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ARTICLE

Morphokinetic characteristics of embryos derived from in-vitro-matured oocytes and their in-vivo-matured siblings after ovarian stimulation

BIOGRAPHY

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KEY MESSAGE

Embryos from in-vitro-matured metaphase I oocytes have a different morphokinetic profile from their sibling oocytes aspirated at the metaphase II stage after completing maturation *in-vivo*. This finding supports their low priority status for embryo transfer. The clinical significance should be prospectively studied in treatments when only such embryos are present.

ABSTRACT

Research question: Does delayed maturation of aspirated metaphase I (MI) oocytes, completed *in vitro*, adversely affect early embryo development?

Design: Time-lapse microscopy was used to compare morphokinetic variables between embryos derived from oocytes with delayed maturation after ovarian stimulation and from in-vivo-matured metaphase II (MII) sibling oocytes from the same IVF and intracytoplasmic sperm injection cycle.

Results: A total of 1545 injected oocytes in 169 cycles from 149 patients were included. The in-vitro-matured oocytes had lower normal fertilization rates than the MII aspirated oocytes (50.2% versus 68.0%; $P < 0.001$). Early key developmental events were significantly delayed in the normally fertilized in-vitro-matured compared with in-vivo-matured oocytes (polar body extrusion: 5.4 ± 3 versus 3.9 ± 1.8 h; $P < 0.001$; pronuclear fading: 27.2 ± 4.7 versus 25.1 ± 4.2 h; $P < 0.001$, respectively) and synchrony of the second cell cycle was adversely affected. The proportions of embryos with optimal second cell cycle length and second cell cycle were similar but with fewer top-quality embryos, based on an algorithm, for the delayed in-vitro-matured oocytes compared with their in-vivo-matured sibling oocytes (14% versus 29.1%; $P < 0.001$).

Conclusions: Embryos derived from oocytes that failed to mature *in-vivo* in standard treatment after ovarian stimulation may show a different morphokinetic profile from their sibling oocytes aspirated at the MII stage after completing maturation *in-vivo*.

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INTRODUCTION

Mammalian oocytes enter meiosis during fetal life and arrest in prophase I, which is characterized by the presence of a distinct germinal vesicle. After puberty and an initial growth phase, and in response to the mid-cycle pre-ovulatory surge of LH, the first meiotic division is reinitiated and culminates in the asymmetric division and extrusion of the first polar body. Maturation is achieved by the ensuing progression to the second division with subsequent arrest at metaphase II (MII). The evident nuclear maturation is accompanied by more elusive changes in the oocyte's cytoplasm, including modifications of the cytoskeleton and reorganization of molecules and organelles fundamental to normal fertilization and support of early embryonic development (*Coticchio et al., 2015*).

Ovarian stimulation IVF treatment protocols are designed to yield an adequate number of mature competent MII oocytes capable of being fertilized. Despite the use of LH surrogates to trigger final maturation, some of the retrieved oocytes are immature: they either retain an intact germinal vesicle or are at a transient metaphase I (MI) stage when neither a germinal vesicle nor a polar body is present. In stimulated cycles, up to an estimated 15% of aspirated oocytes are immature, about 10% are germinal vesicle and about 5% MI (*Rubino et al., 2016*). Those retrieved at the MI stage frequently complete meiotic maturation spontaneously *in vitro*, allowing for delayed intracytoplasmic sperm injection (ICSI). A high proportion of immature oocytes reduces the number of embryos available for selection before embryo transfer, and may even lead to its cancellation. Injecting MI oocytes that spontaneously mature to MII after a short incubation period may be of particular benefit in cases of no or few mature oocytes (*Lee et al., 2016*) as well as in cases of preimplantation genetic testing (PGT) when the eligible embryos are restricted to those that are mutation-free.

Reassuring clinical results have been reported for in-vitro maturation protocols (IVM-IVF) in which germinal vesicle stage oocytes are intentionally aspirated from small antral follicles after short and minimal gonadotrophic priming. In conventional stimulated cycles, however, the use of immature oocytes that have been stripped of cumulus cells in preparation for injection and then matured after in-vitro incubation (sometimes referred to as 'rescued immature oocytes') remains controversial (*Rubino et al., 2016*). Several studies have reported significantly reduced fertilization rates (*De Vos et al., 1999; Strassburger et al., 2004*), higher multinucleation rates (*De Vincentiis et al., 2013*), and lower quality embryos (*Vanhoutte et al., 2005*). Although normal fertilization and embryo development can be achieved, and sporadic pregnancies have been reported (*Lee et al., 2016*), it seems that these embryos should be used with caution as delayed maturation may be associated with an increased risk of aneuploidy (*Emery et al., 2005; Strassburger et al., 2010*).

All these studies were based on static morphological criteria evaluated at pre-defined time points. The recent introduction of time-lapse imaging (TLI) to the IVF laboratory, allowing continuous monitoring and recording, has prompted researchers to search for morphokinetic traits with the potential to predict embryo developmental competence (*Meseguer et al., 2011*). The aim of the present study was to apply this novel technology to assess the effect of delayed maturation on embryo development by comparing early morphokinetic variables and outcome of embryos derived from in-vitro and in-vivo-matured sibling oocytes in stimulated IVF and intracytoplasmic sperm injection (ICSI) cycles.

MATERIALS AND METHODS

Study population and design

We included all ICSI cycles carried out in the IVF unit of a tertiary medical centre between January 2015 and December 2016 in which at least one in-vitro-matured oocyte was injected and the embryos were imaged continuously by time lapse imaging (TLI) (EmbryoScope™, Vitrolife, Sweden). Morphokinetic characteristics of injected MI oocytes that matured *in-vitro* were compared with their sibling oocytes that were MII at time of denudation for ICSI. The study was approved by the local institutional review board (#0704-15 RMC, 16.12.2015).

Ovarian stimulation and oocyte aspiration

Gonadotrophin releasing hormone antagonist (Cetrotide, Merck or Orgalutran, MSD) or agonist (Decapeptyl, Ferring) were used for pituitary suppression and recombinant FSH (Gonal F, Merck or Puregon, MSD) or human menopausal gonadotrophin (Menopur, Ferring) were used for ovarian stimulation. Oocyte retrieval was conducted 36 h after final maturation was triggered with recombinant HCG (Ovitrelle, Merck), gonadotrophin releasing hormone agonist (Decapeptyl, Ferring), or both, when at least three follicles of 17 mm or wider in diameter were present.

ICSI and embryo culture

Oocytes were enzymatically denuded of cumulus cells using hyaluronidase (Irvine scientific, USA) 0.25–3 h after aspiration, and the mature oocytes were inseminated by ICSI. Immature oocytes were left in culture (LifeGlobal Media®, LifeGlobal, Brussels, Belgium or CSC™, Irvine Scientific, Santa Ana, CA, USA) and re-inspected at the end of the injection procedure and again multiple times before the end of the day, according to laboratory routines. As a rule, in-vitro-matured oocytes were injected when fewer than six mature oocytes were present, when the immaturity rate was over 40%, when sperm count or motility were inadequate for regular insemination (n = 108) and in all cases intended for polymerase chain reaction-based PGT (n = 61). Injected oocytes were incubated individually in pre-equilibrated culture medium (Global, LifeGlobal Europe, Brussels Belgium; CSC-C Irvine Scientific, Santa Ana, CA 92705 USA). All sibling oocytes were cultured in the same media in EmbryoSlide® culture dishes (Vitrolife, Sweden), and covered with mineral oil (Irvine Scientific, USA) in an atmosphere of 5.0% O_2, 5.6%CO_2, balanced with nitrogen.

Morphokinetic assessment

Fertilization rates and morphokinetic parameters were compared between 285 MI oocytes and their 1260 sibling oocytes aspirated at the MII stage in the same cycles. The precise timing of second polar body extrusion, pronuclear fading, first cleavage to two cells (t2), and divisions up to the eight-cell stage (t8) were annotated, and the duration from pronuclear fading to the first cleavage

and second cell cycle (CC2 = t3–t2) was calculated as well as the synchrony of the second cleavage cycle (S2 = t4–t3). Optimal CC2 was defined as 5 h or more, and the optimal S2 as 1 h or less (*Meseguer et al., 2011*). As annotation times are automatically calculated from the time of insemination entered for each slide, individual measurements were re-calculated for delayed injections of the in-vitro-matured oocytes. Later, cell divisions were not included in this analysis as they were influenced by blastomere biopsy in the PGT cycles. In most non-PGT cases, embryo transfer and vitrification were conducted on day 3; therefore, the data on later divisions and blastulation in these treatments was too small for analysis.

Embryo grading

Embryos were graded using the KIDScore™ model, which is embedded in the annotation software and offered as a tool for deselection of embryos showing erratic morphokinetic patterns. The model is based on cumulative known clinical outcomes from a large number of IVF centres (*Petersen et al., 2016*). Because it requires day-3 annotations, embryos transferred, vitrified or discarded on day 2 were excluded from this comparison. KIDScore was used as a standardized scale (values ranging from 1 to 5) to assess the effect of in-vitro maturation on cleavage patterns as it uses pronuclear fading and not insemination as starting time point. The suitability of embryos for transfer and vitrification or biopsy (in PGT cycles), which also relied on an in-house selection grading system, based on the Istanbul consensus workshop on embryo assessment (Alpha Scientists in Reproductive Medicine *et al*., *2011*), served as an integrative indicator of adequate embryo quality.

Statistical analysis

SPSS v21.0 package (IBM Corp., USA) was used for statistical analysis. Univariate analysis included chi-squared test for categorical variables and Student t-tests for continuous variables. $P < 0.05$ was considered statistically significant.

RESULTS

A total of 1545 injected oocytes in 169 cycles from 149 patients were included. Demographic and treatment characteristics were similar among the two groups as the groups were sibling oocytes derived from the same patients. The mean age of the study group was 34.4 ± 5.3 years (range: 22–43.3 years); the mean body mass index (BMI) was 25.9 ± 5.1 (range: 17.2–39.2). Forty-one per cent of the study population (61/149) had primary infertility and, in 27.5% of the patients, this was their first IVF cycle.

Oocytes aspirated at the MI stage that matured *in-vitro* were injected 3.4 ± 1.4 h (range: 1–6.8 h) later than their sibling oocytes and added to the culture slide. In-vitro-matured oocytes had a lower normal (two procnuclei [2PN]) fertilization rate than MII-aspirated oocytes (50.2% versus 68.0%; $P < 0.001$), with similar aberrant fertilization rates (one pronucleus or three pronuclei) (TABLE 1). A single pronucleus was detected in 13 (4.6%) versus 53 (4.2%); polyploidy (three pronuclei, presumably resulting from retention of the second polar body) were detected in 12 (4.2%) versus 33 (2.6%) in oocytes that were MI and sibling MII at denudation, respectively (NS).

Analysis of the early key developmental events revealed a significant delay in polar body extrusion ($P < 0.001$), pronuclear fading ($P < 0.001$), t2 ($P < 0.001$), t3 ($P < 0.001$), t4 ($P < 0.001$), t7 ($P = 0.023$), and t8 ($P = 0.016$), with a longer S2 ($P = 0.031$), in embryos developing from normally fertilized in-vitro- compared with sibling in-vivo-matured oocytes, respectively (TABLE 2). The timing of t5 and t6 was not significantly different between the in-vitro and in-vivo-matured oocytes.

Where it could be calculated, the proportion of embryos with optimal CC2 (≥5 h) was 71.8% (102/142) of the in-vitro compared with 76.0% (654/860) of the in-vivo-matured oocytes. Corresponding rates of optimal S2 (≤1 h) were 44.9% (61/136) and 48.2% (404/838). Although these differences were not statistically significant, the proportions of top (KidScore grade 5) and good grade embryos (KidScores grade 5 + grade 4) were significantly higher for the in-vivo-matured oocytes (both $P < 0.001$) (TABLE 3).

In the 108 non-PGT cycles, only 25.6% (45/176) of the injected in-vitro-matured oocytes resulted in embryos eligible for transfer or vitrification, compared with 42.5% (303/713) of the MII oocytes ($P < 0.001$). In the 61 PGT cycles, 41.3% (45/109) of the injected in-vitro-matured and 61.4% (336/547) ($P < 0.001$) of the in-vivo-matured oocytes developed to embryos suitable for biopsy. Currently, our data are not sufficient for comparing pregnancy rates.

DISCUSSION

The underlying causes leading to the failure of some oocytes to mature before follicular aspiration in IVF-stimulated cycles is unclear. It may involve a heterogeneous oocyte quality within a cohort arising from suboptimal cycle synchronization, aspiration of small follicles, diverse follicular microenvironments or intrinsic oocyte factors.

Adequate maturation requires nuclear and cytoplasmic reorganization in the oocyte (*Coticchio et al., 2015*) as well as in the accompanying somatic cumulus cells (*Ouandaogo et al., 2012*). These essential processes may not be imitated when oocytes that failed to mature in-vivo undergo in-vitro maturation. In the present study, oocytes maturing *in vitro* from MI to MII stage were injected 3.4 ± 1.4 h later than their sibling oocytes

TABLE 1 FERTILIZATION RESULTS FOR IN-VITRO- AND IN-VIVO- MATURED OOCYTES

Fertilization result	In-vitro-matured oocytes (MI) (*n* = 285), *n* (%)	In-vivo-matured oocytes (MII) (*n* = 1260), *n* (%)	*P*-value
Normal fertilization (2PN)	143 (50.2)	857 (68.0)	<0.001
No fertilization (0PN),	117 (41.1)	317 (25.2)	<0.001
Aberrant fertilization (1PN + 3PN)	25 (8.8)	86 (6.8)	NS

MI, metaphase I; MII, metaphase II; PN, pronucleus.

TABLE 2 ANNOTATED TIMING OF EARLY DEVELOPMENTAL STAGES IN IN-VITRO- AND IN-VIVO- MATURED OOCYTES

Developmental stage	In-vitro-matured oocytes (MI) (*n* = 285)	In-vivo-matured oocytes (MII) (*n* = 1260)	*P*-value
Polar body extrusion	5.4 ± 3.0	3.9 ± 1.8	<0.001
Pronuclear fading	27.2 ± 4.7	25.1 ± 4.2	<0.001
t2	30.1 ± 6.3	28.2 ±5.8	<0.001
t3	38.9 ± 7.2	37.3 ± 6.5	<0.001
t4	42.8 ± 7.5	40.3 ± 6.5	<0.001
t5	49.7 ± 10.1	49.0 ± 8.3	NS
t6	54.3 ± 9.8	53.1 ± 8.7	NS
t7	58.9 ± 11.7	56.4 ± 8.0	0.023
t8	63.2 ± 10.7	60.7 ± 10.3	0.016
Time from pronuclear fading to t2	2.9 ± 3.7	3.0 ± 3.6	NS
CC2 (t3–t2)	9.2 ± 5.7	9.5±5.1	NS
S2 (t4–t3)	4.1 ± 5.2	3.1 ± 4.8	0.031

Data are presented as mean ± SD. MI, metaphase I; MII, metaphase II; CC2, duration of second cell cycle; S2, synchrony of second cycle divisions.

TABLE 3 KIDSCORE GRADING OF EMBRYOS DERIVED FROM IN-VITRO- AND IN-VIVO-MATURED OOCYTES

KIDScore embryo quality grading	In-vitro-matured oocytes (MI) (*n* = 114)[a], *n* (%)	In-vivo-matured oocytes (MII) (*n* = 745)[a], *n* (%)	*P*-value
Good (grades 4 + 5)	37 (32.5)	378 (50.7)	<0.001
Moderate (grade 3)	12 (10.5)	57 (7.7)	
Poor (grades 1+2)	65 (57)	310 (41.6)	
Top (grade 5 only)	16 (14)	217 (29.1)	<0.001 OR = 2.28 95% CI 1.3 to 3.7

[a] Missing values are due to failure of fertilization, cleavage arrest, or day-2 transfer and vitrification. MI, metaphase; MII, metaphase II.

and added to the culture slide. Our results show that these delayed in-vitro-matured oocytes had lower normal (2PN) fertilization rates than the MII-aspirated oocytes (50.2% versus 68.0%; $P < 0.001$) and that early key developmental events in the first days in culture: second polar body extrusion, pronuclear fading, and timing of first cell-cycle mitotic divisions t2, t3, t4, t7 and t8) were significantly delayed. Although the timing of t5 and t6 was not significantly different between the in-vitro- and in-vivo-matured oocytes (possibly owing to the size and the large variance in both groups), a significant between-group difference in the universal clinically based KIDScore grades that are calculated by optimal cycle durations were observed. The proportion of high-quality embryos was lower and, most importantly, the eligibility of embryos for transfer or vitrification was compromised among embryos derived from in-vitro-matured oocytes compared with embryos derived from their sibling in-vivo-matured oocytes.

A previous study in in-vivo-matured cycles (not in standard ovarian stimulation), which compared morphokinetic characteristics of embryos developing from oocytes surrounded by an expanded cumulus (presumed to be mature) and oocytes enclosed in a compact cumulus (presumed to be at the germinal vesicle stage) found them comparable (*Dal Canto et al., 2016*). In these cases, all oocytes had been cultured encompassed in their cumuli for either 6 or 30 h before denudation. The preserved interaction with the cumulus cells in an appropriate maturing medium (containing hormones) is known to be essential for the development of competent oocytes.

In standard IVF after ovarian stimulation, however, immature oocytes are identified as such only after enzymatic and mechanical removal of cumulus cells and their maturation is expected to be less regulated. In IVM, normally fertilized oocytes showed similar morphokinetic development (*Walls et al., 2015; Dal Canto et al., 2016*); however, in the present study, we show that in-vitro-matured oocytes in regular ICSI cycles significantly differ from their sibling oocytes, at least in the early stages of embryo development.

The common practice of injecting oocytes that attain maturation *in vitro* after a short post-denudation incubation period is considered beneficial especially when few MII oocytes are present or none are available (*Lee et al., 2016*), when treating low responders and in PGT cycles in which a small number of embryos is available. Nevertheless, the available data on live births are scarce as the selection of these embryos is usually avoided or they are co-transferred with embryos from initially mature oocytes (*Rubino et al., 2016*). The use of time-lapse monitoring to estimate the risk of aneuploidy has been suggested by some researchers (*Campbell et al., 2013*) but strongly disputed by others. Therefore, the major concern regarding the chromosomal integrity of these embryos (*Emery et al., 2005; Strassburger et al., 2010*) can currently be addressed only by comprehensive chromosomal analysis.

Our study, using TLI for precise morphokinetic timing, suggests that failure of MI oocytes to complete maturation *in-vivo* leads to a disrupted time frame of early embryo developmental stages after ICSI even if carried out shortly after aspiration. We cannot rule out the possibility that some MII oocytes in our study were MI at aspiration and matured before denudation. Moreover, nuclear and cytoplasmic maturation can be asynchronous (*Coticchio et al., 2015*), which may lead to the injection of developmentally incompetent albeit nuclear mature oocytes in IVF cycles. Yet in both these scenarios, these oocytes have probably attained nuclear maturation in a more physiological manner owing to intact cumulus–oocyte interaction. Tests for cytoplasmic maturation are not available in clinical settings and it was not evaluated in the present study.

In the present retrospective study, the immature oocytes were not incubated in the EmbryoScope and we were, therefore, unable to determine exactly the time between first polar body extrusion and sperm injection, and cannot rule out a negative effect of premature ICSI (*Balakier et al., 2004*) or of the culture conditions up to sperm injection.

In conclusion, our data support the low priority status of embryos derived from delayed maturation after ovarian stimulation. Annotating the dynamic development of these embryos may be useful when a limited number of embryos are available to select from. The clinical significance of our findings should be prospectively studied in treatments in which only such embryos are transferred.

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