in reaching t5 and 2.23h slower in reaching t8 (35). Conversely, an earlier investigation found no difference in morphokinetics in embryos from obese, infertile women compared with normal-weight, infertile women (36). However, the sample size of this analysis was modest, assessing embryos from just 89 patients. It is likely that there is an effect of BMI on an embryo's developmental pattern; however, future research should be directed to determining this effect, specifically in extreme BMIs.

The effect of method of insemination on an embryo's morphokinetic profile has been demonstrated elsewhere (4, 37); however, many used an arbitrary time for t0, the most popular of which is time of insemination or injection. The use of these time points as t0 is obviously confounding as they are ambiguous and could vary by hours from oocyte to oocyte. In support of this, differences observed in embryo morphokinetics have been shown to disappear when an observable time point is used for t0 (38, 39). In the current analysis, time of pronuclear fading was used as t0; therefore, any observed differences in treatment type are more reliable than those using time of insemination or injection. In particular, those embryos created using ICSI had significantly different t2, tSB, tB, cc2, and tM-tSB values when compared with embryos created through IVF. These significant differences of up to 1.5h indicate that, at the very least, ESAs should be developed to accommodate differing treatment types even when a definable t0 is used, as recently corroborated by others (40). There must be further investigations into the rarer treatment types, such as intracytoplasmic morphologically selected sperm injection, physiological-ICSI (P-ICSI), or cycles involving oocyte activation to examine the need for alternative optimum ranges for various morphokinetic parameters. With regards to the consideration of treatment type, specifically IVF and ICSI, pertinent to this investigation is the difference in the incubator used for fertilization; those embryos fertilized using ICSI have over 12 hours longer in the controlled environment of an EmbryoScope compared with those embryos fertilized using standard IVF. This could be considered a confounding variable in this investigation. To control this in future, an incubator shown to have a comparably stable environment to the TLS device of choice should be used, or, in a prospective setting, all embryos should be incubated only in the TLS device post fertilization check to ensure that embryos are consistently exposed to environmental factors.

The current analysis does not include other suspected confounders such as dose of gonadotropins, paternal factors such as age, or endogenous maternal hormone levels and so is by no means exhaustive. In addition, all embryos that reached tB were included in this analysis. Although this increases the likelihood that the most competent embryos were assessed (i.e. blastocysts), it can be argued that poor embryo quality could have a confounding effect on the morphokinetic parameters, and those that do not have the capacity to reach the blastocyst stage may create bias in the data set. The analysis serves to demonstrate the effect of certain patient and treatment parameters to inform future areas of research and highlight that the variability seen in embryo development is not necessarily an effect of embryo viability, as is suggested by those investigators using morphokinetic parameters to predict an embryo's ability to implant. This is also an indication regarding the use of ESAs and their inability to be externally applied with the same efficacy as is observed at the development site (23, 24, 39, 41). It is time for embryo selection methods to be developed with variations in patient and treatment parameters in mind. It is vital that any developed embryo selection methods be prospectively applied in RCTs of adequate sample size and design to eliminate known and unknown confounders. Currently, embryologists select the most viable embryo in a cohort in terms of morphology, morphokinetics, and sometimes chromosomal complement, and yet implantation does not always occur. This highlights the need for relative as well as objective methods for embryo selection. This fact also highlights an obvious confounder that is often overlooked and is likely to be able to be controlled only through RCTs: endometrial receptivity.

Embryo development is seemingly affected in subtle ways by a multitude of factors. The formulation of ESAs using basic morphokinetic information is not likely to be able to account

for the effect of confounders entirely. The differences seen in this analysis appear minimal; however, they are significant. These differences are not able to be detected by the human eye, and the software currently available for the programming of ESAs built using basic morphokinetic parameters is not sensitive enough to account for these seemingly small variations; this computation can only be achieved through machine learning. Until such a time that appropriate models built with machine learning have been tested in robust trials and subsequently become widely available, it may be beneficial to continue to use macromorphokinetic markers that are less variable and potentially less heavily influenced by confounding factors. In the first instance, these parameters can be used to perform effective deselection of those embryos undergoing abnormal division events such as direct and reverse cleavage, both of which are shown to reduce the implantation potential of embryos (15, 42–44).

CONCLUSION

This analysis provides a comprehensive account of the effect of confounding factors on an embryo's morphokinetic profile. It highlights the subtle nature of embryo development and the need to perform appropriate and robust production and validation of ESAs if they are to be employed to perform embryo selection in an IVF laboratory. Where some of the rare infertility diagnoses or treatment types are concerned, conclusions should be considered tentative, however this analysis provides evidence that further investigations should be carried out to clarify the complex relationships between confounders and morphokinetic parameters. Until the development of embryo selection methods via machine learning that consider the effect of confounders and that have been prospectively applied in RCTs, other macromorphokinetic markers should be considered to perform simple but effective deselection using time-lapse imaging.

Acknowledgments: The analyses presented here were carried out using data from the Hewitt Fertility Centre, Liverpool.

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