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ARTICLE

Sperm penetration at the maturing metaphase I stage can trigger oocyte activation in a mouse model

BIOGRAPHY

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KEY MESSAGE

Sperm penetration at the metaphase I stage can trigger oocyte activation. The resulting embryos have high developmental potential, albeit some triploids due to the absence of the second meiotic division.

ABSTRACT

Research question: Can spermatozoa penetrate maturing metaphase I (MI) oocytes, and render subsequent development following conventional IVF in a mouse model?

Design: ICR mice were used in this study. Metaphase II (MII) cumulus–oocyte complexes (COC) harvested 15 h after injection of human chorionic gonadotrophin (HCG) were used for IVF as the control group (Group 1). In the treatment group (Group 2), maturing MI COC harvested 7 h after HCG injection were used for IVF. Fertilization, pronuclear formation, cleavage, blastocyst formation, DNA methylation status, chromosome number and live birth rates were used to evaluate the developmental dynamics and competency of maturing MI oocytes following conventional IVF.

Results: Maturing MI COC were fertilized using conventional IVF, and sperm penetration at MI–telophase I triggered oocyte activation. Most embryos resulting from fertilized MI oocytes developed to blastocyst stage during preimplantation development, albeit a substantial proportion of them were triploids due to the absence of the second meiotic division. Some of the embryos derived from fertilization of maturing oocytes were able to implant and gave rise to full-term development.

Conclusion: Maturing MI COC from follicles before ovulation could be used for mouse IVF, and fertilized MI oocytes had high potential for development. Healthy offspring can be generated from maturing MI COC following conventional IVF. MI COC may represent a valuable source of 'usable' biomaterial in assisted reproduction. However, many embryos derived from MI COC via IVF have abnormal chromosome numbers in the mouse model. The implications of these findings for human IVF remain to be investigated.

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KEY WORDS

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INTRODUCTION

In a routine human oocyte retrieval cycle with ovarian stimulation, around 20% of retrieved oocytes are immature [i.e. metaphase I (MI) and germinal vesicle oocytes] (*Moon et al., 2023*; *Vanhoutte et al., 2005*). As follicles are aspirated prior to rupture, the oocytes retrieved may come from a heterogeneous pool of follicles, thus resulting in the collection of mature and immature oocytes. In clinical practice, the cumulus cells of oocytes are kept when conducting IVF. Hence, it is difficult to distinguish the oocyte stage (mature versus immature), to enable separation, when conducting conventional IVF. As a result, cumulus cells enclosing maturing oocytes (especially MI) are usually inseminated by IVF at the same time as mature MII oocytes. However, the fate of MI oocytes in IVF is unknown. Can spermatozoa penetrate MI oocytes? If so, when? Can fertilized MI oocytes develop normally? Can offspring be derived from fertilized MI oocytes?

It has been reported that in-vitro-matured (IVM) mouse oocytes become impenetrable to spermatozoa due to the hardening of the zona pellucida (*Downs et al., 1986*; *Schroeder et al., 1990*). Hardening of the zona pellucida occurs during the maturation of mouse oocytes in a defined medium (*De Felici and Siracusa, 1982*), and the degree of spontaneous hardening is correlated directly with the failure of the eggs to become fertilized *in vitro* (*Gianfortoni, 1985*). Therefore, it is recommended that cumulus cells and the zona pellucida should be removed from maturing mouse oocytes for insemination (*Abbott et al., 2001*; *Clarke and Masui, 1986*; *Ducibella et al., 1990*; *Ducibella and Buetow, 1994*; *Kryzak et al., 2013*). One problem with this practice is that without the enclosing cumulus cells, MI oocytes are not activated by sperm penetration, which has an adverse effect on embryo development in the mouse model (*Clarke and Masui, 1986*; *McLay and Clarke, 1997*, *2003*). On the contrary to the mouse model, early studies using IVM human oocytes suggested that both the zona pellucida and the oolemma of oocytes were penetrable at all stages of meiotic maturation when immature oocytes enclosed by cumulus cells were used (*Lopata and Leung, 1988*; *Van Blerkom et al., 1994*). As such, it is postulated that maturing MI COC can be fertilized in mouse IVF procedures, and in-vitro and in-vivo experiments were performed to test this claim.

This study found that maturing MI COC can be fertilized, and can support high full-term rates. This study also investigated the specific timing of sperm penetration when maturing MI COC are subjected to IVF.

MATERIALS AND METHODS

Animals

All animal maintenance, care and procedures described in this study were reviewed and approved by the National Taiwan University (NTU) Institutional Animal Care and Use Committee (Protocol No. NTU-110-EL-00114). ICR mice aged 8–12 weeks were used as sperm and oocyte donors for IVF, and female ICR mice were used as recipients and foster mothers.

Superovulation priming of MII and MI COC

COC were collected from ICR female mice subjected to the following hormone priming protocol: superovulation was induced with 5 IU of pregnant mare serum gonadotrophin (HOR-272; ProSpec TechnoGene Ltd, USA), followed 48 h later with 5 IU of human chorionic gonadotrophin (HCG) (SI-CG10, 9002-61-3; Millipore Sigma, USA).

Experimental designs

In Group 1 (control), IVM MII COC were harvested 15 h after the HCG trigger from the oviducts, and freshly collected MII COC and spermatozoa were co-cultured *in vitro* for fertilization.

In Group 2, maturing MI COC were collected 7 h after HCG treatment, and gauge #30 needles were used to puncture tertiary follicles in the ovaries under dissection microscopy (*Chang et al., 2016*). The stage of a mouse oocyte (germinal vesicle COC, MI COC and MII COC) can be distinguished clearly by the morphology of the COC without denudation. Within Group 2, MI COC (7 h post HCG) alone were selected for conventional IVF. The spindles of mouse oocytes can be visualized using a regular optical microscope when mouse oocytes are at the metaphase stage (*Chang et al., 2016*). The MI COC (7 h post hCG) used were all showing visualized spindles after stripping the surrounding cells. In addition, the actual meiotic stage of the enclosed oocytes was validated using α-tubulin immunostaining and DNA staining (Supplementary Figure 1). For time-course analysis, the embryos were collected and fixed 1, 2, 4, 6, 15, 20 and 24 h post IVF.

In-vitro developmental competency was evaluated on day 4 post IVF. The schematic presentation of the study design is shown in FIGURE 1.

IVF and culture

IVF was performed as described by *Behringer et al. (2014)* with some modifications. Briefly, mature spermatozoa were obtained from the epididymis of a 10–12-week-old male ICR mouse. A cauda epididymis was removed and placed in a 3.5-cm Petri dish containing 200 ml of Sydney IVF Sperm Medium (K-SISM-50-AA; Cook Medical, USA) covered with mineral oil, then incubated for approximately 30 min at 37°C in humidified air containing 5% CO_2 to allow the spermatozoa to swim into the medium. IVF was conducted with COC and capacitated spermatozoa with a sperm concentration of approximately 1–2.5 × 10^6/ml in Sydney IVF Fertilization Medium (K-SIFM-20; Cook Medical) under mineral oil for 4 h in a 37°C incubator containing 5% CO_2. Next, cumulus cells were removed from COC, and denuded fertilized eggs (pronuclear stage) were transferred into Continuous Single Culture-NX Complete (CSCM-NXC; Fujifilm Irvine Scientific, USA) under mineral oil for 4 days. Pronuclear, 2-cell, morula and blastocyst stage embryos were recorded 1, 2, 3 and 4 days post culture, respectively. Preimplantation IVF embryos at different stages were collected and fixed, as shown in FIGURE 1. The cell number of blastocysts was evaluated by immunostaining.

Embryo transfer

Blastocyst stage embryos from both groups were transferred into the uteri of day 2.5 pseudopregnant ICR females mated with vasectomized males (Group 1 as control shown in Supplementary Figure 2). Embryos from Group 2 were collected from one surrogate mother on embryonic day 14 by caesarean section. The live pups were obtained through natural birth from recipient mothers around 17 days after transfer, and were raised by recipient mothers. Caesarean section was performed when any recipients from Group 2 were over their due date, with live pups raised by foster mothers. The fertility of offspring derived from Group 2 at 8

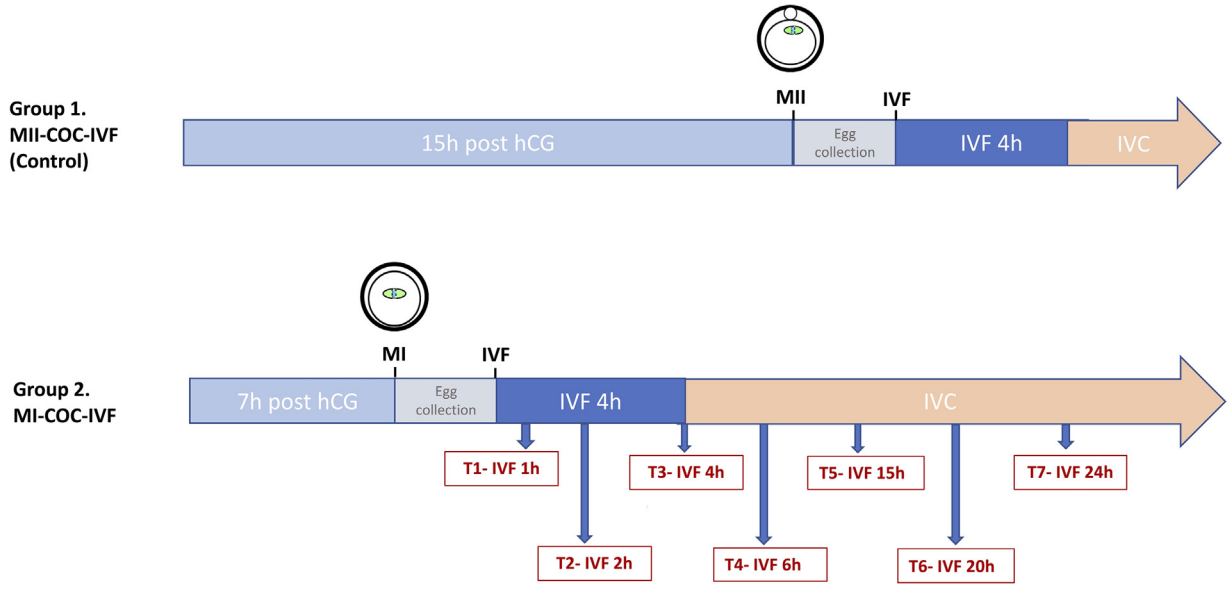


FIGURE 1 Schematic illustration of treatments in metaphase II (MII) cumulus–oocyte complex (COC) IVF (control) and metaphase I (MI) COC IVF groups. In Group 1 (MII COC IVF, control), MII COC were collected 15 h after human chorionic gonadotrophin (HCG) injection and used for IVF. The putative zygotes underwent in-vitro culture (IVC) for 4 days to check their developmental potential. In Group 2 (MI COC IVF), MI COC were collected 7 after HCG injection and used for IVF. Samples were collected 1, 2, 4, 6, 15, 20 and 24 h post IVF for time-course analysis (T1–T7). The putative zygotes underwent IVC for 4 days to check their developmental potential (pronuclear, cleavage, morula and blastocyst stage).

weeks of age was confirmed by mating with 8–12-week-old ICR mice.

Immunohistochemistry and laser scanning confocal microscopy

Mouse oocytes and embryos were collected and fixed with fresh 4% paraformaldehyde (PFA) in Dulbecco's phosphate-buffered saline (DPBS) for 10 min, and stored at 4°C until ready for processing. Permeabilization and blocking were achieved with 2.5% bovine serum albumin (BSA) in Tris-buffered saline solution supplemented with 0.25% Triton-X100 (Sigma-Aldrich, USA) for 20 min.

Metaphase spindle

Immunostaining of metaphase spindles was performed by incubation with the first antibody mouse anti-α tubulin (1:300 dilution, T-5168, RRID: AB_477579; Millipore Sigma, USA) in DPBS with 2.5% BSA (A9647, Sigma-Aldrich) and 0.25% Triton-X100 overnight at 4°C. After rinsing three times in 0.25% DPBS/Tween 20, samples were kept at room temperature for 1 h in the second antibody Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500 dilution, A11029, RRID: AB_2534088; Thermo Fisher, USA) and 4′,6-diamidino-2-phenylindole (DAPI) for 2 h. The negative control with the use of the secondary antibody alone is shown in Supplementary Figure 3.

5mC. and 5hmC

For immunostaining of 5-methylcytosine (5mC) and 5-hydroxy-methylcytosine (5hmC), fixed embryos were denatured with 2N HCl for 10 min and washed several times in DPBS. Next, embryos were incubated in anti-5hmC (1:200 dilution, GTX629765, RRID:AB_2736902; GeneTex, Taiwan) and anti-5mC (1:200 dilution, 28692, RRID: AB_2798962; Cell Signaling Technology, USA) antibodies in DPBS with 2.5% BSA and 0.25% Triton-X100 overnight at 4°C. After washing with DPBS, samples were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500, dilution, A11029, RRID: AB_2534088; Thermo Fisher), Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1:500 dilution, A21207, RRID: AB_141637; Thermo Fisher) and DAPI for 2 h.

Finally, samples were mounted on to glass slides in DPBS with 50% glycerol, and observed with laser scanning confocal microscopy (TCS SP5 II; Leica, Germany). Genomic methylation and DNA were visualized with immunostaining of 5mC and DAPI, respectively.

Mitotic chromosome spread

Mitotic phase embryos were collected following nuclear envelope breakdown approximately 18–20 h post IVF, and subsequently transferred into Tyrode's solution (Sigma-Aldrich) for 45 s to remove the zona pellucida. These embryos were then placed on slides coated with a thin layer of fixative [1% PFA, 0.15% Triton-X100 and 3mM dithiothreitol (Sigma-Aldrich) in H_2O, pH 9.2]. The slides were incubated at room temperature in a closed, humidified chamber overnight. After fixation, the slides were air-dried completely and washed several times with PBS. DAPI staining was performed, and samples were denatured with 2N HCl for 10 min. After washing with PBS, a subsequent immunostaining procedure was performed as described above (5mC and 5hmC staining), and chromosomes were counted manually.

Statistical analysis

Statistical analysis was undertaken using GraphPad Prism with one-way analysis of variance, followed by Tukey's test unless otherwise mentioned. *P*-values <0.05 were considered to indicate significance.

RESULTS

MI COC can be fertilized by IVF and support full-term embryo development

First, this study attempted to determine if MI COC can be fertilized in conventional IVF, and if so, if the resulting embryos are competent to support embryo development and full-term development.

Two groups of mouse COC collected at different time points following

superovulation (FIGURE 1) were used to represent MII COC and MI COC. Briefly, in Group 1, COC were harvested 15 h after HCG injection, and in Group 2, COC were collected 7 h after HCG injection. COC from both groups were subjected to IVF immediately.

The developmental potential of IVF embryos derived from COC in these two groups was compared. As shown in TABLE 1, 80 MII COC and 390 MI COC were collected and used for IVF. As expected, in Group 1, high fertilization and development rates were achieved: 95.2% pronuclear formation rate, 94.0% morula rate, and 91.9% blastocyst rate. However, unexpectedly, in Group 2, approximately 80% of oocytes were fertilized, followed by high morula (77.5%) and blastocyst (67.7%) rates, albeit significantly lower compared with Group 1 ($P = 0.0351$ for pronuclear formation rate, $P = 0.0151$ for morula rate, $P = 0.0039$ for blastocyst rate; FIGURE 2A–D). The morphology of pronuclear, cleavage, morula and blastocyst stage MI COC IVF embryos was akin to the embryos derived from regular IVF using MII COC (FIGURE 2A–D, Supplementary Figure 2). The average number of cells in blastocysts in Group 2 was significantly lower than that of Group 1 ($P < 0.05$). These results demonstrate that MI COC can be fertilized by IVF, and the resulting embryos can develop to blastocyst stage *in vitro*.

Next, embryo transfer was performed to evaluate the in-vivo developmental competency of embryos derived from Group 2. In total, 72 blastocysts from MI COC IVF were transferred to four recipient mice, resulting in 16 term pups (21.0%, FIGURE 2E). Although the term rate achieved by Group 2 oocytes was lower than that achieved by the Group 1 oocytes (75%), this is, to the authors' knowledge, the first evidence that MI COC IVF oocytes support full-term animal development. All the offspring of both sexes from Group 2 survived to adulthood (FIGURE 2F). Normal fertility was confirmed by selecting female and male mice for mating with 8–12-week-old ICR mice, which resulted in eight and 10 offspring, respectively.

Chromatin configurations of MI COC and developmental dynamics in the first 24 h following IVF

Once it was established that MI COC can be fertilized, the authors worked to understand the specific timing for sperm entry, and how spermatozoa interact with maturing oocyte cytoplasm, as well as the subsequent developmental dynamics of the first cell cycle when MI COC are used for IVF.

In Group 2, a total of 152 immature MI COC following IVF were collected at different time points and subjected to immunostaining. At 1 h ($n = 16$) post IVF, all the oocytes remained at the MI phase (FIGURE 3A). Unexpectedly, some spermatozoa started to penetrate MI (30.8%, $n = 4$, FIGURE 3B) and telophase I ooplasms at 2 h (53.8%, $n = 7$, FIGURE 3C) or 4 h (33.3%, $n = 5$) post IVF. Moreover, some swollen spermatozoa were found at 2 h (15.4%, $n = 2$) or 4 h (66.7%, $n = 10$, FIGURE 3D) post IVF, indicating decondensation of the sperm head by the cytoplasm of the maturing oocyte.

No typical MII oocytes were observed with spermatozoa in either group, implying that MII arrest was skipped when spermatozoa penetrated MI COC. Instead, at 6 h post IVF, 11.4% (4/35) of oocytes with condensed chromatins near the first polar body had swollen spermatozoa (FIGURE 3E), 74.2% (26/35) at telophase II had swollen spermatozoa (FIGURE 3F), 2.9% (1/35) at early pronuclear stage had swollen spermatozoa, and 11.4% (4/35) remained at telophase I with swollen spermatozoa.

The majority of embryos reached the pronuclear stage with a large pronucleus and a smaller pronucleus accompanied by two polar bodies (97.2%, 35/36, FIGURE 3G) at 15 h post IVF. One embryo reached syngamy (2.8%, 1/36, FIGURE 3H).

At 20 h post IVF, 54.5% ($n = 12$), 27.3% ($n = 6$) and 18.2% ($n = 4$) of embryos reached the pronuclear stage, syngamy and first mitotic metaphase cell cycle (FIGURE 3I), respectively.

All embryos cleaved to 2-cell embryos at 24 h post IVF ($n = 15$, FIGURE 3J). The developmental dynamics and interaction between the spermatozoon and maturing oocyte cytoplasm in MI COC following IVF over the first 24 h post IVF are shown in FIGURE 3K. The nuclear and cytoskeletal dynamics of the maturing MI oocyte in the first 24 h post IVF are summarized in FIGURE 3L.

Developmental arrest during gestation of MI COC IVF embryos

Term development in Group 2 was only one-third of that in Group 1. To gain

TABLE 1 DEVELOPMENTAL COMPETENCY OF MURINE METAPHASE II AND METAPHASE I CUMULUS–OOCYTE COMPLEXES FOLLOWING IVF

Group	No. of mice used for oocyte collection	No. of starting[a] oocytes	No. (%) of embryos with PN (based on starting oocytes)	No. (%) of early cleavage embryos (based on PN embryos)	No. (%) of morulae (based on PN embryos)	No. (%) of blastocysts (based on PN embryos)	No. of cells in a blastocyst	No. of embryo recipients	Total no. of blastocysts transferred	Total no. (%) of live births (based on blastocysts transferred)	No. of surviving pups[c]
Group 1 (MII COC IVF)	3	80	75 (95.2 ± 4.3)	73 (97.9 ± 1.8)	71 (94.0 ± 2.7)	69 (91.9 ± 2.0)	53.8 ± 14.6	1	12	9 (75.0)[b]	9
Group 2 (MI COC IVF)	7	390	300 (80.1 ± 9.6)	283 (94.2 ± 4.5)	235 (77.5 ± 8.8)	201 (67.7 ± 10.0)	39.7 ± 21.6	4	72	16 (21.0±8.8)	16
P-value			**0.0351**	0.2123	**0.0151**	**0.0039**	**0.0422**				

Percentage data were arcsine transformed and analysed using *t*-test in Graphpad Prism (v6.02). Mean and standard deviation are shown. Bold indicates significant *P*-values.

[a] MII oocytes for Group 1; MI oocytes for Group II.

[b] Standard deviation is not shown because only one recipient mouse was used in Group 1.

[c] Defined as number of pups that survive to adulthood, living beyond 8 weeks of age.

PN, pronuclear stage; COC, cumulus–oocyte complexes; MI, metaphase I; MII, metaphase II.

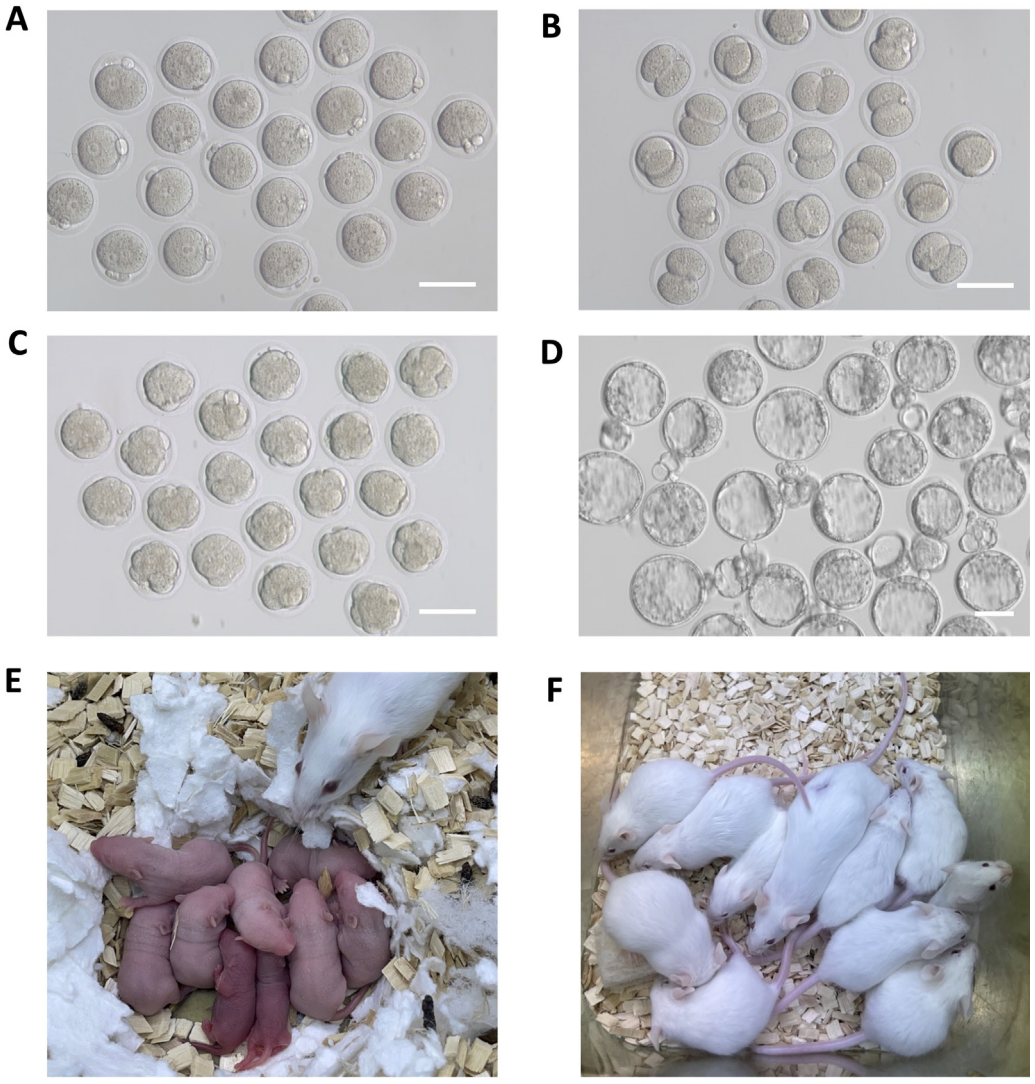


FIGURE 2 In-vitro and in-vivo developmental competency of metaphase I (MI) cumulus–oocyte complexes (COC) following IVF. Morphology of (A) pronuclear, (B) 2-cell, (C) morula and (D) blastocyst stage of MI COC IVF embryos after 1, 2, 3 and 4 days of culture, respectively. MI COC IVF offspring at (E) 2 days old and (F) 26 days old. Scale bars = 100 μm.

insights into the low term rate in the MI COC IVF group, a caesarean section was performed in the middle of gestation (embryonic day 14) on one recipient mouse that had received 22 MI COC IVF blastocysts. In total, 20 implantation sites were found. Only four fetuses were still alive, and the other 16 (80%) had arrested during gestation (TABLE 3, FIGURE 4A,B). In another recipient that received 14 MI COC IVF blastocytes, three live pups and six (66.7%) arrested fetuses were found after caesarean section on embryonic day 21, when the recipient was 2 days overdue (FIGURE 4C,D). These findings indicate that a substantial percentage (66.7–80%) of MI COC IVF embryos failed to develop to term after embryo transfer.

Substantial percentage of MI COC IVF embryos have abnormal pronuclear numbers

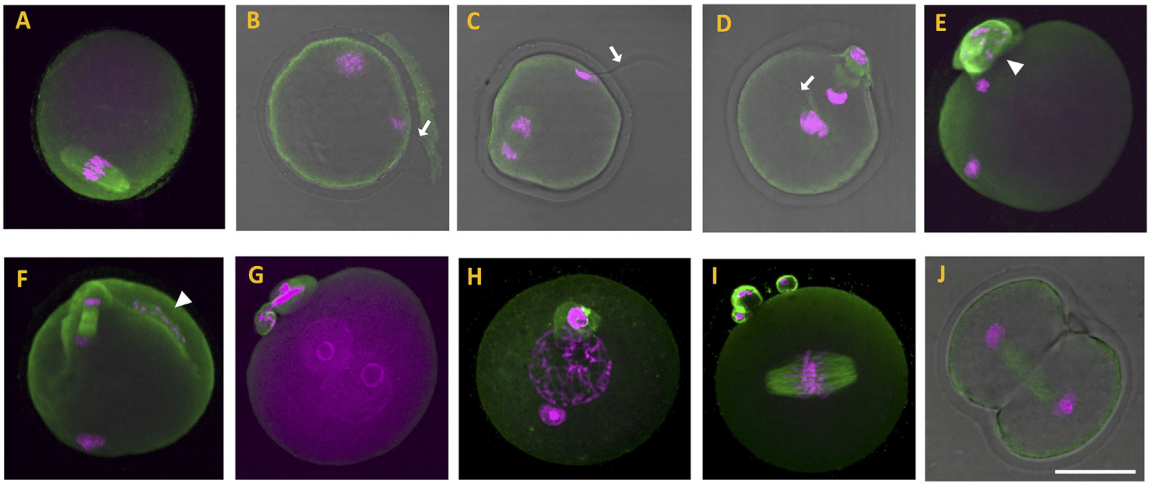
It was reasoned that the lower term rate of MI COC IVF embryos compared with MII COC IVF embryos may be attributed to at least two factors: (i) abnormal fertilization, leading to abnormal pronuclear numbers; and (ii) incomplete meiosis, leading to abnormal chromosome numbers.

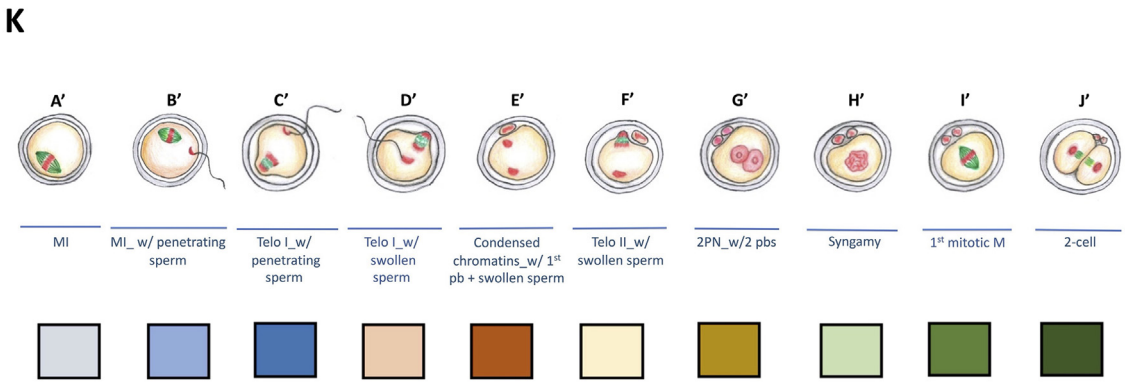
First, the pronuclear formation status of MI COC IVF embryos was investigated. In total, 216 MI COC were used for IVF, and 152 (70.4%) of them had formed pronuclei at 16 h post IVF. Among the embryos that formed pronuclei, the majority (n = 113, 74%) had two pronuclei, and had high cleavage (99%), morula (82%) and blastocyst (66%) rates. In the remaining embryos, 36 (24%) had only one pronucleus; these embryos had high cleavage rates (92%) but relatively low morula (39%) and blastocyst (28%) rates. A very small percentage (n = 3, 2%) of the embryos that formed pronuclei had three pronuclei. Interestingly, three-pronuclei embryos failed to develop into morula and blastocysts (TABLE 2).

These data indicate that a substantial percentage of MI COC IVF embryos have one pronucleus, likely a result of parthenogenetic activation.

Substantial percentage of MI COC IVF embryos have abnormal ploidy numbers

Fifteen two-pronuclear embryos derived from MI COC IVF were collected at 18–20 h post IVF for immunostaining to





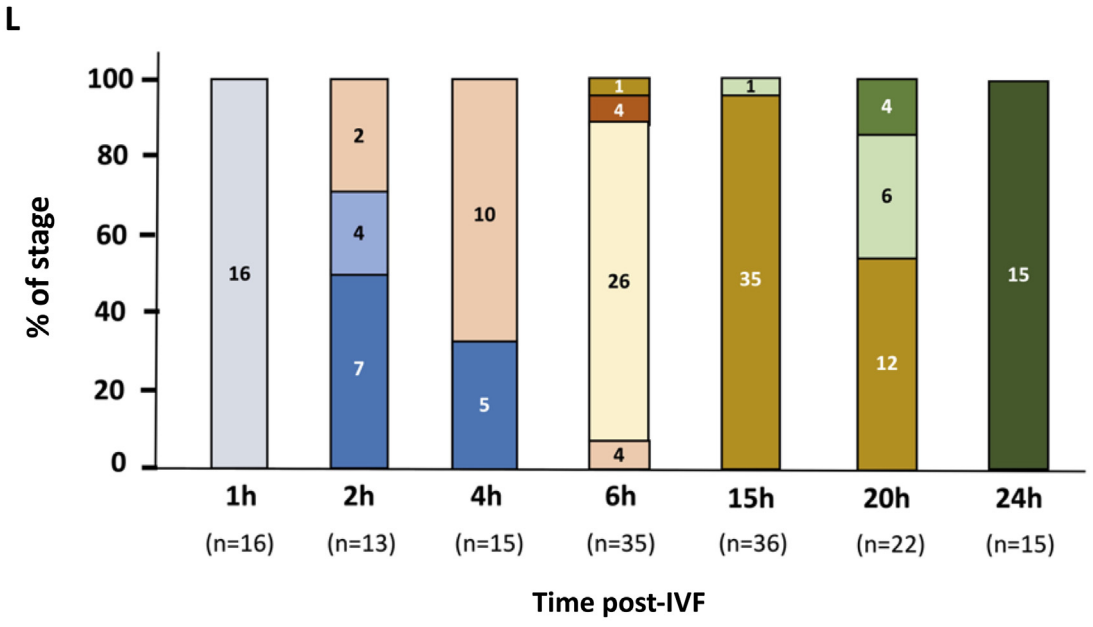


FIGURE 3 Interaction between spermatozoon and maturing oocyte cytoplasm and developmental dynamics in metaphase I (MI) cumulus–oocyte complexes (COC) following IVF over first 24 h. (A) MI oocyte, (B) MI oocyte with penetrating spermatozoon (arrow indicates sperm tail), (C) telophase I oocyte with penetrating spermatozoon (arrow indicates sperm tail), (D) telophase I (Telo I) oocyte with swollen sperm head (arrow indicates sperm tail), (E) condensed chromatins near the first polar body (indicated by arrowhead) with swollen spermatozoon, (F) telophase II oocyte with swollen spermatozoon (first polar body indicated by arrowhead), (G) pronuclear zygote with two pronuclei (2PN) and two polar bodies [(2)pbs], (H) syngamy, (I) first mitotic metaphase (1st mitotic M) embryo, and (J) 2-cell embryo in MI COC IVF group. Red, DAPI; green, microtubules. Scale bar = 50 μm. (K) Summary of developmental dynamics of MI COC IVF embryos over first 24 h; A'– J' and colour bars illustrate the phenomenon corresponding to A–J. (L) Distribution of developmental dynamics and interaction between spermatozoon and maturing oocyte cytoplasm in MI COC following IVF over first 24 h. Only fertilized oocytes were used for this figure. w/, with.

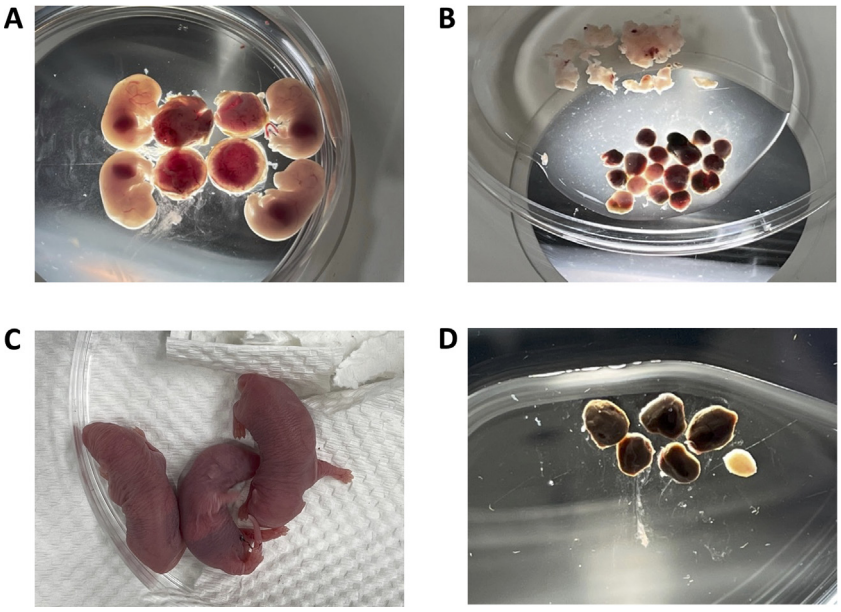


FIGURE 4 Arrested fetuses found at mid-gestation and at term in metaphase I (MI) cumulus–oocyte complex (COC) IVF group. Four live fetuses with their placentas (A) and 16 arrested fetuses (B) were collected by caesarean section from the recipient on day 14. Three healthy pups (C) and six arrested fetuses (D) were collected by caesarean section on day 21 when the gestational recipient was 2 days overdue.

determine the levels of 5mC (red) and 5hmC (green) in male and female pronuclei of MI COC IVF embryos. As shown in FIGURE 5A, a higher 5mC signal was detected in the female pronucleus but not in the male pronucleus. In contrast, 5hmC was shown in the male pronucleus of the two-pronuclear embryos.

By combining DNA with 5mC staining, the paternal and maternal chromosomes can be counted and distinguished at the moment of nuclear envelope breakdown. Most MI COC IVF embryos were found to contain diploid parental genomes (65.8%, $n = 25$, FIGURE 5B). However, other embryos had abnormal chromosome numbers: 7.9% ($n = 3$) had a chromosome count of 50, and 26.3% ($n = 10$) had a chromosome count of 60 (26.3%, $n = 10$). In those embryos with 60 chromosomes, the maternal genome showed a higher 5mC signal than zygotes containing 40 chromosomes, indicating that the second meiotic division could be missed in these MI COC IVF embryos (FIGURE 5C).

DISCUSSION

In IVF practices, it is documented that up to 20% of oocytes collected after superovulation are immature (*Moon et al., 2023*; *Vanhoutte et al., 2005*). These immature oocytes are generally discarded for intracytoplasmic sperm injection (ICSI), a process in which embryologists have the opportunity to judge the maturation status after removing the cumulus cells from the oocytes. However, in conventional IVF practice, such pre-fertilization evaluation cannot be made because COC, with intact cumulus cell complexes, are co-incubated with spermatozoa to allow fertilization. Hence, the question remains whether MI COC can be fertilized in conventional IVF, and if so, can they – and at what percentage – support embryo development and, ultimately, term development?

The current work provides evidence using a mouse model that maturing oocytes (MI to telophase I) can be fertilized in conventional IVF (FIGURE 2). Further, this study demonstrated that embryos derived from MI COC IVF support pre- and post-implantation embryo development, with blastocyst and term rates >60% and >20%, respectively (TABLE 1).

Mammalian embryonic development is initiated by the successful activation of an oocyte by a fertilizing spermatozoon. The fusion of additional spermatozoa with the oocyte is prevented by the early events of

TABLE 2 DEVELOPMENTAL COMPETENCY OF MURINE METAPHASE I CUMULUS–OOCYTE COMPLEX IVF EMBRYOS CONTAINING DIFFERENT NUMBERS OF PRONUCLEI

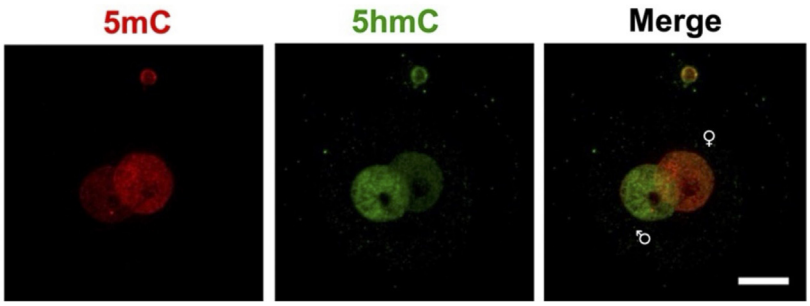
No. of MI used	No. of embryos with PN formed (%)	No. of PN type		No. (%) of cleaved (% of PN)	No. (%) of morula (% of PN)	No. (%) of blastocyst (% of PN)
216	152 (70.4%)	1PN	36	33 (91.7)[a]	14 (38.9)[a]	10 (27.8)[a]
		2PN	113	112 (99.1)[a]	93 (82.3)[b]	74 (65.5)[b]
		3PN	3	3 (100)[a]	0[c]	0[a]

Percentage data were arcsine transformed and subjected to Tukey's multiple comparisons tests.

[a,b,c] Different superscripts within the same column indicate signidicant differences ($P < 0.05$). Detailed comparisons are provided in Supplementary Table 1.

MI, metaphase I; PN, pronucleus(pronuclei).

A



B

PN type	No. of PN used	Chromosome number (n)		
		20	40	>40
		No. of embryos (%)		
2PN	38	0	25 (65.8)	13 (34.2)

C

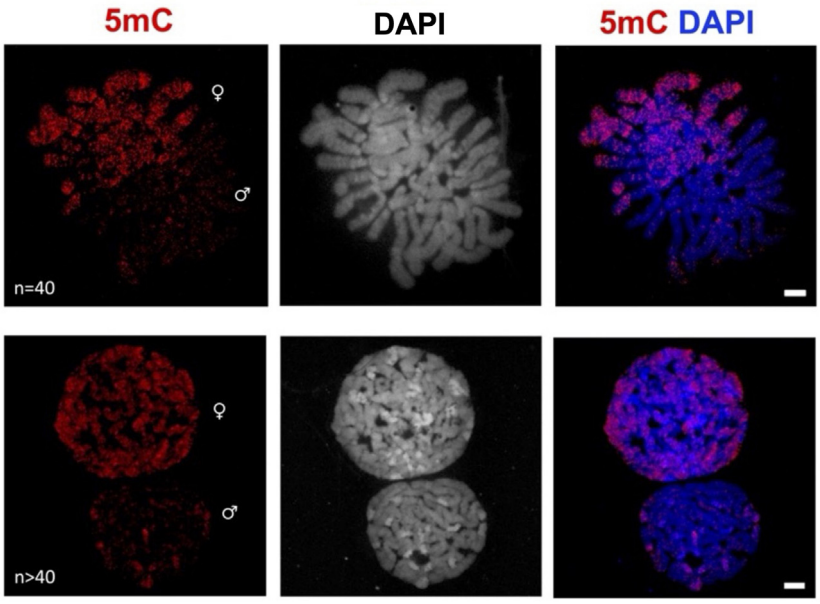


FIGURE 5 Pronuclear number, DNA methylation, and ploidy status of metaphase I (MI) cumulus–oocyte complexes (COC) following IVF. (A) The DNA methylation status of 5-methylcytosine (5mC) and 5-hydroxy-methylcytosine (5hmC) in two-pronuclear (2PN) zygote. Red, 5mC; green, 5hmC. ♂, paternal pronuclei; ♀, maternal pronuclei. Scale bar = 20 μm. (B) Measure of the chromosome number of 2PN zygotes. Expected normal chromosome number = 40. (C) Representative 5mC expression and chromosome number analysis in 2PN zygotes. Red, 5mC; blue, DAPI. Scale bar = 5 μm.

cortical granule exocytosis and subsequent block of the zona pellucida to polyspermy. The ability of oocytes to undergo cortical granule exocytosis arises very late in oocyte maturation, before ovulation and after the completion of oocyte growth (*Ducibella et al., 1990*, *1993*; *Ducibella and Buetow, 1994*). The ability to prevent polyspermic fertilization develops during the oocyte-to-egg transition (*Berrios and Bedford, 1979*; *Iwamatsu and Chang, 1971*; *Mehlmann and Kline, 1994*; *van der Ven et al., 1985*); cortical maturation is a component of cytoplasmic maturation during the oocyte-to-egg transition, and the egg cortex has to be appropriately primed and tuned to be responsive to a fertilizing spermatozoon (*Kryzak et al., 2013*). After fertilization, dynamic epigenetic modification of the genome, such as genome-wide DNA demethylation, involves global TET3-mediated oxidation of 5mC to 5hmC in the paternal pronucleus (*Iqbal et al., 2011*; *Santos et al., 2002*; *Zhang et al., 2012*). This methylation asymmetry between the maternal and paternal pronucleus allows the male and female pronuclei of MI COC IVF embryos to be distinguished using 5mC and 5hmC immunostaining. This study found that the polyspermy rate was low, as evidenced by the low ratio of multi-pronucleus embryos (>2) (**TABLE 2**) when MI COC were used for IVF. It is very likely that the ability of oocytes to undergo cortical granule exocytosis is functional to prevent the

entry of multiple spermatozoa into the oocyte.

This study showed that sperm penetration at the maturing MI stage did not disrupt the completion of meiosis I and extrusion of the first polar body. Sperm penetration at the maturing MI stage was found to trigger oocyte activation and pronuclear formation, and progression to the first mitotic division was similar to regular MII oocyte fertilization. These results have clearly demonstrated that it is possible for maturing MI oocytes to be fertilized *in vitro* prior to ovulation. These results are also supported by the findings of others, which demonstrated that neither nuclear nor cytoplasmic maturation is required for sperm penetration and sperm head decondensation in MI oocyte cytoplasm. *Eppig et al. (1994)* demonstrated that prepubertal immature oocytes (18 days old) arrested prematurely at MI upon insemination could extrude the first polar body, form pronuclei, cleave, and develop to blastocyst stage. Sperm penetration can trigger oocyte activation when oocytes are arrested prematurely at MI, and fail to complete polar body extrusion. Those prematurely arrested MI oocytes treated with calcium ionophore also underwent parthenogenetic activation, and the pattern of protein synthesis was comparable to that in MII oocytes. This phenomenon occurred without the need for the oocyte to complete its first meiotic division in LT/sv mice; female mice regularly ovulate both primary (MI) and secondary (MII) oocytes (*O'Neill and Kaufman, 1987*).

Strassburger et al. (2004) demonstrated that spermatozoa could be injected into the cytoplasm of 'MI oocytes' (represented by the absence of a polar body and no discernible germinal vesicle nucleus) using the human ICSI model. In their study, the outcomes of 'MI oocytes' that were injected with spermatozoa immediately after denudation were compared with the outcomes of rescue IVM oocytes (MI–MII, oocytes retrieved as 'MI oocyte' stage and matured to the stage showing the presence of a polar body when left in culture). Interestingly, 28% of the 'MI oocytes' injected with spermatozoa immediately after denudation had two pronuclei. These results indicated that a proportion of oocytes can be activated when spermatozoa are introduced forcefully into the cytoplasm of immature human oocytes (*Bilibio et al., 2021*; *Strassburger et al., 2004*). The live birth of a healthy baby from a blastocyst formed after ICSI of an MI oocyte has been documented (*Bilibio et al., 2021*). The finding of pronuclear formation after human 'MI oocyte' ICSI coincided with the outcome found in mouse conventional IVF in the present study, where sperm entry into MI COC also triggered oocyte activation. However, the lower two-pronuclei rate from human 'MI oocyte' ICSI was possibly due to the undefined stage of immaturity, spanning from germinal vesicle breakdown to MI when spermatozoa were injected. Hence, the injection of rescue IVM oocytes is preferred to the injection of 'MI oocytes' as rescue IVM oocytes have a higher fertilization rate (44 versus 28%) and a higher rate of embryo development (*Strassburger et al., 2004*). Indeed, the outcome of immature oocytes in ICSI can be improved by rescue IVM. Therefore, the utilization of human 'MI oocytes' was primarily employed after rescue IVM (*Bilibio et al., 2021*; *De Vincentiis et al., 2013*; *Friden et al., 2005*; *Mandelbaum et al., 2021*; *Moon et al., 2023*; *Shu et al., 2007*; *Strassburger et al., 2010*; *Vanhoutte et al., 2005*). The developmental competence of human 'MI oocytes' with delayed maturation *in vitro* (rescue IVM model) has been widely documented in terms of indicators such as fertilization, blastocyst formation, euploidy and live birth rate (*Bilibio et al., 2021*; *De Vincentiis et al., 2013*; *Moon et al., 2023*; *Shu et al., 2007*). On the contrary, MI COC were not cultured to wait for polar body extrusion before insemination in the present study. A specified stage of mouse MI oocytes was used to conduct conventional IVF. This study found that the timing of sperm penetration occurs between MI and telophase I (**FIGURE 3**). Two hours post insemination, the sperm head can be found in the cytoplasm of MI oocytes (**FIGURE 3**). After penetration, the spermatozoon initiates the cascade of events that depletes maturation promoting factor activity, and promotes entry into anaphase II (*Hashimoto and Kishimoto, 1988*; *Weber et al., 1991*). The results presented in this study show that the spermatozoon started to decondense in the first few hours after penetration of the maturing cytoplasm (**FIGURE 3B–F**). M-phase cytoplasm is generally permissive for sperm chromatin dispersion and recondensation, and assembly of histones (*Maleszewski et al., 1999*; *McLay and Clarke, 2003*). However, the extensive decondensation of the chromatin within the male pronucleus and the import of nuclear proteins requires transition from M phase to interphase. The presence of the sperm head in the cytoplasm of the oocyte can force the cell cycle from metaphase towards interphase; as such, no oocytes arrested at MII were observed, as sperm penetration had occurred.

Based on the chromosome staining and chromosome counting outcomes, it became apparent that a proportion of the fertilized maturing oocytes may end up being triploid due to failure to extrude a second polar body (**FIGURE 5C**). However, it is unclear how some embryos with a diploid chromosome complement complete their second meiotic divisions without progressing through MII. It remains to be tested which factors contributed to the difference. Interestingly, the same phenomenon with the generation of triploid and diploid embryos was observed in a previous study, where sperm penetration was found to trigger oocyte activation when oocytes were arrested prematurely in MI (*Eppig et al., 1994*). One possibility is that the subtle difference in timing of sperm entry may affect the ploidy outcome of fertilization while meiotic maturation is ongoing, as sperm entry could occur both prior to and after extrusion of the first polar body.

Also, the in-vivo outcome of maturing MI COC following IVF was investigated, and the estimated implantation rate was 80.5% (29 implantation sites out of 36 embryos transferred) based on the caesarean section outcome of two gestational carriers; this is very close to the live birth rate of the control group using MII COC for IVF (75%; 9/12) (**TABLE 1**). However, approximately 75.8% (22 developmental arrests out of 29 implantation sites found in the caesarean section) of the implanted embryos were arrested and absorbed during gestation (**TABLE 3**). This indicates that a proportion of the implanted embryos derived from MI COC IVF were genetically abnormal (**FIGURE 5B**). To ascertain whether this is the case, the ploidy evidence was confirmed by the outcomes of chromosome staining and chromosome number counting, and 34% of the tested embryos were found to be genetically abnormal (77% of the abnormal embryos showed digynic triploidy) (**FIGURE 5B**).

It should be noted that the animal model used in this study does not represent all IVF situations. Ovarian stimulation for patients undergoing IVF often results in

TABLE 3 DEVELOPMENTAL ARREST DURING GESTATION IN GROUP 2

ET no.	No. of recipients used	No. of blastocysts transferred	No. of alive fetuses or pups (%)[a]	No. of arrested embryos (%)[b]	No. of implantation sites[c] (%)[d]
MI-D14	1	22	4 (20)	16 (80)	20 (90.9)
MI-D21	1	14	3 (33.3)	6 (66.7)	9 (64.3)
Total	2	36	7 (24.1)	22 (75.8)	29 (80.5)

Data presented as *n* (%).

[a] % = alive fetuses/implantation sites.

[b] % = arrested embryos/implantation sites.

[c] No. of implantation sites = alive fetuses + arrested embryos.

[d] % = implantation sites/blastocysts transferred.

ET, embryo transfer; MI-D14, caesarean section performed on embryonic day 14 of gestation in Group 2; MI-D21, caesarean section performed on embryonic day 21 of gestation in Group 2.

unsynchronized follicular growth. Hence, aspiration of small follicles that contain immature eggs in clinical IVF is not unusual. In the present study, maturing MI mouse oocytes were retrieved 7 h after HCG injection. At this stage, the follicles are relatively small, thus mimicking the situation of aspirating immature eggs from small follicles, but not those which are already fully developed, in fertility clinics. The primary objective of this study was to investigate whether maturing MI oocytes can be fertilized by spermatozoa, and support embryonic development; and the model system provided insights. The results strongly support the notion that MI COC can be fertilized and support term development, albeit with much lower success rates than those achieved by fully matured MII COC. Therefore MI COC may represent a valuable source of 'usable' biomaterial in assisted reproduction.

DATA AVAILABILITY

Data will be made available on request.

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AUTHOR CONTRIBUTIONS

C.C (Ching-Chien Chang) and L.S. conceived and designed the study. M.P., C.W. and L.S. performed the experiments. L.T., C.C (Chia-Chun Chang), C.C (Chin-Cheng Chien) and C.L (Chia-Jung Li) contributed to the data collection and analysis. Z.N. and C.L. (Chi-Hong Liu) provided the discussion, interpreted the data, and revised the manuscript. C.C. (Ching-Chien Chang), J.X., C.L. (Chung-Hao Lu) and L.S. interpreted the data and wrote the article. All the authors approved the final version of the manuscript.

SUPPLEMENTARY MATERIALS

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