Zygote

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Does rescue *in vitro* maturation of germinal vesicle stage oocytes impair embryo morphokinetics development?

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Research Article

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Summary

Currently, rescue *in vitro* maturation (IVM) is not a routine method in assisted reproductive treatment (ART) programmes but is a promising procedure for ART to improve IVM. The aim of this study was to compare embryo morphokinetics of germinal vesicles (GV) with metaphase II (MII) oocytes from controlled ovarian hyperstimulation (COH) cycles by time-lapse photography monitoring (TLM). Morphokinetics of the same number of embryos derived from the *in vivo* (group I) and rescue of *in vitro* matured oocytes (group II) from 310 patients were analyzed and compared retrospectively. The time to form second PB extrusion (tPB2), time of pronuclei appearance (tPNa), time of pronuclei fading (tPNf) and time of two to eight discrete cells (t2–t8) were assessed. Abnormal cleavage patterns such as uneven blastomeres at the two-cell stage, cell fusion (Fu), trichotomous mitoses (TM), and the rates of embryo arrest were assessed. These data showed that tPB2, tPNa, tPNf, t2, t3 and t4 stages took place later in group II compared with group I ($P < 0.001$, $P = 0.017$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, respectively). The rates of uneven blastomeres, Fu, TM, and embryo arrest were increased significantly in group II compared with group I ($P = 0.001$, $P < 0.001$, $P = 0.003$, $P < 0.001$, respectively). Based on the exact annotation of timing parameters and cleavage patterns, the present data agreed with the concept that rescue IVM of oocytes negatively influences embryo morphokinetics. Therefore, cautious use of embryos derived from rescue IVM of GV oocytes should be made.

Introduction

Following controlled ovarian hyperstimulation (COH), approximately 20% of oocytes are immature (Ashourzadeh *et al.*, 2015). These oocytes are usually discarded in the majority of the IVF programmes (Lee *et al.*, 2016). However, rescue *in vitro* maturation (IVM) of the immature oocytes can increase embryo quantity, particularly in poor responder women (Omidi *et al.*, 2014). In these patients, immature oocytes can be a source of urgently required extra embryos, although their implantation and pregnancy outcomes are considered low (Shin *et al.*, 2013). IVM is a well known technique in ART, especially in women with polycystic ovary syndrome (PCOS) or who are at high risk of ovarian hyperstimulation syndrome (OHSS) during COH (Omidi *et al.*, 2013). Nevertheless, IVM is carried out in only a few ART centres, as it entails intensive work with low clinical outcomes. Over recent years there have been extensive studies undertaken to improve outcomes (Guzman *et al.*, 2013). As rescue IVM has only limited expectation for success, this type of procedure has not been widely used in common clinical practice (Lee *et al.*, 2016).

The developmental potential of *in vitro* matured oocytes is controversial. Possible detrimental developmental competence in IVM oocytes may be affected by IVM conditions, which do not exactly mimic *in vivo* conditions. Chromosomal segregation fidelity or essential cytoplasmic changes occur during germinal vesicle (GV) to MII stage oocyte transition (Coticchio *et al.*, 2013) (Smitz *et al.*, 2011). Full imaging of the developmental competence of the embryos derived from IVM oocytes is an important task to ascertain feasible ways to improve the IVM technique as a clinical approach. It is clear that the final proof of oocyte developmental competence is its ability to develop a viable pregnancy (Dal Canto *et al.*, 2016).

Over recent decades, the competence of embryo development has been commonly assessed by static conventional morphological criteria including blastomere number and symmetry, anucleated fragmentation rate, multinucleation, etc. These morphological evaluations of

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embryos are limited at daily discrete time points (Faramarzi *et al.*, 2017c). Conventional morphological assessments have provided limited information about how embryos are generated from conventional IVM and rescue IVM oocytes (Son *et al.*, 2008, Lee *et al.*, 2016). However, non-invasive time-lapse monitoring (TLM) using timing of embryo developmental events and imaging of dynamic morphology has provided another dimension to complement conventional morphological assessment (Meseguer *et al.*, 2011; Faramarzi *et al.*, 2015).

To the best of our knowledge, there are only limited amounts of literature that report morphokinetics of embryos generated following rescue IVM in a clinical setting. The assessment of embryo developmental competency derived from rescue IVM may be important for poor responder women. It is possible that MII oocytes derived from rescue IVM lead to embryos with different developmental potential, which can be assessed by the TLM system. Therefore, the aim of this study was to compare the cleavage kinetics and abnormal phenotypic events of embryos derived from *in vivo*-matured with rescue IVM from the same patients using TLM.

Materials and methods

Study design

In this retrospective study, 620 GV oocytes and 315 MII oocytes were included from 310 infertile patients (29 ± 5 years) undergoing oocyte retrieval for ICSI at the Yazd institute for reproductive sciences. The couples were referred to the ICSI programme due to male factor aetiology. Only women less than 38 years old with basal follicle-stimulating hormone (FSH) <10 IU/ml and at least six mature oocytes were included. Exclusion criteria were women with endometriosis and polycystic ovaries as well as couples with sperm concentrations of less than 1×10^6/ml, frozen or surgically retrieved spermatozoa. The study population included patients who had both mature (MII) and immature (GV) oocytes. In addition, the same numbers of embryos derived from *in vivo* (group I) and *in vitro* matured (group II) oocytes from each patient were included in this study to diminish possible couple specific biases. According to the normal fertilized *in vitro* matured GV oocytes, the numbers of normal fertilized *in vivo* matured oocytes were selected from each patient. Also, normal fertilized *in vivo* matured oocytes were selected randomly.

Ovarian stimulation

The patients were stimulated with the standard GnRH antagonist protocols. In the antagonist protocol, 150 IU/day of follicle-stimulating hormone (Gonal F, Serono, Geneva, Switzerland) was administered on day 2 of the menstrual cycle. When at least one follicle reached 14 mm, 0.25 mg of a GnRH antagonist (Cetrotide, Merck Serono, Darmstadt, Germany) was administered and continued until the day of human chorionic gonadotropin (hCG) injection. When at least two 17-mm follicles were seen on transvaginal ultrasound, recombinant hCG (rhCG; Merck Serono, Darmstadt, Germany) was injected to trigger final maturation and ovulation. 36 h later, ultrasound-guided oocyte collection was done using a single lumen aspiration needle (Wallace; Smiths Medical International, UK).

Oocyte preparation, rescue IVM and injection

Approximately 2–3 h after retrieval, the cumulus–oocyte complexes (COCs) were denuded of cumulus cells by exposure to HEPES-buffered medium containing 80 IU/ml hyaluronidase (Irvine Scientific, CA, USA), and by pipetting the COCs with a Pasteur pipette. Nuclear maturity of the denuded oocytes was checked. MII oocytes were injected and GV oocytes were cultured in blastocyst medium (G2; Vitrolife Co., Sweden) supplemented with 75 IU/l of human menopausal gonadotropin (Ferring) in a triple gas incubator at 5% O_2 and 6% CO_2 and 89% N_2. The GV oocytes were assessed for maturity after 24 h of IVM programme. Only 24 h matured oocytes were included in the study and injected using the partner's spermatozoa.

Embryo culture

After injection, the oocytes were individually placed in a culture slide containing pre-equilibrated G-1 medium (Vitrolife, Sweden) covered with sterile mineral oil and cultured in the time-lapse microscope (Primo Vision Time-lapse Embryo Monitoring System, Goteborg, Sweden) in a triple gas incubator. Injected oocytes were cultured under a time-lapse microscope immediately after ICSI for 3 days.

Time-lapse analysis

The images of each embryo were taken in seven focal planes every 10 min. Only embryos with normal fertilization (presence of two pronuclei) were included in the study. All annotations were done by one embryologist. Embryo morphokinetics, including time of second PB extrusion (tPB2), time of pronuclei appearance (tPNa), time of pronuclei fading (tPNf), time of two to eight discrete cells (t2–t8) were assessed. In addition, abnormal cleavage patterns such as: (a) uneven blastomeres embryos at two cells stage (more than one third different from sibling blastomeres in size was described uneven) (Ciray *et al.*, 2014); (b) cell fusion (Fu), in which fusion or merging of blastomeres entails reduction in embryo cells number, which gives the reverse cleavage appearance to embryos or a blastomere reabsorbed after cleavage; or (c) trichotomous mitoses (TM), in which a single blastomere divided directly from 1 ± 3 cells. Also, the rate of embryo arrest was monitored.

Statistics

Results are expressed as mean ± standard deviation (SD) for normal numeric variables, median ± interquartile range and percentage for categorical variables. The means of samples with normal distribution and of sufficient size were compared by independent samples parametric test (independent samples t-Test). For non-normal distribution, the medians were compared by Mann–Whitney *U*-test non-parametric test. Categorical variables were compared using chi-squared test. *P*-values <0.05 was considered as significant. Statistical analysis was performed using the Statistical Package for the Social Sciences 20 (SPSS Inc., Chicago, IL, USA).

Results

Out of 620 GV oocytes, 411 (66.29%) were matured during 24 h, and underwent ICSI. Table 1 shows the characteristics of oocytes and derived embryos. In total, 218 (53.04%) oocytes were fertilized normally (group II). Also, of the 315 MII oocytes that were injected, 218 (69.20%) were fertilized normally (group I). Immature oocyte selection was based on GV oocytes that were matured up to 24 h, as well as normally fertilized ones. Some of

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the normally fertilized oocytes were excluded from our analysis due to the early developmental arrest or ample fragmentation at different stages of development. The embryos derived from *in vivo* matured oocytes according to other embryos and patients conditions were either transferred or cryopreserved. All embryos derived from IVM oocytes were cryopreserved.

Time-lapse analysis showed that some early developmental events including tPB2, tPNa, tPNF, t2, t3 and t4 occurred significantly later in group II than in group I ($P < 0.05$; Table 2). But, no differences were observed between timings of t5, t6, t7 and t8 ($P > 0.05$).

There was significant difference regarding uneven blastomeres between group II and group I. As shown in Table 3, group I had a lower rate of uneven blastomeres when compared with group II (5.5% vs 16.1%, respectively) ($P = 0.001$).

There was significant difference regarding cell fusion (Fu) between groups I and II. As stated in Table 4, embryos within group II had higher rate of Fu when compared with group I (51.6% vs 27.8%, respectively) ($P < 0.001$).

There was significant difference regarding trichotomous mitosis (TM) embryos between groups I and II. As mentioned in Table 5, group I had lower rate of TM compared with embryos derived from rescue IVM (14.0% vs 25.7% respectively) ($P = 0.003$).

There were significant differences in the rates of arrest between embryos derived from group I and group II (Table 6). Group II had higher rates of arrest when compared with group I (31.7%% vs 5.5% respectively) ($P < 0.001$).

Table 1. Summary of the characteristics of GV and MII oocytes and derived embryos

Characteristics	Number (%)
No. of cycles	310
No. of retrieved oocytes	4132
No. of GV oocytes	620
No. of MI oocytes	206
No. of MII oocytes	3299
No. of degenerated oocytes	7
GV maturation rate in 24 h	411 (66.29%)
Fertilization rate of GV	218 (53.04%)
Fertilization rate of MII oocytes	2283 (69.20%)
No. of embryos derived from GV oocytes (2 cells)	198
No. of embryos derived from MII oocytes (2 cells)	216
No. of embryos derived from GV oocytes (3 cells)	172
No. of embryos derived from MII oocytes (3 cells)	214
No. of embryos derived from GV oocytes (4 cells)	171
No. of embryos derived from MII oocytes (4 cells)	214
No. of embryos derived from GV oocytes (5 cells)	169
No. of embryos derived from MII oocytes (5cells)	213
No. of embryos derived from GV oocytes (6 cells)	165
No. of embryos derived from MII oocytes (6 cells)	206
No. of embryos derived from GV stage of oocytes (7 cells)	158
No. of embryos derived from MII oocytes (7 cells)	206
No. of embryos derived from GV oocytes (8 cells)	149
No. of embryos derived from MII oocytes (8 cells)	206

GV, germinal vesicle; MII, metaphase II.

Discussion

Despite optimizing the COH protocols, nearly 20% of the retrieved oocytes remain immature at the GV or MI stages (Ashourzadeh *et al.*, 2015). Such immature eggs are considered unsuitable for clinical use and they are commonly discarded from the IVF programme (Escrich *et al.*, 2011). A good source of embryos can originate from immature oocytes that undergo IVM procedure for poor responders. However, these embryos are generally involved with poor clinical outcomes (Shin *et al.*, 2013). To our knowledge, this is the first study to compare the morphokinetics of the embryos derived from rescue IVM and *in vivo* matured oocytes in a ICSI setting. We found that morphokinetics timings, including tPB2, tPNa, tPNf, t2, t3 and t4, occurred later in embryos generated from rescue IVM oocytes.

Our data are in contrast with the previous studies reported by Walls *et al.* (2015). Dal Canto *et al.* (2016) and Roesner *et al.* (2017). Both the Wall and Dal Canto groups did not report any

Table 2. Comparisons of cleavage timings in groups I and II

Morphokinetics timing	Group I	Group II	*P*-value
tPB2* (h)	2.21 ± 0.41; N = 218	2.35 ± 0.31; N = 218	< 0.001**
tPNa* (h)	7.21 ± 2.37; N = 218	8.25 ± 3.15; N = 218	0.017**
tPNf* (h)	22.34 ± 4.16; N = 217	24.38 ± 5.00; N = 218	< 0.001**
t2* (h)	23.43 ± 3.06; N = 216	24.58 ± 2.46; N = 198	< 0.001**
t3# (h)	35.38 ± 9.01; N = 214	34.58 ± 9.39; N = 172	< 0.001**
t4* (h)	40.06 ± 4.51; N = 214	42.37 ± 3.09; N = 171	< 0.001**
t5# (h)	47.42 ± 6.19; N = 213	48.06 ± 6.00; N = 169	0.28
t6# (h)	51.25 ± 5.39; N = 206	51.04 ± 5.01; N = 165	0.88
t7# (h)	53.06 ± 6.14; N = 206	54.14 ± 6.18; N = 158	0.74
t8* (h)	62.46 ± 11.32; N = 206	62.00 ± 11.58; N = 149	0.12

Results are expressed as mean ± standard deviation (SD) for normal numeric variables, median ± Interquartile range and percentage for categorical variables. 2nd PB extrusion (tPB2), pronuclei (PN) appearance (PNA), PN fading (PNF), t2 = first cleavage (2-cell stage); t3 = second cleavage (3-cell stage); t4 = 4-cell stage; t5 = 5-cell stage; t6 = 6-cell stage; t7 = 7-cell stage t8 = 8- cell stage.
Data were presented as *: median ± IQ.
Data were presented as #: mean ± SD.

Table 3. Comparisons of uneven blastomeres between groups I and II

	Uneven blastomeres			
Embryos	Yes	No	OR (95% CI)	*P*-value
Group I	12 (5.5%)	205 (94.0%)	3.01 (1.54–5.87)	0.001
Group II	35 (16.1%)	183 (83.9%)		

CI, confidence interval; OR, odds ratio.

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Table 4. Comparisons of cell fusion (Fu) between group I and group II

	Cell fusion			
Embryos	Yes	No	OR (95% CI)	*P*-value
Group I	60 (27.8%)	156 (72.2%)	2.77 (1.86–4.14)	< 0.001
Group II	111 (51.6%)	104 (48.4%)		

OR, odds ratio; CI, confidence interval.

Table 5. Comparison of rate of trichotomous mitosis (TM) embryos between groups I and II

	TM			
Embryos	Yes	No	OR (95% CI)	*P*-value
Group I	30 (14.0%)	185 (86.0%)	2.13 (1.30–3.50)	0.003
Group II	54 (25.7%)	156 (74.3%)		

CI, confidence interval; OR, odds ratio.

Table 6. Comparisons of the rates of arrest between embryos group I and group II

	Arrest			
Embryos	Yes	No	OR (95% CI)	*P*-value
Group I	12 (5.5%)	206 (94.5%)	7.95 (4.15–15.20)	<0.001
Group II	69 (31.7%)	149 (68.3%)		

CI, confidence interval, OR, odds ratio.

differences in morphokinetics time points between embryos derived from *in vitro* or *in vivo* matured oocytes (Walls *et al.*, 2015; Dal Canto *et al.*, 2016). Also, Roesner and colleagues showed that there were no differences in the tPB2, tPNf, t2, t3, t4 and t5 time points. However, they reported that tPNA was shorter and t6, t7 and t8 were longer in the embryos derived from *in vitro* matured oocytes compared with the *in vivo* matured oocytes (Roesner *et al.*, 2017). These results do not concur with our findings and were mainly due to differences in the sources of the oocytes, e.g. unstimulated vs. stimulated cycles. Also, Dal Canto and colleagues used IVM with the surrounding cumulus cells, but we used denuded oocytes. However, our data are somewhat in accordance with Kim and colleagues (2016), who observed longer morphokinetic timing (time point) from tPB2 to t8 in the embryos developed from MI oocytes after COH (Kim *et al.*, 2016). Several have studies reported that cleavage kinetic timings may predict the outcome of embryo development (Meseguer *et al.*, 2011; Hashimoto *et al.*, 2012). Also, Dal Canto's group showed that embryos with high competence were related to the earlier time point of cleavage during the first three rounds of mitosis divisions (Dal Canto *et al.*, 2012).

Oocyte maturation can be one of the determining factors for cleavage timing (Gardner *et al.*, 2012). The reasons for these delays in cleavage timings may be related to the inadequate maturation of IVM oocytes or poor quality immature oocytes from COH cycles (Kim *et al.*, 2000). Oocytes undergoing IVM protocols may not synchronize orchestrated nuclear and cytoplasmic maturations as probably *in vitro* conditions may not be able to completely mimic intrafollicular conditions. Conventional assessment shows no sign of cytoplasmic maturation. Unlike nuclear maturation, this is determined by first PB extrusion. Poor outcomes of IVM were mostly related to poor cytoplasmic maturity (Liu *et al.*, 2010). However, diminished developmental potential can be associated with post-meiotic chromosomal errors that have been induced by oocyte cytoplasmic immaturity (Álvarez *et al.*, 2013; Shin *et al.*, 2013). In addition, rescue IVM changes the mitochondria distribution (Liu *et al.*, 2010) and morphology of oocytes (Shahedi *et al.*, 2013) that may be potential factors for diminished development.

In addition, this study demonstrated no differences between t5, t6, t7 and t8 in *in vivo* or *in vitro* matured oocytes that were normally fertilized. However, the maternal genome controls the early stages of embryo development. Embryonic genome activation (EGA) is vital for late embryo development. Furthermore, decay of maternal RNAs starts after fertilization. EGA starts at the end of the second division that dominates during third cytokinesis (Wong *et al.*, 2010). Therefore, rescue IVM treatment may influence embryo development during this transition and before EGA completion. The genome of embryos after activation at the end of the third cleavage could affect timing of cleavage, so reverting these to normal status.

Moreover, we found that the rates of uneven blastomeres, Fu and TM embryos were significantly increased in embryos derived from rescue IVM oocytes, compared with in vivo matured oocytes. Also, the rate of arrest increased in the embryos derived from rescue IVM oocytes when compared with *in vivo* matured oocytes. Concurred with our data, Walls and teammates showed that PCOS-IVM group showed significantly more uneven two cells embryos, when compared with the control-ICSI group (Walls *et al.*, 2015). Aneuploidies and genetic disorders are possible causes for embryos to fail from normal cleavage pattern (Faramarzi *et al.*, 2018). So, it could be a strategy to properly not selecting the poor rescue IVM embryos. Further, it has been indicated that multiple aneuploidies are in the majority of embryos that do not develop normal cell divisions timing (Meseguer *et al.*, 2011). Several previous studies showed that cleavage pattern, such as embryos with uneven blastomere size at 2-cell stage, direct cleavage, reverse cleavage that resulted in the lower developmental competence as well as implantation potential (Rubio *et al.*, 2012) (Meseguer *et al.*, 2011) (Liu *et al.*, 2014). The molecular mechanisms that entail the abnormal cleavage pattern are not obvious. Yet, it is assumed that mitotic fault may play a major role in these events (Almagor *et al.*, 2015). In our recent works, it was shown that oocyte quality can affect the embryo cleavage patterns in clinical programme (Faramarzi *et al.*, 2017b) (Faramarzi *et al.*, 2017a, Faramarzi *et al.*, 2017d). As, quality of the rescue IVM oocytes was lower when compared with *in vivo* matured oocytes, it may be one of the reasons for increased abnormal cleavage patterns and arrest.

This is the first study that evaluated the development of embryos derived from *in vitro* matured GV oocytes obtained from COH by TLM. The limitations of this study were the small number of data sets, and lack of information on blastocyst development and clinical outcomes. However, future larger studies are needed to validate the present findings. Based on the exact annotation of timing parameters and cleavage patterns made possible by TLM, the present data agree with the concept that rescue IVM GV stage oocytes negatively influence embryo morphokinetics as well as increase embryo arrest. Therefore,

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cautious use of embryos derived from rescue IVM of GV oocytes should be made. Additional studies for improvement of the IVM medium in order to enhance overall embryo development and implantation rates are needed. It is suggested that IVM of MI stage oocytes may improve the generated embryo development.

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Ethical standards. This study was approved by Ethics Committee of our institution.

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