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Original Research Articles

Understanding the implications of follicular output rate (FORT) and follicle to oocyte index (FOI) on human embryo morphokinetics

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Objective

To study if there are any effects of follicular output rate (FORT) and follicle to oocyte index (FOI) on embryos morphokinetics.

Study design

Kinetic data of 8,376 embryos, cultured in a time-lapse imaging incubator, derived from 2,470 patients undergoing ICSI cycles were analysed. Embryos were split into groups according to FOI value: Low FOI (n=247 cycles and 894 embryos) and High FOI (n=2,223 cycles and 7,482 embryos) and according to the FORT value: Low FORT (n= 753 cycle and 2,556 embryos), Medium FORT (n=874 cycles and 2,970 embryos), and High FORT (n=843 cycles and 2,850 embryos). Morphokinetic data were compared among the groups.

Results

Embryos derived from cycles with a low FOI presented slower development, a significantly lower KID score D5, blastocyst formation, and implantation rates when compared with those from cycles with high FOI. For the FORT, an increased time to complete morphokinetic events, significantly lower rates of blastocyst formation and implantation was observed among embryos derived from cycles with low FORT, followed by those with medium FORT, while embryos derived from cycles with high FORT presented a better development competence. However, no significant differences were noted in clinical pregnancy, miscarriage, or livebirth rates when the low, medium, and high FORT groups were compared.

Conclusion

FORT and FOI correlate with faster embryo development and may be a valuable approach to predict embryo developmental potential.

INTRODUCTION

Utilizing controlled ovarian stimulation (COS) is essential to maximize the success of in vitro fertilization (IVF). The aim is to produce an ideal number of mature oocytes to maximize success as best as possible. However, the ovarian response to COS may be poor, suboptimal, or even excessive, all of which can negatively impact patients.[1,2]

The ovarian response is influenced by various factors, which can make predicting oocyte yields less obvious. For example, some patients exhibit a low response to COS, despite displaying normal biomarkers of ovarian reserve, such as antral follicle count (AFC) and anti-Mullerian hormone (AMH) serum levels, the hypo-responder patients,[2-4] showing that the response to COS could possibly be correlated with the sensitivity of the ovaries to externally adminis-

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tered gonadotropins, which may be influenced by genetic factors.[5] The quantity of oocytes obtained from these patients after stimulation is not congruous with the number of follicles present at the beginning of COS or the AFC.[6] Therefore, the use of conventional ovarian reserve biomarkers to predict the ovarian response may be inadequate.

Alternative approaches to investigate the gonadotropin stimulation resistance in ovaries are the follicular output rate (FORT)[7] and follicle-to-oocyte index (FOI).[6] The FORT and FOI, which are qualitative markers of the response to gonadotrophins, reflecting the follicular development dynamic more accurately.[6]

The FORT, defined as preovulatory follicle (with a mean diameter between 16 and 22 mm) count divided by AFC × 100, was linked with increased pregnancy rate, number of mature oocytes, and better embryo quality.[8-11]

The FOI represents the ratio of the number of retrieved oocytes and AFC before COS, and may be the most reliable parameter to represent follicular development under FSH stimulation.[6] On addition, FOI is a key approach to identify hypo-responders, women with a diminished ovarian response to COS, despite of having normal ovarian reserve parameters.[12] FOI < 50% was described to represent normal ovarian responsiveness, however, the connection of FOI with clinical outcomes is still controversial. While higher FOI was associated with increased implantation rate,[13] the same was not observed in women of advanced age.[14]

It has been described that hypo-responders present an initial slow increase in estradiol levels and development of follicles,[15,16] and it has been postulated that this group of patients may have a certain genotype that influences their response to ovarian stimulation.[17] Patients presenting with specific homozygosis may exhibit impaired FSH receptor function,[17,18] leading to a higher consumption of gonadotropin.[19,20] However, the exactly mechanism, by which, hypo-responders present an unexpected ovarian resistance to exogenous gonadotropins remains to be elucidated. BY examining the correlation between FOI and FORT and embryo biology would be an interesting way to understand by which pathways hyporesponsiveness occurs

The precise evaluation of embryo morphokinetics plays a pivotal role in determining in IVF success rates, as it provides valuable insights into the dynamic cellular events during early embryonic development, contributing to the selection of embryos with higher implantation potential (Ref: Sfontouris et al., 2015; Racowsky et al., 2019)."

Time-lapse imaging (TLI) has emerged as a powerful tool, offering a dynamic and detailed perspective into the process of embryo development. Unlike traditional static observations, TLI involves capturing a series of images at regular intervals, allowing continuous monitoring of embryonic growth and morphological changes. This provides invaluable insights into the kinetics of critical events during early embryogenesis, proving a comprehensive understanding of the temporal dynamics of embryonic progression.[21,22]

Here, we hypothesized that some follicles may present with a specific genotype profile that influences not only their response to ovarian stimulation but also the quality of the oocyte and embryos. Consequently, TLI may be an advantageous approach to evaluate the correlation between both FORT and FOI and the competences of developing embryos, considering morphokinetic parameters. Therefore, the goal of the present study was to investigate the impact of FORT and FOI on embryo morphokinetics.

MATERIALS AND METHODS

PATIENTS AND EXPERIMENTAL DESIGN

This historical cohort study was performed in a private university-affiliated IVF centre between February 2019 and December 2021. Kinetic data were analysed in 8,376 embryos, individually cultured in a time-lapse imaging (TLI) incubator (EmbryoScope+, Unisense Fertilitech, Aarhus, Denmark) until day five of development, and derived from 2,470 patients undergoing ICSI cycles using ejaculated fresh sperm. The timing of specific events from the point of insemination was determined using TLI. The FORT and FOI were determined, and the effects of the levels of these parameters on morphokinetic events and ICSI clinical outcomes were investigated.

Embryos were split into groups according to FOI value: FOI< 50 (Low FOI, n=247 cycles and 894 embryos) and FOI⩾ 50 (high FOI, n=2,223 cycles and 7,482 embryos) and according to the FORT value: FORT values below the 33rd percentile, FORT< 27.3 (low FORT, n= 753 cycle and 2,556 embryos), FORT values between the 33rd and the 67th percentile, FORT: 27.3–47.6 (medium FORT, n=874 cycles and 2,970 embryos), and FORT values above the 67th percentile, FORT> 47.6 (high FORT, n=843 cycles and 2,850 embryos). Embryo morphokinetics and ICSI outcomes were compared among the FOI and FORT groups.

Each patient provided written consent acknowledging their willingness to share the results of their reproductive cycles for research, and local Institutional Review Board approval was granted for the study.

CONTROLLED OVARIAN STIMULATION AND LABORATORY PROCEDURES

Controlled ovarian stimulation started on day three of the cycle, by the injection of r-FSH, daily (Gonal-F®, Serono, Geneva, Switzerland; or Rekovelle®, Ferring, Saint-Prex, Switzerland). A gonadotropin-releasing hormone (GnRH) antagonist (GnRHa, Cetrotide®; Merck KGaA, Darmstadt, Germany) was used to prevent premature LH surge, when the largest follicle in the cohort reached 14 mm.

When three or more follicles were ⩾17 mm, r-hCG (Ovidrel®, Merck KGaA, Darmstadt, Germany) was used to trigger final follicular maturation. Whenever the patient was at risk of developing OHSS, a GnRH agonist (0,2 mg triptorelin acetate, Gonapeptyl daily®, Ferring GmbH, Kiel, Germany) was administered SC instead of r-hCG Oocyte pick up was accomplished in 35 hours. Oocytes in metaphase II were selected for ICSI.

SEMEN ANALYSIS AND PREPARATION

Semen samples were collected through masturbation, in the laboratory. Samples liquefied within 30 minutes and sperm count and motility were evaluated using a count chamber (Leja® slide, Gynotec Malden, Nieuw-Vennep, the Netherlands). The total sperm concentration was defined as the number of spermatozoa in the ejaculate.

For sperm motility assessment, 100 spermatozoa were characterized as progressively motile, nonprogressively motile, or immotile.

Moreover, the motility results are expressed as percentages.

For sperm morphology assessment, air-dried smears were fixed and stained using the quick-stain technique (Diff-Quick, Quick-Panoptic, Amposta, Spain). Sperm cells (200) were characterized as morphologically normal or abnormal and the results were expressed as percentages.

For ICSI sperm samples were prepared by using a two-layered density gradient centrifugation technique (50% and 90% Isolate, Irvine Scientific, Santa Ana, CA, USA).

INTRACYTOPLASMIC SPERM INJECTION

Intracytoplasmic sperm injection was performed according to Palermo et al.[23] Sperm were selected at 400x magnification using an inverted Nikon Eclipse TE 300 microscope and injected into the oocytes in a microinjection dish prepared with buffered medium (Global w/HEPES, LifeGlobal, Guilford, USA) covered with paraffin oil (Paraffin oil P.G., LifeGlobal) on an inverted microscope heated stage (37.0 °C ± 0.5 °C).

EMBRYO CULTURE

Injected oocytes were individually cultured in a 16-well culture dish (Embryoslide, Unisense Fertilitech, Aarhus, Denmark) in 360 µl of continuous single-culture media (Global® total®, LifeGlobal) overlaid with 1.8 ml of mineral oil (Paraffin oil P.G., LifeGlobal) in a TL-monitored incubator (EmbryoScope+, Unisense Fertilitech, Aarhus, Denmark) set at 37 °C with an atmosphere of 6% O_2 and 7.2% CO_2 until day five of embryo development. The incubator high-definition camera was set up to record embryo images in eleven focal planes every 10 minutes. Recorded kinetic markers were timing to pronuclei appearance (tPNa) and fading (tPNf), timing to two (t2), three (t3), four (t4), five (t5), six (t6), seven (t7), and eight cells (t8), and timing morulation (tM), timing to start blastulation (tSB) and to blastulation (tB). The durations of the second (cc2, t3-t2) and third cell cycles (cc3, t5-t3) and the timing to complete synchronous divisions t2-tPNf (s1), t4-t3 (s2) and t8-t5 (s3) were calculated. Data generated from EmbryoScope+ were analysed using EmbryoViewer software (Vitrolife, Denmark). The incidences of multinucleation at the 2- and 4-cell stages and of abnormal cleavage patterns (direct or reverse cleavage) were recorded for each embryo.

FORT AND FOI DETERMINATION

FORT was defined as the ratio between the number of pre-ovulatory follicles (16–22 mm in diameter) obtained in response to FSH administration and the preexisting pool of small antral follicles (3–8 mm in diameter) at baseline (Genro et al., 2011).

The FOI was assessed as the ratio between the total number of oocytes retrieved at oocyte pick-up and the number of antral follicles available at the start of stimulation.[6]

FORT=Pre–ovulatory follicle count on the day of hCG trigger ÷ AFC

FOI=The number of oocytes retrieved at ovum pick–up ÷ AFC*100

CLINICAL FOLLOW-UP

Embryo transfer was performed on Day 5 of embryo development, and one or two embryos were transferred per patient.

Women with a positive pregnancy test, performed 10 days post embryo transfer, had a transvaginal ultrasound scan 2 weeks later. The clinical pregnancy was diagnosed upon detection of foetal heartbeat. The pregnancy rate was calculated per embryo transfer. The implantation rate is the number of gestational sacs with foetal heartbeats divided by the number of transferred embryos. Miscarriage was defined as clinical pregnancy loss before 20 weeks.

DATA ANALYSIS AND STATISTICS

The primary outcome measure was tB since it is the most advanced key stage of embryonic development recorded in our centre. Post hoc power analysis was calculated, given an α of 5%, a sample size of 554 embryos that reached the blastocyst stage at Day 5 of development, and an effect size for tB. The achieved power was superior to 80%. The calculation was performed using the Hotelling Lawley Trace test in the GLIMMPSE App for multilevel data,[24] which accounted for the correlation between embryos from the same cycle.

Generalized mixed models (GMM) adjusted for potential confounders, as maternal and paternal age and female body mass index (BMI), followed by Bonferroni post hoc for the comparison of means between groups were used to study the impact of FOI and FORT on embryo morphokinetics. The models were generated using FOI and FORT as independent variable and kinetic markers as dependent variables. Maternal and paternal ages were included as covariates in all models to control for their influence. A random effect was added to account for the correlation between the embryos within the same cycle, with linear distribution for morphokinetic data in hours (h) and known implantation diagnosis score (KIDScore) ranking. For clinical outcomes, which were based on a single observation per couple, regression models (generalized linear models) followed by Bonferroni post hoc for the comparison of means between groups were used without random effects, with linear

Table 1. Comparison of demographic and cycle characteristics between low and high follicle-to-oocyte index (FOI) groups

		Low FOI	High FOI	P value
n		247	2,223	
Female age (years)		38.6 ± 3.4	38.3 ± 3.8	0.213
Male age (years)		39.5 ± 5.9	39.5 ± 5.7	0.683
Female BMI (kg/m^2)		24.9 ± 4.4	24.3 ± 3.8	0.081
Total dose of FSH	Follitropin alfa (IU)	2531.5 ± 1000.6	2574.9 ± 774.0	0.241
	Follitropin delta (mcg)	148.0 ± 32.3	152.0 ± 29.9	0.124
Oestradiol level (pg/mL)		1351.7 ± 1002.0	1546.4 ± 384	**0.001**
Follicles (n)		7.5 ± 6.3	16.4 ±10.4	**<0.001**
Retrieved oocytes (n)		3.9 ± 3.4	12.5 ± 8.4	**<0.001**
Mature oocyte (n)		3.0 ± 2.7	9.2 ± 6.8	**<0.001**

Note: Values are means ± standard error. unless otherwise noted. ICSI – intracytoplasmic sperm injection; BMI – body mass index; FSH – follicle stimulating hormone; hCG – human chorionic gonadotropin.

Table 2. Comparison of demographic and cycle's characteristics between low, medium, and high follicular output rate (FORT) groups

		Low FORT	Medium FOI	High FORT	P value
n		753	874	843	
Female age (years)		36.9 ± 3.3	37.1 ± 3.4	37.2 ± 2.9	0.651
Male age (years)		39.0 ± 4.7	39.3 ± 3.9	39.4 ± 5.6	0.615
Female BMI (kg/m^2)		24.1 ± 3.3	24.5 ± 3.4	24.6 ± 4.1	0.162
Total dose of FSH	Follitropin alfa (IU)	2539.7 ± 785.6	2558.5 ± 958.0	2545.6 ± 789.9	0.953
	Follitropin delta (mcg)	151.0 ± 32.3	149.0 ± 35.4	151.5 ± 29.8	0.457
Oestradiol level (pg/mL)		1495.9 ± 547.6^a	1545.2 ± 475.7^b	1639.2 ± 547.9^c	0.001
Follicles (n)		7.3 ± 5.9^a	12.3^b ± 5.6	14.1 ± 9.3^c	<0.001
Retrieved oocytes (n)		4.1 ± 3.6 ^{a}	10.1 ± 7.9 ^{b}	11.1 ^{c} ± 9.1	<0.001
Mature oocyte (n)		3.4 ± 2.5 ^{a}	7.9 ± 6.3 ^{b}	8.9 ^{c} ± 5.1	<0.001

Note: Values are means ± standard error. unless otherwise noted. ICSI – intracytoplasmic sperm injection; BMI – body mass index; FSH – follicle stimulating hormone; hCG – human chorionic gonadotropin. a≠b≠c

distribution for implantation rate and binomial distribution for clinical pregnancy, miscarriage, and livebirth rates.

The results were expressed as percentages or means ± standard errors (SEs) and p values or as power coefficient (B or OR) with 95% confidence interval (CI) and p-values. *P* < 0.05 was considered statistically significant. Data analysis was conducted using the Statistical Package for the Social Sciences (SPSS) 21 (IBM, New York, NY, USA).

RESULTS

The average maternal and paternal age, body mass index, FOI, and FORT in the study population were 37.7 ± 3.78 years old, 39.7 ± 5.81 years old, 24.2 ± 23.71 kg/m2, 73.8 ± 24.96%, and 38.2 ± 25.92%, respectively.

Demographic data concerning male and female partners and the cycle characteristics of patients in the FOI and FORT groups are shown in Tables 1 and 2, respectively.

Patients in the Low FOI Group presented a lower oestradiol level, number of follicles, number of retrieved oocytes and number of retrieved mature oocytes.

A significant difference was noted in almost all morphokinetic parameters, where embryos derived from cycles with an FOI <50 presented slower development than embryos derived from cycles with an FOI <50, considering tPNf, t2, t4, t6, t7, t8, tM, tB, s1, s2, s3, and cc2 (**Table 3**).

A significantly higher KID score D5 was observed among embryos derived from cycles with an FOI ⩾ 50 compared to those with an FOI < 50 (**Table 4**).

This finding was confirmed by the regression analysis, which demonstrated a positive influence of the FOI on the KID score D5 (B: 0.490, IC: 0.88–0.691, p=0.001)

Additionally, increased blastocyst formation and implantation rates were noted among cycles with higher FOIs. However, no significant differences were noted in fertilization, pregnancy, miscarriage, or livebirth rates when the low and high FOI groups were compared (**Table 4**).

Table 3. Comparison of morphokinetic parameters between the low and high Follicle-To-Oocyte (FOI) index groups.

Morphokinetic parameters (h)	Low FOI	High FOI	p value	B	95% CI
n	894	7532			
tPNa	6.9 ± 0.1	6.7 ± 0.41	0.144	0.006	-0.002 – 0.014
tPNf	25.3 ± 0.2	24.3 ± 0.0	**< 0.001**	-0.013	-0.025 – 0.00
t2	27.8 ± 0.2	26.8 ± 0.0	**< 0.001**	-0.007	-0.023 – 0.065
t3	37.9 ± 0.2	37.5 ± 0.8	0.071	-0.007	-0.026 – 0.008
t4	40.2 ± 0.2	39.2 ± 0.1	**< 0.001**	-0.023	-0.044 – -0.003
t5	50.7 ± 0.3	50.0 ± 0.1	0.062	-0.030	-0.060 – 0.001
t6	53.6 ± 0.3	52.9 ± 0.1	**0.040**	-0.029	-0.060 – -0.014
t7	56.8 ± 0.4	55.7 ± 0.1	**0.004**	-0.038	-0.072 – -0.04
t8	61.2 ± 0.4	59.3 ± 0.1	**< 0.001**	-0.027	-0.068 – -0.018
tM	90.1 ± 0.5	88.9 ± 0.1	**0.023**	-0.033	-0.089 – -0.023
tSB	101.8 ± 0.5	100.8 ± 0.1	0.065	-0.029	-0.077 – 0.019
tB	111.2 ± 0.5	109.9 ± 0.2	**0.028**	-0.039	-0.085 – 0.028
s1	2.7 ± 0.0	2.6 ± 0.0	**0.046**	-0.002	-0.004 – 0.000
s2	2.4 ± 0.1	1.8 ± 0.0	**< 0.001**	-0.008	-0.014 - -0.003
s3	10.8 ± 0.3	9.6 ± 0.1	**< 0.001**	-0.016	-0.029 - -0.003
cc2	10.2 ± 0.1	10.7 ± 0.052	**< 0.001**	-0.008	-0.030 – -0.002
cc3	12.9 ± 0.2	12.6 ± 21.48	0.262	-0.001	-0.009 – 0.009

Note: Generalized mixed models (GMM) adjusted for potential confounders, as maternal and paternal age, and female body mass index (BMI). P: Post-hoc Bonferroni. Values are means ± standard error. unless otherwise noted. FOI - follicle-to-oocyte (FOI). H – hours. tPNa – timing to pronuclei appearance. tPNf – timing to pronuclei fading. t2 – timing to two cells. t3 – timing to three cells. t4 – timing to four cells. t5 – timing to five cells. t6 – timing to six cells. t7 – timing to seven cells. t8 – timing to eight cells. tSB – timing to start blastula-
tion. tB – timing to blastulation. s1 – timing to complete t2-tPNf synchronous divisions. s2 – timing to complete t4-t3 synchronous divisions. s3 – timing to complete t8-t5 synchronous divisions. cc2 – c

Table 4. Comparison of known implantation diagnosis (KID) score D5 and intracytoplasmic sperm injection (ICSI) outcomes between the low and high follicle-to-oocyte (FOI) index groups.

Variable	Low FOI	High FOI	p value	B/OR	95% IC
Cycles	247	2.223			
Embryos	894	7532			
Kid score	5.1 ± 0.09	5.60 ± 0.03	**< 0.001**	-0.490*	-0.691 - -0.288
Fertilization rate (%)	70.0 ± 1.39	71.71 ± 0.72	0.299	-1.61*	-1.4 – 4.7
Blastocyst rate (%)	53.6 ± 0.92	44.85 ± 1.87	**< 0.001**	-8.8*	-12.9 - -4.7
Implantation rate (%)	24.8 ± 0.32	26.08 ± 0.53	**0.037**	-0.051	-0.099 – -0.004
Pregnancy rate (%)	32.4± 4.9 (80/247)	34.9 ± 3.22 (778/2.223)	0.573	1.157**	0.692 – 1.93
Miscarriage rate (%)	13.7 ± 3.6 (11/80)	16.2 ± 3.2 (130/800)	0.451	1.145**	0.847 – 1.547
Livebirth (%)	27.5 ± 2.1 (68/247)	30.2 ± 2.4 (672/2.223)	0.547	1.547**	0.475 – 1.654

Note: Generalized mixed models (GMM) adjusted for potential confounders, as maternal and paternal age, and female body mass index (BMI). P: Post-Hoc Bonferroni. Values are percentage ± standard error. unless otherwise noted. Effect size: *: B (continuous variables) and ** OR (binary variables), considering Low FOI as reference group.

An increased time to complete morphokinetic events was observed among embryos derived from cycles with low FORT, followed by those with medium FORT, while embryos derived from cycles with high FORT presented a faster development competence: tPNa, t2, t4, t5, t6, t7, t8, tsB, s2, s3 (**Table 5**).

Embryos derived from cycles with high FORT presented a higher Kid Score D5, followed by those derived from cycles with medium FORT, and embryos from cycles with low FORT presented the lowest KID score (Table 6). This finding was confirmed by the regression analysis, which demonstrated a positive influence of the FORT on the KID score D5 (B: 0.208, IC: 0.050–0.366, p=0.01)

Significantly higher rates of blastocyst formation and implantation were observed in embryos derived from cycles with high FORT, followed by those with medium FORT, while embryos from cycles with low FORT presented the lowest blastocyst formation and implantation rates. However, no significant differences were noted in the fertilization, pregnancy, miscarriage, or livebirth rates when the low, medium, and high FORT groups were compared (**Table 6**).

Table 5. Comparison of morphokinetic parameters between the low, medium and high follicular output rate (FORT) groups

Variable	Low FORT	Medium FORT	B	95% CI	High FORT	B	95% CI	p value
n	2,556	2,970			2,850			
tPNa	7.1 ± 0.7[a]	6.6 ± 0.6 [b]	-0.540	-0.781 - -0.397	6.5 ± 0.0[c]	-0.589	-0.781 - -0.397	**< 0.001**
tPNf	24.6 ± 0.1	24.3 ± 10.3	-0.334	-0.631 - - 0.036	24.4 ± 0.1	-0.227	-0.527 – 0.073	0.084
t2	27.4 ± 0.1[a]	26.9 ± 0.1 [b]	-0.531	-0.851 - -0.0211	26.7 ± 0.1[c]	-01730	-0.408 - -0.211	**< 0.001**
t3	37.8 ± 0.1	37.6 ± 0.1	-0.256	-0.638 – 0.127	37.3 ± 0.1	-0.452	-0.837 - -0.066	0.071
t4	39.6 ± 0.1[a]	39.6 ± 0.1[b]	-0.049	-0.446 – 0.348	38.9 ± 0.1[c]	-0.681	-1.080 - -0.281	**< 0.001**
t5	50.3 ± 0.2[a]	50.3 ± 0.2[b]	-0.018	-0.571 – 0.535	49.7 ± 0.2[c]	-0.627	-1.184 - -0.070	**0.036**
t6	53.0 ± 0.2[a]	53.4 ± 0.2[a]	0.370	-0.201 – 0.940	52.5 ± 0.2[b]	-0.550	-1.124 – 0.024	**0.004**
t7	56.4 ± 0.2[a]	55.6 ± 0.2[b]	-0.768	-1.371 - -0.165	55.3 ± 0.2[c]	-0.273	-0.880 – 0.335	**0.001**
t8	60.1 ± 0.2[a]	59.2 ± 0.2[b]	-0.955	- 1.641 - -0.268	59.0 ± 02[c]	-0.183	-0.873 – 0.507	**< 0.001**
tM	89.5 ± 0.3	89.2 ± 0.2	-0.348	-1.097 – 0.402	88.6 ± 0.2	-0.863	-1.611 - -0.115	0.073
tSB	101.6 ± 0.3[a]	100.6 ± 0.2[b]	-0.942	-1.689 - -0.195	100.6 ± 0.2[b]	-1.001	-1.743 - -0.259	**0.014**
tB	110.3 ± 0.3	110.1 ± 0.3	0.181	-0.660 – 1.022	109.9 ± 0.3	-0.252	-1.089 – 0.585	0.574
s1	2.6 ± 0.0	2.6 ± 0.0	-0.023	-0.090 – 0.043	2.0 ± 0.0	-0.078	-0.145 - -0.011	0.063
s2	2.0 ± 0.0[a]	1.8 ± 0.8[a]	- 0.236	-0.445 - -0.027	1.6 ± 0.0[b]	-0.203	-0.413 – 0.008	**< 0.001**
s3	2.1 ± 0.7[a]	1.8 ± 0.0[b]	-0.925	-1.444 - -0.406	1.6 ± 0.7[c]	-0.403	-0.924 - 0.119	**0.002**
cc2	10.6 ± 0.9	10.7 ± 0.0	0.070	-0.171 – 0.310	10.7 ± 0.0	0.089	-0.154 – 0.331	0.756
cc3	12.6 ± 0.1	12.9 ± 0.1	0,239	-0.132 – 0.610	12.4 ± 0.1	-0.197	-0.571 – 0.176	0.057

Note: Generalized mixed models (GMM) adjusted for potential confounders, as maternal and paternal age, and female body mass index (BMI). P: Post-hoc Bonferroni. Values are means ± standard error, unless otherwise noted. H – hours, tPNa – timing to pronuclei appearance, tPNf – timing to pronuclei fading, t2 – timing to two cells, t3 – timing to three cells, t4 – timing to four cells, t5 – timing to five cells, t6 – timing to six cells, t7 – timing to seven cells, t8 – timing to eight cells, tSB – timing to start blastulation, tB – timing to blastulation, s1 – timing to complete t2-tPNf synchronous divisions, s2 – timing to complete t4-t3 synchronous divisions, s3 – timing to complete t8-t5 synchronous divisions, cc2 – duration of th

DISCUSSION

The existing markers for ovarian reserve, namely AFC and AMH, do not accurately forecast ovarian response, particularly for a specific group of patients. Despite presenting a normal ovarian reserve, hypo-responders present a lower ovarian sensitivity to FSH, leading to a lower ovarian response to stimulation (16). Other methods, such as the use of FOI[6] and FORT,[7] can identify hypo-responders.

Although a positive relationship between FOI and FORT and treatment success has been demonstrated,[17,25-27] whether these results are exclusively related to a decreased number of retrieved oocytes or to potential impacts on oocyte quality and embryo development remains unexplored. For the current research, we formulated a hypothesis that the FORT and FOI could potentially affect the speed of embryonic cell division in TLI incubators, a subtlety that would remain undetected through traditional morphological evaluation of embryos.

Our results demonstrate that decreasing FORT and FOI negatively impacts embryo morphokinetics by decreasing the time to achieve several of the investigated kinetic events.

Statistically significant associations between each FORT and FOI and the outcomes of KIDScore ranking, blastocyst formation, and implantation rates were also noted. Although we noticed a significant difference for several factors when the Bonferroni Post Hoc test compared the groups, the effect sizes in many cases were relatively

Table 6. Comparison of known implantation diagnosis (KID) score D5 and intracytoplasmic sperm injection (ICSI) outcomes between the low and high follicle-to-oocyte (FOI) index groups.

Variable	Low FORT	Medium FORT	High FORT	p value
Cycles	753	874	843	
Embryos	2,556	2,970	2,970	
Kid score	5.4 ± 0.5[a]	5.5 ± 0.5 [a,b]	5.6 ± 0.6 [b]	**0.021**
Fertilization rate (%)	63.7 ± 1.1	71.4 ± 1.1	73.3 ± 1.1	0.299
Blastocyst formation rate (%)	49.2 ± 1.4[a]	50.8 ± 1.4[a]	55.5 ± 1.4[b]	**< 0.001**
Implantation rate (%)	23.6 ± 0.4[a]	24.5 ± 0.4a	27.1 ± 0.5b	**< 0.001**
Pregnancy rate (%)	30.9 ± 4.4 (233/753)	35.9 ± 4.9 (314/874)	36.9 (311/843)	0.538
Miscarriage rate (%)	16.3 ± 3.45 (38/233)	16.2 ± 4.1 (51/314)	16.7 ± 3.9 (52/311)	0.457
Livebirth (%)	27.2 ± 2.1 (205/753)	30.2 ± 2.1 (264/874)	32.1 ± 2.2 (271/843)	0.345

Note: Generalized mixed models (GMM) adjusted for potential confounders, as maternal and paternal age, and female body mass index (BMI). P: Post-Hoc Bonferroni. Values are percentage ± standard error. unless otherwise noted. a ≠ b ≠ c. Effect size: *: B (continuous variables) and ** OR (binary variables), considering Low FOI as reference group.

small.[28-30] Even though the coefficients are small, it has been reported that slower embryos have lower implantation potential, and even minimal differences in the developmental speed may impact clinical outcomes.[31-33]

Both of these indices serve as a straightforward indication of the personal reaction to ovarian stimulation, and their relationship with poor clinical outcomes in patients with normal ovarian reserve can be easily explained by the low oocyte yield and therefore low number of embryos available for transfer. However, the reason why FORT and FOI impact embryo developmental competence quantitatively and qualitatively remains to be elucidated.

The complete understanding of the pathophysiological mechanisms behind hypo-response still needs to be discovered. It has been argued that a hypo-response may be associated with a genetic polymorphism in FSH and LH and their receptors. Indeed, A wide range of studies. is being conducted to understand the effects of polymorphic variants of gonadotropins and their receptor genes on reproductive health.[17,34,35]

Why these genetic variations may have detrimental effects on embryo developmental competence is unknown. One could argue that, although altered expression of certain genes in cumulus and granulosa cells, leading to a greater resistance to FSH, by itself may compromise follicle development, follicles that achieve a diameter that allows follicular aspiration may contain oocytes developed under gonadotropin deprivation, affecting their further development, as demonstrated here by the slower cell divisions, lower kid score, lower formation of blastocysts and lower implantation. Indeed, FSH is responsible for the proliferation, growth, and differentiation of granulosa cells. It also regulates the expression of oocyte-derived BMP-15 and GDF-9,[36] which are at play in controlling the glycolysis process and the production of cholesterol throughout follicular development, ultimately influencing crucial stages such as oocyte development, ovulation, fertilization, and embryonic competence.[37]

Another explanation, apart from the genotypic characteristic, involves an association between serum and intrafollicular presence of environmental contaminants and ovarian response to gonadotrophin stimulation. There is a hypothesis suggesting that certain pollutants may result in a lack of FSHR transduction, which in turn causes toxicity. In fact, Higher levels of baseline FSH were observed in women with increased intrafollicular benzene concentrations, compared to those with low intrafollicular benzene levels.[38,39] Since these pollutants are present in the follicular fluid, there are reasons to believe that they may also have a detrimental effect on oocyte quality and further embryo development.

Finally, there is some evidence suggesting that oxidative stress negatively affects the ovarian response to controlled ovarian stimulation. The administration of antioxidants prior to ovarian stimulation led to an improvement in the response to the gonadotrophin stimulus by increasing the number of retrieved oocytes and increasing the number of mature oocytes.[40] It is necessary to conduct further investigation into the role of oxidative stress in the pathogenesis of hypo-response. On the other hand, oocytes are situated within the ovarian follicle and subsist in a low-oxygen (hypoxic) environment, relying on the follicular fluid and cumulus cells to furnish them with oxygen.[41] The follicular fluid encompassing the oocyte serves as an antioxidant buffer to sustain the equilibrium of the redox state. At the physiological concentration hydrogen peroxide present in follicular fluid, appears to serve as an indicator for the optimal development of oocytes[42,43]; therefore, oxidative stress directly affects folliculogenesis[44-48] and influences the quality of oocytes and embryos.[49]

Our findings align with previous research that suggests that quantitative markers of ovarian reserve, such as the AMH level, may also be correlated with oocyte quality in stimulated cycles.[50(p2006)] Fanchin et al.[51] proposed a direct correlation between the capacity of granulosa cells to generate AMH and the functioning of oocytes. Moreover, in unpublished data, our group observed that increasing AMH levels positively impact embryo quality and morphokinetics. Positive correlations between AMH concentrations and KIDScore ranking, as well as with implantation rate, were also noted.

Here, we detected an influence of FORT and FOI as early as at tPNa (for FORT) and tPNf (for FOI), which progressed to a cumulative earlier development until tSB (for FORT) and tB (for FOI). In fact, it has been previously demonstrated that embryos with high blastulation and implantation potential cleave from the 2- to 8-cell stage progressively earlier than those with low potential.[52,53(p:@267188)]. Additionally, shorter t4, which here was positively influenced by FOI and FORT, has been previously correlated with euploidy.[54]

In the present study, despite a positive correlation between FORT and FOI and faster developmental kinetics, KID score D5 and implantation potential, there was no impact on clinical pregnancy outcome. In another recently published report FOI and FORT were also significantly related to the number of MII oocytes obtained but not cumulative clinical pregnancy.[55] This finding, together with the fact that despite the significant difference observed with the Bonferroni post hoc test, the effect size was relatively small for many variables, may suggest that the morphokinetic differences noted here are only numerically, but without biological relevance. Another hypothesis would be that the "non-selection" of embryos exhibiting aberrant morphokinetic development patterns may have positively impacted the quality of the implanted embryos, thereby increasing the pregnancy rate across all analyzed cohorts.

The retrospective nature limits our study. Other limitations are: the study was conducted in a single university-affiliated IVF center, which may not represent the broader population of patients undergoing IVF. Additionally, it is unknown whether significant impacts in clinical pregnancy were not noted because of the deselection of slow embryos or because there was no association between the variables at all. Therefore, a cautious interpretation is necessary. The results presented here contribute to the knowledge of the correlation between FORT and FOI and embryo morphokinetic development and provide a rationale for the culture of embryos of hypo-responder patients in TLI incubators.

Our data demonstrate that the hypo-response to COS goes beyond a reduced number of retrieved oocytes. Indeed, embryo morphokinetics appear to be influenced by the same factors that contribute to this diminished response. Significant positive relationships were observed between embryo development and FOI and FORT, which would not be detected in embryos cultured in conventional incubators. Together with existing literature, our findings emphasize the importance of alternative markers and advanced evaluation techniques as TLI system, especially in hypo-responders.

In conclusion, this study highlights the association between FORT and FOI and embryo morphokinetic development. FOI and FORT can serve as reliable predictors of embryo developmental potential in patients undergoing IVF It is possible that the deselection of slow embryos may have prevented an effect on clinical pregnancy, but additional research is needed to validate this assumption and also to further research is warranted to validate the clinical implications of FORT and FOI and their potential impact on IVF success rates and live birth outcomes.

CONFLICT OF INTEREST STATEMENT

The authors declare no financial or personal conflict of interest

AUTHOR CONTRIBUTIONS

Conceptualization: Daniela Braga (Lead). Methodology: Daniela Braga (Equal), Christina Morishima (Equal), Assumpto Iaconelli (Equal). Investigation: Daniela Braga (Equal), Amanda Setti (Equal), Assumpto Iaconelli (Equal). Writing – original draft: Daniela Braga (Lead). Formal Analysis: Amanda Setti (Lead). Software: Amanda Setti (Lead). Writing – review & editing: Amanda Setti (Equal), Christina Morishima (Equal), Assumpto Iaconelli (Equal), Edson Borges (Equal). Supervision: Edson Borges (Lead).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available and may be accessed if needed.

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