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The developmental competence of human metaphase I oocytes with delayed maturation *in vitro*

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Abstract

Objective: To evaluate if metaphase I (MI) oocytes completing maturation *in vitro* to metaphase II ("MI-MII oocytes") have similar developmental competence as the sibling metaphase II (MII) oocytes that reached maturity *in vivo*.

Design: Retrospective cohort study.

Subjects: A total of 1124 intracytoplasmic sperm injection (ICSI) cycles from 800 patients at a single academic center between April 2016 and Dec 2020 with at least one MII oocyte immediately after retrieval and at least one sibling "MI-MII oocyte" that was retrieved as MI and matured to MII in culture before ICSI were included in the study.

Main Outcome Measures: A total of 7865 MII and 2369 sibling MI-MII retrieved from the same individuals were compared for fertilization and blastocyst formation rates. For patients undergoing single euploid blastocyst transfers (n=406), the clinical pregnancy, spontaneous pregnancy loss rate and live birth rate were compared between the two groups.

Results: The fertilization rate was significantly higher in MII oocytes than delayed matured MI-MII oocytes (75.9% *vs* 56.1%, p<0.001). Similarly, the blastocyst formation rate was higher in embryos derived from MII oocytes as compared to those from MI-MII oocytes (53.8% vs 23.9%; p<0.001). The percentage of euploid embryos derived from MII oocytes was significantly higher

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Authors contribution statement:

JM conceived and designed the study. JM and BY drafted the manuscript. QZ, JZ collected and analyzed the data. VR, JH, YC, NZ, JD, LN, BB edited the manuscript. BY obtained funding. All authors have read and approved the final manuscript.

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than those from MI-MII oocytes (49.2% *vs* 34.7%; p<0.001). Paired comparison of sibling oocytes within the same cycle showed higher developmental competence of the MII oocytes than MI-MII oocytes. However, the pregnancy, spontaneous pregnancy loss and live birth rate after a single euploid blastocyst transfer showed no statistically significant difference between the two groups (65.7% *vs* 74.1%: 6.4% *vs* 5.0%: 61.5% *vs* 70.0%, respectively, MII vs MI-MII group, p>0.05).

Conclusion: Compared to oocytes that matured *in vivo* and were retrieved as MII, the oocytes that were retrieved as MI and matured to MII *in vitro* before ICSI showed lower developmental competence, including lower fertilization, blastocyst formation, and euploidy rates. However, euploid blastocysts from either cohort resulted in similar live birth rates, indicating the MI oocytes with delayed maturation can still be useful even though the overall developmental competence was lower than their *in vivo* matured counterparts.

Capsule

Compared to sibling MII oocytes, the MI oocytes that were matured to MII *in vitro* showed lower developmental competence but equivalent live birth rates after single euploid blastocyst transfers.

Keywords

Metaphase I (MI); Metaphase II (MII); oocyte maturation; single euploid blastocyst; frozen embryo transfer (FET)

Introduction

After controlled ovarian hyperstimulation (COH), approximately 20% of oocytes retrieved are found to be immature in the metaphase I (MI) or germinal vesicle (GV) stage following the removal of the cumulus cells (1, 2). MI oocytes have undergone the process of germinal vesicle breakdown *in vivo* and may spontaneously complete maturation and reach metaphase II (MII) stage within several hours of *in vitro* culture (3). In order to reach the mature MII stage, oocyte maturation involves two equally important steps: nuclear and cytoplasmic maturation (1, 4, 5), which are required for successful fertilization and embryo development. Nuclear maturation is concomitant with cytoplasmic maturation, which requires a reorganization of numerous subcellular compartments and organelles including mitochondria membrane, endoplasmic reticulum, and cytoskeleton (6, 7). Maturation *in vitro* can alter these processes and affect fertilization and embryo development. Indeed, oocytes that are retrieved at the MI stage and mature to the MII stage when left in culture ("MI-MII oocytes") have been shown to result in lower fertilization rates (1, 8–12), high embryo multinucleation rate (13), higher abnormal embryonic development rates (4) compared to *in vivo* matured oocytes that are retrieved at the MII stage. However, successful pregnancies have been reported after transferring embryos derived from MI-MII oocytes (11, 14–17).

Preimplantation genetic testing for aneuploidy (PGT-A) following trophectoderm biopsy is a widely used procedure to select euploid embryos for transfer and has significantly improved live birth rates and decreased early pregnancy losses for women with more advanced age who have a higher percentage of aneuploid oocytes and embryos. Selecting euploid embryos derived from MI-MII oocytes using PGT-A may be a potential pathway to increase the

number of available embryos and to achieve higher cumulative live births in a single patient. This could be especially beneficial in patients with poor response to COH or in patients with an unsynchronized cohort of follicles that yields a high number of immature oocytes. However, no previous study has systematically studied the developmental competence of MI-MII oocytes or reported the pregnancy rate after single euploid embryo transfers with embryos derived from MI-MII oocytes. Our hypothesis is that MI-MII oocytes may have lower developmental competence and result in lower live birth rates after single euploid embryo transfers compared to MII oocytes.

Here, we conducted a side-by-side comparison of the developmental competence of the MI-MII oocytes with their sibling MII oocytes from the same individuals and the same retrievals. We compared the fertilization rate after intracytoplasmic sperm injection (ICSI), biopsied blastocyst rate, and euploidy rate after PGT-A. After single euploid blastocyst transfers, we compared the clinical pregnancy rate, early pregnancy loss rate, and live birth rate between the two groups. We found that the MI oocytes with delayed maturation (MI-MII oocytes) can result in similar live birth rates if they are euploid, even though the overall developmental competence is lower than their *in vivo* matured counterparts (MII oocytes).

Materials and methods

Source of oocytes

This retrospective study was approved by the Institutional Review Board at Stanford University. The patients undergoing fertility treatments in our center from April 2016 to December 2020 who met the inclusion criteria were included in the study. Gonadotropin-releasing hormone (GnRH) antagonist, GnRH agonist down-regulation, and micro-dose flare protocols were used for controlled ovarian hyperstimulation (COH). Oocyte retrieval was performed by transvaginal ultrasound-guided follicle aspiration 35–36 hours after ovulation trigger with human chorionic gonadotropin (hCG) and/or Leuprolide (9). Intracytoplasmic sperm injection (ICSI) was used as the fertilization method for the oocytes that were mature at the time of fertilization. “MI-MII oocytes” are defined as oocytes that were retrieved at the MI stage and left to mature to the MII stage in culture before ICSI. “MII oocytes” are defined as oocytes that were retrieved at the MII stage. “Sibling oocytes” are defined as oocytes retrieved from the same patient after the same COH cycle and from the same oocyte retrieval procedure. Only cycles with at least one MII oocyte and one or more sibling MI-MII oocytes that underwent ICSI were included in this study. The COH cycles that resulted in no sibling MII and MI-MII oocytes, did not use ICSI as the fertilization method, or did not create embryos such as oocyte cryopreservation cycles were excluded from this study. Developmental competence parameters including the fertilization rate, blastocyst formation and biopsy rate, euploidy rate were compared between the MII and the sibling MI-MII oocytes. For the pregnancy outcome part of the study, we only included the FET cycles with single euploid blastocyst transfers and excluded all other embryo transfer cycles within the study cohort. No COH or FET cycles were excluded based on the fertility diagnosis or the cycle characteristics such as the number of mature and immature oocytes retrieved.

Preparation of oocytes

Oocytes were collected 35–36 hours after ovulation trigger. After oocyte retrieval, the cumulus cells were immediately removed using 80 IU/ml hyaluronidase (SAGE, Cooper Surgical Inc, Trumbull, CY) and the oocyte maturation status was determined for each oocyte. The MII and MI oocytes were incubated separately for 2–4 hours in fertilization medium (SAGE, Cooper Surgical Inc, Trumbull, CY). In cases with low oocyte maturity at the time of retrieval, i.e., less than 50% of retrieved oocytes were MII, MI oocytes were cultured for up to 6 hours before maturity was reassessed.

ICSI procedure and embryo processing

In 2–6 hours after oocyte retrieval, the MI oocytes were reassessed for maturity. Those that matured to the MII stage were collected and labeled as “MI-MII oocytes”, and those that remained as MI oocytes from the time of retrieval were labeled as “MI oocytes”. Immature oocytes were discarded after the maturity reassessment. No overnight culture of immature oocytes was performed in our center. Intracytoplasmic sperm injection (ICSI) was performed on all mature oocytes, including MI-MII and MII oocytes, at that time. Fertilization was examined 16–19 hours after ICSI and confirmed by the appearance of two distinct pronuclei and two polar bodies. The fertilization rate was calculated by the number of zygotes with two pronuclei (2PN) divided by the number of oocytes undergoing ICSI. The 2PN zygotes, regardless of the original oocyte maturity, were cultured in single-step media (SAGE, Origio, Malov, Denmark) with 5.6% CO_2 and 5.0% O_2 at 37°C (pH 7.28–7.32) until day 7 after fertilization.

The blastocyst formation was evaluated each day from day 5 to day 7. Blastocyst quality was evaluated for the level of expansion (from 1 to 6), the morphology of inner cell mass (ICM) graded from A to D, and trophectoderm (TE) graded from A to D based on the development of ICM and TE adapted from Gardner et al., 2000 (18). Blastocyst formation rate was calculated by dividing the total number of blastocysts by the number of 2PN zygotes.

Each day from day 5 to 7 of embryo development, TE biopsy was performed on expanded blastocysts with a grade above 3CC. The biopsied blastocyst formation rate was calculated by dividing the number of biopsied blastocysts by the number of 2PN zygotes. The biopsied TE samples were sent to an outside company for preimplantation genetic testing for aneuploidy (PGT-A) (19). The blastocysts were then vitrified using Cryotec vitrification kit (Cryotech, Japan) following the instructions of the manufacturer. During the subsequent frozen embryo transfer (FET) cycles, the selected euploid blastocysts were thawed using a standard published protocol (20) before transfer.

FET and outcome

A single euploid blastocyst was selected, thawed, and transferred for each FET cycle. The euploid embryos derived from the MII oocytes, regardless of the morphology grades, were prioritized over those derived from the MI-MII oocytes. The FET cycle protocols and transfer procedures were previously published by our center (21). Briefly, both natural and programmed protocols were used for endometrial preparation, with the aim to achieve an endometrial thickness of at least 7 mm. In the natural FET cycles, luteal support was

provided with vaginal progesterone (100 mg Endometrin, Ferring Pharma, Inc.) two times per day. In programmed FET cycles, luteal support was provided with estradiol 2 mg orally three times daily, intramuscular progesterone in oil 50 mg per night, starting five days prior to transfer. Luteal phase support was continued through 10 weeks of gestation if pregnancy was accomplished. A clinical pregnancy was defined by the presence of an intrauterine gestation sac on transvaginal ultrasound at or above 6–7 weeks of gestation. A spontaneous pregnancy loss was defined as the loss of an intrauterine pregnancy before 20 weeks of gestation after a clinical pregnancy was previously established on ultrasound. A live birth was defined as the birth of a neonate at or beyond 24 weeks gestation as dated by the date of embryo transfer. The clinical pregnancy rate was determined per embryo transfer and the pregnancy loss rate was calculated per clinical pregnancy.

Statistical analysis

The exposure is the *in vitro* culturing to facilitate delayed maturation. The two cohorts are MI-MII oocytes and MII oocytes as defined above. The outcomes of interest are fertilization rate, blastocyst formation rate, pregnancy rate, spontaneous pregnancy loss rate, and live birth rate. The frequency tables were built to describe the distribution of each outcome. Fisher Exact tests were conducted to test the difference between MII oocytes and the sibling MI-MII oocytes for each outcome. To account for the heterogeneity among cycles, the generalized estimating equation (GEE) approach with exchangeable covariance structure was applied to conduct paired comparisons of the probability of fertilization, biopsied blastocyst formation, and euploid blastocyst formation between the MII and the sibling MI-MII oocytes within the same cycle. The analysis was conducted by the geepack package in R. All statistical tests were two sided and a p value of < 0.05 was considered statistically significant.

Results

This study included a total of 800 patients and 1124 cycles with at least one MII oocyte immediately after retrieval and at least one sibling “MI-MII oocyte” that was retrieved as MI and matured to MII in culture before intracytoplasmic sperm injection (ICSI). The mean age of the patients was 37 ± 3 years (average age ± standard deviation). Only 7% of included cycles had >50% MI oocytes at the time of the retrieval and these MI oocytes were incubated for up to 6 hours to enable maturation. ICSI was performed on 7865 MII oocytes and 2369 sibling MI-MII oocytes from the same patients after the same COH cycles and oocyte retrieval procedures.

The fertilization rate was significantly higher in MII oocytes than delayed matured MI-MII sibling oocytes (75.9% *vs* 56.1%, p<0.001) (Table 1). Similarly, the blastocyst formation rate was higher in embryos derived from MII oocytes as compared to those from sibling MI-MII oocytes (53.8% vs 23.9%; p<0.001) (Table 1). After PGT-A, the percentage of euploid embryos derived from MII oocytes was significantly higher than those from MI-MII oocytes (49.2% *vs* 34.7%; p<0.001) (Table 1). Twelve cycles had only one MII and only one MI-MII available, and the fertilization, blastocyst formation, and euploid blastocyst

rates followed similar trends as the other cycles, with MI-MII oocytes showing lower developmental competence than MII oocytes.

To account for the heterogeneity among cycles, the developmental competence between the MII and the sibling MI-MII oocytes within the same cycle was compared using the generalized estimating equation (GEE) approach. The MII oocytes had higher probability of fertilization, biopsied blastocyst formation, and euploidy in all biopsied embryos than their sibling MI-MII oocytes (Table 2), which was consistent with the group comparisons (Table 1). For example, the probability of fertilization of the MII oocytes was statistically significantly higher than their sibling MI-MII oocytes across all cycles (odds ratio 2.43, 95% confidence interval [2.19, 2.70], $p< 0.01$) (Table 2). The probability of euploidy in day 6 and day 7 blastocysts were not statistically significantly different between MII oocytes and sibling MI-MII oocytes (Table 2).

We further studied those patients who underwent single euploid blastocyst transfers during the study period (n=406). We compared the outcomes of the pregnancies that resulted from euploid embryos derived from MII versus MI-MII oocytes. The pregnancy rate, spontaneous pregnancy loss rate and live birth rate after a single euploid blastocyst transfer showed no statistically significant difference between the two groups (65.7% *vs* 74.1%: 6.4% *vs* 5%: 61.5% *vs* 70.0%, respectively, MII vs MI-MII group, $p>0.05$) (Table 3). Five patients had euploid embryos derived from both MII and sibling MI-MII oocytes transferred, four of whom had concordant pregnancy outcomes from MII versus sibling MI-MII oocytes (with two pregnant both cycles and two failed both). The patient with discordant pregnancy outcomes was not pregnant with MII-derived embryo but had live birth after MI-MII-derived euploid embryo transfer in the subsequent FET cycle.

Discussion

In this large study, we compared the developmental competence of MII oocytes and the sibling oocytes retrieved as MI with delayed maturation to MII after 2–6 hours of culture, and followed the pregnancy outcomes after single euploid blastocyst transfers from the embryos derived from these oocytes. We showed that the oocytes that were retrieved as MI and matured to MII *in vitro* before ICSI (“MI-MII oocytes”) possessed lower developmental competence, including lower fertilization, blastocyst formation, and euploidy rates, when compared to oocytes that matured *in vivo* and were retrieved as MII (“MII oocytes”). However, euploid blastocysts derived from either cohort of oocytes resulted in similar live birth rates after single blastocyst transfer, indicating the MI oocytes with delayed maturation can still be useful even though the overall developmental competence is lower than their *in vivo* matured counterparts.

MI oocytes retrieved after COH can complete the maturation process when left in the conventional culture media without any hormone supplementation (4). In our study, the MI oocytes were cultured for up to 6 hours prior to ICSI instead of overnight extended culture, which can lead to oocyte aging and result in poor embryo development and a high rate of chromosomal abnormality (4, 11, 22). Due to this reason, we do not culture immature oocytes overnight in our center nor perform ICSI on the day after retrieval. Despite a shorter

duration in culture, the delayed *in vitro* matured oocytes (or MI-MII oocytes) still showed lower fertilization rates and poor embryo development, consistent with previous publications (1, 2, 4, 11, 23, 24). Successful fertilization and subsequent embryo development require both nuclear and cytoplasmic maturation of the oocyte (1, 4, 5, 25, 26). Cytoplasmic maturation involves the relocation of organelles, such as the mitochondria or ribosomes, and the assembly of microtubules for development and calcium release (27). The lower fertilization rates and poor embryo development from the MI-MII oocytes may be associated with cytoplasmic immaturity despite the nuclear maturity or resumption of meiosis to the MII stage. Oocytes with cytoplasmic immaturity are unlikely to respond to the activation signal provided by the spermatozoa (28), which leads to a high rate of abnormal sperm de-condensation or premature chromosome condensation (PCC) (4, 29). A sign of PCC under polarized light microscopy (Polscope™) is the second spindle derived from sperm chromatin (7, 30–35). We found that most MI-MII oocytes that failed to fertilize showed the second spindle, indicating incomplete cytoplasmic maturation may be the reason for the lower fertilization rate (36).

The major strength of our study is that we followed the pregnancy outcomes after single euploid embryo transfers using the embryos derived from either MI-MII oocytes or MII oocytes. This approach eliminated the uncertainty of transferring multiple embryos and is superior to relying on the embryo grades, which was done in published studies (1, 10). The pregnancy outcomes using this approach best reflect the true potential differences between the MI-MII oocytes with delayed maturation and the *in vivo* matured MII oocytes, after minimizing the potential confounding factors such as chromosomal abnormalities of transferred embryos or the number of embryos transferred. To the best of our knowledge, such data is not previously available. Another strength of our study is the comparison between the sibling oocytes for all the parameters of developmental competence we investigated. This approach minimized the variabilities introduced by the inter-individual differences, COH protocol, oocyte retrieval, and culture condition variations. We also used ICSI as the fertilization method for all oocytes in this study. Therefore, the observed differences in fertilization or blastocyst formation rates between the sibling MII and MI-MII oocytes were from well controlled comparisons between the two group of oocytes. No COH or FET cycles were excluded based on the fertility diagnosis or the cycle characteristics, and our findings could potentially be applicable to various types of patients and cycles.

Our study has some limitations. It is a retrospective study and thus lacks the power of answering our study question more definitively like a randomized controlled trial could. Due to prioritized selection of embryos derived from MII oocytes, the number of single embryo transfers using embryos derived from sibling MI-MII oocytes was lower. However, when a patient exhausted all euploid embryos derived from MII oocytes, these additional euploid embryos derived from MI-MII oocytes offered additional opportunities for embryo transfers and in some cases, successful implantation and live births. We did not compare the embryo grades between the two groups, as it is still controversial whether embryo grades add any clinically useful information beyond the embryo ploidy status that is obtained from the PGT-A (37–40). However, among the euploid embryos available for transfer, we routinely select the embryos derived from MII oocytes regardless of the grades over those derived from the MI oocytes; therefore, the two groups may have an uneven distribution of embryo

grades, which was not shown to affect the pregnancy outcomes after single euploid embryo transfers. Despite our practice of preferentially transferring euploid embryo derived from MII oocyte over sibling MI-MII oocyte, the pregnancy and live birth rates were similar between the two groups. This may indicate that an euploid embryo from an MI-MII oocyte could have a higher potential for resulting in a live birth because they were transferred only after euploid embryos from MII oocytes failed. However, the number of euploid embryo transfers in the MI-MII group was not high enough to draw such conclusions. Even though we compared the fertilization, blastocyst formation and euploidy rates between the sibling oocytes from the same cycle, it was not possible to do a sibship-based comparison of the pregnancy outcomes from the embryos derived from the sibling oocytes because most patients did not have the availability of transferrable embryos from the sibling oocytes nor the opportunity of transferring both during the study period. Therefore, only the pregnancy outcomes of the two groups were compared.

Conclusions

In this large retrospective cohort study comparing the developmental competence of sibling oocytes, we found that compared to MII oocytes that matured *in vivo*, the sibling MI-MII oocytes that were retrieved as MI and matured to MII *in vitro* before ICSI showed lower developmental competence, including lower fertilization, blastocyst formation, and euploidy rates. However, after single euploid embryo transfers, similar live birth rates and spontaneous pregnancy loss rates were observed from either cohort. Our study suggests that the euploid MI oocytes with delayed maturation can still be useful even though the overall developmental competence is lower than their *in vivo* matured counterparts.

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Table 1.

The fertilization, blastocyst formation, and euploid blastocyst formation rates of MII and MI-MII oocytes.

	MII oocytes	**MI-MII oocytes**	***P* value**
Fertilization rate (%) (# 2PN/ # ICSI)	75.9%[a] (5970/ 7865)	56.1%[a] (1329/ 2369)	<0.001
Biopsied blastocyst rate (%) (# biopsied blastocysts/ # 2PN)	53.8%[b] (3209/ 5970)	23.9% [b] (317/ 1329)	<0.001
Euploidy rate in day 5 embryos (%) (# euploid/ # biopsied on day 5)	58.2%[c] (762/ 1309)	33.9% [c] (38/ 112)	<0.001
Euploidy rate in day 6 embryos (%) (# euploid/ # biopsied on day 6)	43.5% (781/ 1796)	36.3% (69/ 190)	0.06
Euploidy rate in day 7 embryos (%) (# euploid/ # biopsied on day 7)	35.6% (37/ 104)	20% (3/ 15)	0.38
Euploidy rate in all biopsied embryos (%) (# euploid/ # total biopsied on day 5–7)	49.2%[d] (1580/ 3209)	34.7%[d] (110/ 317)	<0.001

a-d: $P < 0.001$

*P calculation used Fisher's exact test.

Table 2.

Paired comparisons of the probability of fertilization, biopsied blastocyst formation, and euploid blastocyst formation between MII and sibling MI-MII oocytes within the same cycle.

Outcome	Odds Ratio	Confidence Interval	*P* value
Fertilization	2.43	2.19, 2.70	<0.01
Biopsied blastocyst	2.77	2.39, 3.21	<0.01
Euploidy in day 5 embryos	2.1	1.39, 3.18	<0.01
Euploidy in day 6 embryos	1.04	0.76, 1.41	0.83
Euploidy in day 7 embryos	1.99	0.49, 8.16	0.339
Euploidy in all biopsied embryos	1.51	1.17, 1.93	<0.01

Table 3.

The clinical pregnancy, spontaneous pregnancy loss and live birth rates after single euploid embryo transfers using embryos derived from MII or MI-MII oocytes.

	MII oocytes	MI-MII oocytes	*P value
Pregnancy rate of day 5 euploid blastocyst transfer (%) (# pregnancy/ # transfer)	67.7% (132/ 195)	55.6% (5/ 9)	0.48
Pregnancy rate of day 6 euploid blastocyst transfer (%) (# pregnancy/ # transfer)	64.3% (117/ 182)	83.3% (15/ 18)	0.12
Pregnancy rate of day 7 euploid blastocyst transfer (%) (# pregnancy/ # transfer)	0% (0/ 2)	N/A (0/ 0)	1.00
Total pregnancy rate of euploid blastocyst transfer (%) (# pregnancy/ # transfer)	65.7% (249/ 379)	74.1 % (20/ 27)	0.41
Pregnancy loss rate after day 5 single euploid blastocyst transfer (%) (# loss/ # pregnancy)	3.0% (4/ 132)	0% (0/5)	1.0
Pregnancy loss rate after day 6 single euploid blastocyst transfer (%) (# loss/ # pregnancy)	10.3% (12/ 117)	6.7% (1/15)	1.0
Pregnancy loss rate after day 7 single euploid blastocyst transfer (%) (# loss/ # pregnancy)	N/A (0/0)	N/A (0/0)	N/A
Pregnancy loss rate after all single euploid blastocyst transfers (%) (# loss/ # pregnancy)	6.4% (16/ 249)	5.0% (1/20)	1.0
Total live birth rate (%) (# live births/ # transfer)	61.5% (233/ 379)	70.0% (19/27)	1.0

*P calculation used Fisher's exact test.