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Fertilization and early embryonic development of *in vitro* matured metaphase I oocytes in patients with unexpected low oocyte maturity rate

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Research Article

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Summary

To determine the fertilization and embryonic potential of immature metaphase I (MI) oocytes in patients with low oocyte maturity rate in whom the percentage of mature oocytes obtained was less than 75% of the total retrieved ones. *In vivo* matured metaphase II (MII) oocytes (MII-ICSI, $n = 244$), and *in vitro* matured MI oocytes (MI-MII-ICSI, $n = 202$) underwent an intracytoplasmic sperm injection (ICSI) procedure. Maturation rate, fertilization rate and early embryonic development were compared in both groups. In total, 683 oocytes were collected from 117 ICSI cycles of 117 patients. Among them, 244 (35.7%) were mature MII and 259 (37.9%) were MI after the denudation process. Of those 259 MI oocytes, 202 (77.9%) progressed to MII oocytes after an incubation period of 18–24 h. The maturation rate was 77.9%. Fertilization rate was found to be significantly higher in the rescued *in vitro* matured MI oocyte group when compared with the *in vivo* matured MII oocyte group (41.6% vs 25.8%; $P = 0.0006$). However, no significant difference was observed in terms of cleavage rates on days 2 and 3 between the groups ($P = 0.9126$ and $P = 0.5031$, respectively). There may be unidentified *in vivo* factors on the oocyte maturation causing low developmental capacity in spite of high fertilization rates in the group of patients with low oocyte maturity rate. Furthermore, studies are needed to determine the appropriate culture characteristics as well as culture period and ICSI timing of these oocytes.

Introduction

The process of controlled ovarian stimulation (COH) aims to promote multifollicular development and to recruit higher rates of mature oocytes in assisted reproductive techniques (ART). Although 85% of retrieved oocytes from COH cycles are mature metaphase II (MII) oocytes, the remaining 15% are still immature, either at metaphase I (MI, 4%) or germinal vesicle (GV, 11%) stage (Cha and Chian, 1998; De Vos *et al*., 1999). In the Vienna consensus by the ESHRE Special Interest Group of Embryology and Alpha Scientists, oocyte maturity rate, which is generally related to nuclear maturity, was defined as the proportion of oocytes at MII stage. In their consensus report, the competency rate of retrieved mature oocytes was stated as 75% (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). In standard *in vitro* fertilization (IVF) cycles, these immature oocytes are sacrificed. However, the ovarian response to stimulation is variable. In cases of poor ovarian reserve and asynchronous follicle development, the probability of retrieving immature oocytes is high. Other than the above-mentioned groups, there may be patients with low oocyte maturity rate in whom unexpectedly low numbers of mature oocytes are obtained on retrieval when compared with ultrasonographic appearance. In these circumstances, these immature oocytes are of value to increase the number of embryos obtained. Immature oocytes have the capacity of *in vitro* spontaneous maturation and subsequent progression in different mammalian species, including humans (Edwards, 1965). They are allowed to progress up to the MII stage in *in vitro* cultures and serve as a source of oocytes for intracytoplasmic sperm injection (ICSI) cycles.

Immature oocytes can mature in different time periods. It is proposed that *in vitro* oocyte maturation is a relatively fast event that can occur shortly after the retrieval of the oocytes (Balakier *et al*., 2004). In the study by Balakier and colleagues the MI oocytes were cultured *in vitro* for a maximum of 3 h and they were monitored every 30 min for the progression of their maturation. Among 468 immature MI oocytes, 90 (19%), 182 (39%), 168 (36%) and 28

(6%) oocytes underwent maturation during 30 min, 1 h, 2 h and 3 h of *in vitro* culture, respectively (Balakier *et al.*, 2004).

Previous studies have shown controversial results about the rates of fertilization and embryonic development for immature oocytes. These studies focused on the cycles in which at least one immature oocyte was obtained. In the present study, we aimed to investigate the fertilization rates and early embryonic development of *in vitro* matured MII oocytes retrieved from stimulated cycles of patients with an unexpected poor response, with the percentage of mature oocytes less than 75% of those retrieved. The study was designed to determine the fertilization and embryonic potential of these immature oocytes and if *in vitro* matured MII oocytes could have a clinical application in this selected group of patients with low oocyte maturity rate. To our knowledge, this issue has not been investigated in any other studies in this group of patients.

Materials and methods

Patient population, stimulation protocol and oocyte retrieval

This retrospective study was approved by the Ethical Committee of the Ankara City Hospital (reference number E1-20-275). The study included 117 ICSI cycles of 117 women between January 2010 and December 2018 with unexpected low oocyte maturity rate in which the percentage of obtained mature oocytes was less than 75% of the retrieved ones. The study group was selected according to the Vienna consensus report in which the competency rate of retrieved mature oocytes was stated as 75% (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). The indications for ART were unexplained infertility, tubal factor, poor ovarian reserve and male factor. To be able to evaluate the fertilization and the early embryonic development, patients over the age of 38 or patients with severe male factor were excluded from the study.

The COH protocol consisted of gonadotropin-releasing hormone (GnRH) antagonist or agonist administered for pituitary supression. Recombinant FSH (r-FSH) or human menopausal gonadotropin (hMG) followed by hCG were used during the COH cycles. Oocyte retrieval was performed under the guidance of transvaginal ultrasound 36 h after 10,000 IU hCG injection to trigger ovulation. All ultrasonographically identifiable follicles larger than 14 mm were aspirated. Oocyte retrieval and embryology laboratory processes were performed by the same team of senior doctors.

Oocyte preparation, ICSI and embryo culture

Cumulus–oocyte complexes (COCs) were collected from the aspirated follicular fluid, cultured, and transferred to fertilization medium. They were stored in an incubator at 37°C, 6% CO_2, 5% O_2, and 90% humidity for 1–2 h. Following the incubation, denudation of the oocyte by removal of the surrounding cumulus and corona cells included enzymatic (HYASE-10X Vitrolife, Sweden, AB) and mechanical step (Flexipet Denuding Pipette, Cook, USA). After the denudation process, the oocytes were evaluated for the nuclear maturational stages defined as GV (oocytes in which the meiosis was not initiated), MI (oocytes with a GV breakdown without the extrusion of first polar body), and MII (oocytes with the presence of an extruded first polar body). ICSI was performed on the MII oocytes (Group 1, MII-ICSI, $n = 244$). MI oocytes were incubated to mature *c.* 18–24 h more after denudation process. During the 8 h after the denudation process, which coincides with workday hours, controls for the meiotic maturation were performed hourly. ICSI was performed on the mature oocytes detected on controls until the end of the workday. For the still immature oocytes, the control period was extended until 18–24 h after denudation. At the end of this period, ICSI was performed on the *in vitro* matured MI oocytes (rescued *in vitro* matured MI oocytes) (Group 2, MI-MII-ICSI, $n = 202$).

Injected oocytes were assessed for fertilization *c.* 16–18 h after ICSI. The presence of two pronuclei (PN) confirmed successful fertilization. Oocytes with 1PN or ≥3PN were considered as abnormally fertilized. Embryo cleavage was assessed 24 h after PN was detected. All embryos are graded on a scale of 1–4 according to the blastomere number, size, irregularity and the presence of fragmentation (Gardner and Schoolcraft, 1999).

Fertilization rates were calculated as oocytes with 2PN/oocytes ICSI performed. Embryo cleavage rates were calculated as number of cleavage embryos/oocyte with 2PN.

Table 1. Baseline clinical characteristics of patients

Characteristics	$n = 117$ patients, $n = 117$ cycles
Age (years)	33 (28–37)
Paternal age (years)	35 (31–38)
BMI (kg/m^2)	27 (20–36.5)
Duration of infertility (years)	5 (3–9)
Duration of ovarian stimulation (days)	10 (9–11)
E2 level on ovulation trigger day (ng/ml)	1279 (754.25–1918.5)
P level on ovulation trigger day (ng/ml)	0.6 (0.3–1.1)

Data given as median (25–75% percentile).
BMI, body mass index; E2, estradiol; P, progesterone.

Statistical analysis

Continuous variables were summarized as mean ± standard deviation or median (minimum–maximum) values. In categorical variables, the data are summarized in numbers and percentages. The chi-squared test or Fisher's exact test was applied by evaluating the comparison of assumptions in the comparison of two groups independent of a categorical variable.

A type I error rate of alpha 0.05 was accepted in the calculations. Statistical analysis was performed using R v.3.5.0 (R Core Team, 2018) software. Tables were created with Microsoft Excel.

Results

In total, 117 patients were included in the study. Baseline clinical characteristics of patients are listed in Table 1. The median age of the patients was 31.9 years. The indications for ART were as follows: unexplained infertility (41%, 48/117), male factor (34%, 40/117), poor ovarian reserve (15.3%, 18/117), and other (9.4%, 11/117).

Here, 683 oocytes were collected from 117 ICSI cycles of 117 patients. Among them 244 (35.7%) were mature MII, 259 (37.9%) were MI and 180 were GV (26.3%) after the denudation process. Of those 259 MI oocytes, 202 (77.9%) progressed to MII oocyte, and remaining 57 (22%) were arrested at MI stage after an incubation period of 18–24 h. Only five oocytes obtained matured in the first 3 h period. The maturation rate was 77.9%. Group 1 (MII-ICSI, $n = 244$) constituted the injected oocytes determined at MII stage after denudation process, Group 2

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Table 2. Fertilization and cleavage rates in groups

	Group 1 (MII-ICSI) ($n = 244$)	Group 2 (MI-MII-ICSI) ($n = 202$)	*P*-value*
Fertilization rate (2PN)	63 (25.8%)	84 (41.6%)	0.0006
Day 2 cleavage embryo rate	60 (24.6%)	51 (25.2%)	0.9126
Day 3 cleavage embryo rate	33 (13.5%)	32 (15.8%)	0.5031

*$P < 0.05$, statistically significant.

Table 3. Quality of cleavage embryos in groups

	Group 1 (MII-ICSI) ($n = 244$)	Group 2 (MI-MII-ICSI) ($n = 202$)	*P*-value*
Day 2			
Grade 1	16 (26.7%)	4 (7.8%)	0.053
Grade 2	34 (56.7%)	33 (64.7%)	
Grade 3	7 (11.7%)	11 (21.6%)	
Grade 4	3 (5%)	3 (5.9%)	
Day 3			
Grade 1	4 (12.1%)	1 (3.2%)	0.037
Grade 2	23 (69.7%)	17 (53.1%)	
Grade 3	4 (12.1%)	13 (40.6%)	
Grade 4	2 (6.1%)	1 (3.1%)	

*$P < 0.05$, statistically significant.

(MI-MII-ICSI, n = 202) were the injected oocytes that had reached the MII stage from MI after incubation period (rescued *in vitro* maturated oocytes).

Table 2 demonstrates the fertilization and cleavage rates of the oocytes. Normal fertilization rate in Group 1 was 25.8%, conversely it was 41.6% in Group 2. Fertilization rate was found to be significantly higher in the rescued *in vitro* matured MI oocyte group when compared with the *in vivo* matured MII oocyte group ($P = 0.0006$). However, no significant difference was observed in terms of cleavage rates in early embryonic development on days 2 and 3 between the groups ($P = 0.9126$ and $P = 0.5031$, respectively).

Embryo quality and the percentages of embryos in G1–G4 are listed in Table 3. On day 2, the percentage of G1+G2 embryos was 83.4% in Group 1 and 72.5% in Group 2. Although the percentage of G1+G2 embryos was higher in Group 1, its statistical significance was borderline ($P = 0.053$). Conversely, on day 3, the percentage of G1+G2 embryos was 81.8% in Group 1 and 56.2% in Group 2. The percentage of G1+G2 embryos was significantly lower in the *in vitro* matured MI oocyte group when compared with the *in vivo* matured MII oocyte group. None of the cleavage embryos had progressed to the blastocyst stage. As embryos from Group 1 and Group 2 patients cannot be transferred to patients on the same cycle separately, data related to implantation and pregnancy cannot be given.

Table 4 lists fertilization and embryonic development rates according to the ART indications. No statistically significant differences were observed in terms of fertilization and embryonic development within and between the groups when individual ART indications were considered.

Discussion

In the present study, we aimed to evaluate the fertilization and early embryonic developmental capacity of rescued *in vitro* matured MI oocytes in ICSI cycles of patients with unexpectedly low numbers of mature oocytes obtained on retrieval. To be more specific, in this group of patients, the percentage of mature oocytes obtained was less than 75% of the total retrieved oocytes, and was low according to the ultrasonographic expectation on the day of oocyte retrieval. The results from this study demonstrated that immature oocytes can mature *in vitro* and fertilization rates of *in vitro* matured oocytes were significantly higher than for *in vivo* matured oocytes. However, this increased fertilization rate did not support better embryonic development from these oocytes. Indeed, there was no difference in cleavage rates, an even lower percentage of G1+G2 embryos was detected in the *in vitro* matured oocyte group.

The ovarian responses in COH cycles are not always optimal, especially in patients with low ovarian reserve and asynchronous follicle development, varying percentages of oocytes at different stages of maturity can be observed (Escrich *et al.*, 2018). In addition, in some cycles, lower numbers of mature oocytes than expected with respect to the folliculometric follow-up are obtained on retrieval. Recently, the interest for the clinical use of these immature oocytes has been increasing, as these oocytes can spontaneously mature *in vitro* (Edwards, 1965).

The reported maturation rates of these immature oocytes vary among studies. Chen and colleagues studied the progression to maturation of MI oocytes at various time intervals (Chen *et al.*, 2000). Maturation rates were 16.4% and 88.3% after 9 h and 30 h of incubation. Similarly, Vanhoutte and colleagues reported a time-dependent increase in the maturation rate, ranging from 41% after 2–4 h to 75% after 19–26 h of *in vitro* maturation (Vanhoutte *et al.*, 2005). In the study of Li and co-workers, the maturation rate of MI oocytes after 3–5 h culture was 40.1% and was significantly lower than for MI oocytes cultured overnight that had a maturation rate of 87.2% (Li *et al.*, 2011). In general, maturation rates are higher after prolonged culture periods. Overall maturation rates of MI oocytes in different studies were 26.7% (De Vos *et al.*, 1999), 62.8% (Shin *et al.*, 2013), 46% (Strassburger *et al.*, 2004), 43% (Vanhoutte *et al.*, 2005), and 54% and 65.1% (Shu *et al.*, 2007). Variations in reported maturation rates in studies might be explained by several factors. One of these factors is the difference in culture periods and culture conditions. Another proposed factor for this variation in maturation rates is the time interval between retrieval and oocyte denudation. Shu and colleagues stated that, in the studies in which oocyte denudation was not performed immediately, oocytes that were actually matured *in vitro* prior to denudation were assumed to be *in vivo* matured oocytes, therefore the maturation rate might be underestimated and the maturational profile would be inaccurate (Shu *et al.*, 2007). Moreover, ovarian stimulation conditions with high doses of gonadotropins in COH cycles may also lead to defects in maturation (Li *et al.*, 2011). The maturation rate of MI oocytes in the present study was 77.9% and was slightly higher than reported in the published literature, possibly due to the relatively longer culture period.

Previous studies have reported lower fertilization rates from *in vitro* matured oocytes compared with mature oocytes (De Vos *et al.*, 1999; Strassburger *et al.*, 2004; Vanhoutte *et al.*, 2005; Shin *et al.*, 2013; Bilibio *et al.*, 2021). In contrast, we found

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Table 4. Fertilization and embryonic development according to the indications for ART

	Unexplained infertility		Male factor		Poor ovarian reserve		Other		
	Group 1 MII-ICSI	Group 2 MI-MII-ICSI	Group 1 MII-ICSI	Group 2 MI-MII-ICSI	Group 1 MII ICSI	Group 2 MI-MII-ICSI	Group 1 MII-ICSI	Group 2 MI-MII-ICSI	*P*-value
Number of oocytes	113	84	75	85	28	21	28	12	NS
Fertilization rate	25 (10.2%)	33 (16.3%)	23 (9.4%)	36 (17.8%)	8 (3.3%)	9 (4.5%)	7 (2.9%)	5 (2.5%)	NS
Day 2 cleavage embryo rate	24 (9.8%)	21 (10.4%)	23 (9.4%)	21 (10.4%)	7 (2.9%)	6 (3%)	5 (2%)	3 (1.5%)	NS
Day 3 cleavage embryo rate	14 (5.7%)	16 (7.9%)	14 (5.7%)	13 (16.4%)	2 (0.8%)	3 (1.5%)	3 (1.2%)	0	NS

NS, non-significant.

significantly higher rates of fertilization from *in vitro* matured oocytes (fertilization rates, MII oocytes 25.8%, MI-MII oocytes 41.6%). In all these cited studies, ICSI was performed on both *in vivo* matured MII oocytes and *in vitro* matured MI oocytes. In line with our findings, Shu and co-workers failed to find a significant difference in fertilization rates between *in vitro* and *in vivo* matured oocytes (fertilization rates for MII oocytes 69.5% and for MI-MII oocytes 62.2%) (Shu *et al.*, 2007). They proposed that the discrepancy in fertilization rates between their findings and those of other studies could be explained by the timing of the denudation process. They stated that the immediate denudation process in their study enabled them to obtain a proper maturational profile of oocytes at the time of retrieval, and therefore the oocytes that were actually matured *in vitro* before denudation were accepted as *in vitro* matured oocytes (Shu *et al.*, 2007). In a latter study by Li and co-workers, fertilization rates from *in vitro* matured oocytes that were cultured for 3–5 h were comparable with the results for mature MII oocytes (fertilization rates for MI-MII oocytes 68.6% vs for MII oocytes 74.5%) (Li *et al.*, 2011). Similarly, Balakier and colleagues reported a 61% fertilization rate for matured oocytes cultured for 3–6 h *in vitro* (Balakier *et al.*, 2004). One might think that more positive outcomes are associated with oocytes found not to have extruded a polar body at denudation for ICSI, but do so within a shorter-3–4 h culture period, indicating that they were actually at MI and close to MII at retrieval. However, higher fertilization rates in the present study cannot be solely explained by this issue, as only five oocytes matured in the first 3 h period. The higher fertilization rates of *in vitro* matured oocytes in the present study of selected group of patients in whom the retrieved mature oocyte number were less than the expected rates (mature oocyte percentage <75% of the total retrieved oocytes) and may be related to intrinsic factors, leading to an unfavourable environment for the oocyte *in vivo*. Another factor may be related to the duration of culture before ICSI. In the study by Balakier and colleagues, fertilization rates of *in vitro* matured oocytes injected at different time periods after polar body extrusion were compared (Balakier *et al.*, 2004). It was found that, as the time in culture was prolonged, the frequency of binuclear zygotes increased considerably. They proposed that an appropriate duration of MII arrest was essential for matured oocytes to develop the capacity for full activation and pronuclear formation and a minimum of 3 h of MII arrest was required to obtain reasonable fertilization rates (Balakier *et al.*, 2004). In the present study, the matured oocytes were subjected to an ICSI procedure within a 18–24 h period, which might have allowed the *in vitro* matured oocytes to gain optimal fertilization capacity. Conversely, in the study by Goud and co-worker, it was shown that *in vitro* matured oocytes are susceptible to post-maturation ageing, and delayed sperm injection results in a higher incidence of zygotes that bear only one pronucleus, with pronucleus size asynchrony that exhibited cleavage arrest. They concluded that the timing of ICSI was critical for *in vitro* maturation oocytes (Goud *et al.*, 1999). Studies on the MII spindle in cultured human oocytes showed that a progressive destabilization of the kinetochore and polar astral microtubules occurred with increased time in culture beyond what is considered the post-ovulation/aspiration 'fertilization window'. As a result, monovalent chromosomes become detached from the spindle generating an aneuploid gamete. This phenomenon is frequently termed 'postmaturity'. It is the known major negative factor associated with rescue ICSI for patients with a high proportion of immature oocytes at aspiration when examined for fertilization. However, increased fertilization rates of *in vitro* matured MI oocytes in the present study showed that ICSIs were performed in a timely manner.

Despite the higher fertilization rates observed, there was no difference in cleavage rates and even lower rates of G1+G2 embryos were obtained from *in vitro* matured oocytes. Experiments on bovine oocytes also indicated that, as the duration of MII arrest is prolonged before insemination, the ability to support fertilization and embryo development is gradually lost (Dominko and First, 1997). Similarly, in the study of Li and colleagues on the overnight culture of an *in vitro* matured oocyte group, high cleavage rates (84.1%) were observed; however, the high quality embryo rate was significantly low (19.1%) (Li *et al.*, 2011). As the culture times for *in vitro* maturation of immature oocytes increased, the incidence of abnormal cytoskeletal organization and chromosomal imbalance also increased, therefore the developmental potential of these embryos was decreased (Strassburger *et al.*, 2004; Li *et al.*, 2011). Use of a time lapse system may help to improve the evaluation of developmental capacity of these embryos in the present study.

One limitation of the study was the absence of a time lapse monitoring system to determine the exact time of meiotic maturation of the oocytes and the embryonic developmental capacity. However, due to the increased fertilization rates in *in vitro* matured MI oocytes, absence of a time lapse system does not seem to be a limitation for ICSI timing. In the present study, there may be unidentified *in vivo* factors on the oocyte maturation causing low embryonic developmental capacity in spite of high fertilization rates in this group of patients with low oocyte maturity rate.

In conclusion, we may encounter patients with low oocyte maturity rate in whom the percentage of mature oocytes was obtained less than 75% of the total retrieved ones. Immature MI oocytes can mature spontaneously *in vitro*. Fertilization rates of *in vitro* matured oocytes were found to be significantly higher than

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for *in vivo* matured oocytes. However, this increased fertilization rate did not support better embryonic development from these oocytes. Therefore, the developmental competency of the embryos from immature oocytes was significantly reduced. Studies are needed to determine the optimal culture characteristics such as culture period by using time lapse monitoring system. Most importantly, unidentified *in vivo* factors for oocyte maturation and the probability of chromosomal abnormalities causing low embryonic developmental capacity requires further research.

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