Research Square

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Analysis of the genetic material of immature oocytes derived embryos after short-term insemination in vitro

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Abstract

Fertilization is a very complex process, and efforts to understand its mechanisms are ongoing. Our retrospective analysis showed that immature oocytes could develop into blastocysts after routine in vitro insemination. The incidence of immature oocytes developing into blastocysts was about 0.59%. The source of genetic material and clinical use value of these embryos are still controversial. Herein, quantitative parental contamination test (qPCT) was used to analyze the genetic material of immature oocyte embryos with potential parental contamination. Fluorescence in situ hybridization (FISH) was used to detect XY chromosomes to further validate the sequencing results. After short-term insemination of immature oocytes, the sperm has entered the immature oocytes, and complete fertilization after immature oocytes further mature. Sequencing and FISH indicated that the incidence of pronuclear zygote abnormality was significantly increased, and most of them were maternal alloploidy, which may be related to the asynchronous maturation of oocyte nuclei rather than polyspermia. Our results may serve as a basis for further studies to investigate the mechanism of cortical reaction and fertilization.

INTRODUCTION

Fertilization is a critical biological process. In this process, a capable sperm recognizes the oocyte and binds to the zona pellucida (ZP), fuses with the egg membrane and activates the oocyte. In a series of coordinated molecular and cellar events that culminate with the generation of a unique zygote(1, 2, 3, 4, 5, 6). For the zygote, in addition to providing half of the chromosomal genes, the sperm also contribute centrioles to the zygote. The oocyte contains enzymes, mRNAs, organelles, and metabolic substrates that are crucial for fertilization and early embryonic development(7, 8). Oocyte development competence is usually considered to be the ability of a female gamete to mature. Oocyte maturation is characterized as the resumes and complete the first meiotic division, under the surge of luteinizing hormone (LH) from the pituitary or external source triggers with accompanying chromosome condensation, spindle formation and extrusion of the first polar body(9, 10). Therefore, mature oocyte is essential in fertilization and embryonic development, and even pregnancy.

Oocyte maturation in vivo is thought to be the result of natural selection. During the process of controlled ovarian hyperstimulation (COH), the administration of supra-physiologic doses of exogenous gonadotropin induces multiple follicles growth and maturation to obtain a considerable number of available oocytes, and subsequently increase the chance of pregnancy(11, 12). Nearly 85% of mature oocytes at retrieval, whereas the remaining 15% are still at the metaphase I(MI) or germinal vesicle （GV）stage (MI [4%] or GV [11%])(13). In general, the immature oocytes are usually discarded, but some of the immature oocytes may extrude the first polar body during in vitro culture and be considered for intracytoplasmic sperm injection (ICSI). The duration of meiosis maturation of human oocytes in vitro is about 20–22 h. In previous studies, immature oocytes obtained in ICSI cycles were cultured in vitro matured (IVM) to MII, and sperm injected to obtain more embryos after the oocytes matured(14, 15, 16). There have been many reports of successful pregnancy of immature oocytes through in vitro maturation and ICSI insemination(17, 18).

In the clinical work of assisted reproductive technology (ART), some oocytes were immature and discard after denudation for 4–6h, or 16–18h after routine IVF. However, we have occasionally observed the development of immature oocytes into blastocysts after IVF. It is not clear what happens to immature oocytes during the IVF cycle. We know that mature oocytes can ensure that only one sperm enters. Na^+ influx reaction is activated within 1-3s after sperm contact with the egg membrane to prevent polyspermia. At the same time, cortical granules react to harden the zona pellucida (zona pellucida reaction) under intracellular Ca^{2+} excitation, which provides a protective layer for the zygotic and the embryo, preventing the recombination and penetration of excess sperm. Whether these immature oocytes could be fertilized and triggered Na^+ influx reaction and cortical granules reaction to prevent polyspermia or parthenogenesis due to in vitro manipulation. It is not clear whether embryos developed from these oocytes are clinically useful.

The purpose of this study is to investigate the development competence of immature oocytes-derived embryos in short-time insemination. (Fig. 1).

MATERIALS AND METHODS

Patients and data collection

First, this study collected and analyzed data from 1504 ART patients who underwent routine short-term insemination. Subjects included female patients aged at least 20 years who underwent IVF procedures between 2017 and 2022 in the Second Affiliated Hospital of Fujian Medical University. 5957 embryos developed to the blastocyst stage were included. Morphological evaluations were performed on days 0, 1, 3, 5, and 6 of the culture. All of the morphological evaluations at the cleavage and blastocyst stages were conducted by two of the authors.

On day 3, the embryo quality was assessed through a combination of cell number, blastomere size, and degree of fragmentation. Grade I embryos (good quality) were defined as eight-cell embryos with evenly sized blastomeres and no more than 5% fragmentation. Grade II embryos (fair quality) were deemed 7–10-cell embryos with minor defects in blastomere size or moderate fragmentation (10%–15%). Grade III embryos (poor quality) were embryos with any two of the following defects: cell number < 7 or > 10, abnormally sized blastomeres, and a fragmentation rate of ≥ 15%. Grade IV embryos (extremely poor embryo) were embryos with any two of the following defects: cell number ≤ 4, abnormally sized blastomeres, and a fragmentation rate of ≥ 25%. Embryos with severe fragmentation (≥ 50%) or those with a combination of more than two major defects were not considered for transfer.

The Gardner grading system (1) was utilized for the blastocyst assessment. The degree of expansion (1: the blastocoel is < 50% volume of the embryo; 2: the blastocoel is ≥ 50% volume of the embryo; 3: the blastocoel fills the whole embryo; 4: the blastocoel volume is increased with zona thinning; 5: the TE partially escapes from the zone; 6: the blastocyst completely hatches from the zona), the morphology of the ICM (A: many cells, tightly packed; B: fewer cells, loosely packed; C: very few cells), and the morphology of the TE (A: a cohesive epithelium with many cells, typically > 12 cells at the equator of the blastocyst; B: a loose epithelium with fewer cells, typically 7–12 cells at the equator of the blastocyst; C: very few large irregularly sized cells, typically < 7 cells at the equator of the blastocyst) were evaluated accordingly. Blastocysts with poor morphological scores (≤ CC) or low expansion grades (grades 1–2) were not considered

for cryopreservation or transfer. To facilitate the comparison of the various results, quality categories similar to those employed in a previous study (4) were used in the present study: top quality: AA; good quality: AB and BA; average quality: AC, CA, and BB; and poor quality: BC and CB; Unavailable quality: CC or grades 1–2.

Immature oocyte embryos biopsy

In order to investigate the in vitro fertilization of immature oocytes, we collected embryos that developed further after insemination. All Oocytes were cultured in vitro for 2–4 hours before insemination. Oocytes denudation were performed immediately after 4–6 hours insemination. Fertilized oocytes were checked for the release of the second polar body and perivitelline space (PVS) following the removal of cumulus cells. GV oocytes had the presence of germinal vesicle. MI oocytes were represented by the absence of a PB and no discernible GV nucleus. Immature oocytes for GV or MI were incubated in cleavage medium for 3 days. Immature oocyte derived embryos with pronucleus formation or further development will be biopsied.

We collected 9 samples: C-1 ~ C-9. C-1 was observed to be MI post insemination 4-6h, D1 developed to MII, and biopsy was performed after multiple pronuclei were observed in D3. C-2 was observed to be GV in D0, MI in D1, and D3 was biopsied after multiple pronucleus occurrences. C-3 was GV in D0, progressed to MI in D1, and was biopsied after two pronucleus appeared in D3. C-4 was GV in D0, developed to MI in D1, and biopsy was performed after two pronuclei were observed in D3. MI was observed for C-5 in D0, MI in D1, and D3 was biopsied after two pronucleus occurrences. C-6 was MI in D0, progressed to MII in D1, and was biopsied after two pronucleus appeared in D3. C-7 was GV in D0, developed to MI in D1, and biopsy was performed after two pronuclei were observed in D3. C-8 was observed to be GV in D0, MI in D1, and D3 was biopsied after multiple pronucleus occurrences. C-9 was MI in D0, progressed to MII in D1, and biopsy was performed after 2Cell were observed in D3 (Table 1) .

Table 1
Evaluation and WGA results of immature oocyte derived embryos after routine short-term insemination. (n = 9)

Sample ID	Post-insemination 4-6h	Day1	Polar number	Day3	WGA results(ng/ul)
C-1	MI	MII	1	> 3PN	18.56
C-2	GV	MI	0	> 3PN	1.23
C-3	GV	MI	0	2PN	20.43
C-4	GV	MI	0	2PN	18.06
C-5	MI	MI	0	2PN	33.15
C-6	MI	MII	1	2PN	36.25
C-7	GV	MI	0	2PN	11.36
C-8	GV	MI	0	3PN	19.01
C-9	MI	MII	1	2Cell	18.29

Biopsy was performed in 10 µl drops of G-MOPS-PLUS (Vitrolife, Sweden). Zygote or cleavage embryo cytoplasm biopsy was performed with the laser method. Before the operation, biopsy dishes and embryo protective fluid tubes (Yikon, Shanghai, China) were prepared and labeled with patient’s name, medical record number and embryo number. The zona pellucida was perforated by a laser for 2.0 ms, after which the zona pellucida gap was induced before biopsy to loosen cell connections. By entering the zona with the micropipette, the cytoplasm was aspirated with soft suction or slight tugs. The biopsied cells were washed and placed in 0.2 ml PCR tubes with embryo protective fluid. The couple's saliva was collected and used to trace the origin of parental DNA in the biopsy cells. All the samples stored at − 20°C until further processing.

Table 2
Karyotype analysis of 8 immature oocyte derived embryos.(n = 9)

sample ID	Karyotype result
C-1	XX,-2p(pter→p22.3,~35Mb,×1,mos,~52%)
C-2	N/A
C-3	XY,+Xp(×2,mos,~34%),+Xq(q21.1→q28,~73Mb,×2,mos,~39%)
C-4	XX
C-5	XY,+Xp(×2,mos,~44%),+Xq(×2,mos,~46%)
C-6	XY,+X(×2,mos,~41%)
C-7	XY,+Xp(×2,mos,~43%),+Xq(q11.1→q21.31,~28Mb,×2,mos,~43%),+Xq(q22.1→q28,~56Mb,×2,mos,~49%),-15(×1,mos,~63%),+16q(q12.1→q22.1,~
C-8	47,XY,+15(×3),-21(×1,mos,~48%),+22(×3,mos,~37%)
C-9	XX,-16p(p13.3→p12.2,~20Mb,×1,mos,~38%),-20p(p12.2→p11.23,~11Mb,×1,mos,~34%)

"N/A" indicates that the test results of this sample do not meet the quality control standards, and the condition of embryo chromosomes cannot be judged. T uncontrollable objective factors such as sample loss, biopsy sample fragmentation or denuclearization during the biopsy process.

FISH analysis

In this part, we collected 5 samples for FISH analysis: E-1 ~ E-5. E-1 was observed to be GV post insemination 4-6h, D1 developed to MI, two pronuclei were observed in D3. E-2 was MI in D0, progressed to MII in D1, D3 was multiple pronucleus. E-3 was MI in D0, MII in D1, and wo pronuclei were observed in D3. E-4 was observed to be GV in D0, MI in D1, and D3 was multiple pronucleus. E-5 was MI in D0, progressed to MII in D1, and 4Cell were observed in D3 (Table 3) .

Table 3
Characterization and sex chromosome karyotype analysis of five immature oocyte derived embryos.(n = 5)

Sample ID	Post-insemination 4-6h	Day1	Polar number	Day3	FISH results
E-1	GV	MI	0	2PN	X
E-2	MI	MII	1	3PN	XY
E-3	MI	MII	1	2PN	XXY
E-4	GV	MI	0	> 3PN	XY
E-5	MI	MII	1	4Cell	N/A
“N/A” indicates that the sample fails to be detected.					

For FISH analysis, glass slides were immersed in denaturant solution for 2 min (70% formamid/2× SSC). After drying the slides, a mixture of fluorescence-labeled probes specific for DXZ1 (CEP X spectrum green, VYSIS) and DYZ3 (CEP Y spectrum red, VYSIS), were applied. Slides were then hybridized at 37°C for 15–20 h. After washing, chromosomes were counterstained by DAPI (blue). Slides were examined under a fluorescence microscope equipped with a triple band pass filter. X and Y chromosomes were identified by green and red fluorescence, respectively. If two signals of the same color, size and intensity were separated by at least one domain, disomy was diagnosed.

Whole-genome amplification of biopsied cells

Single-cell whole genome amplification (WGA) was performed for 9 samples of immature oocytes embryos. The multiple annealing and loop-based amplification cycle (MALBAC)-based single-cell WGA kit (Cat. No. KT110700324, Yikon Genomics Ltd, Suzhou, China) was utilized to amplify DNA from biopsied cells according to the manufacturer’s instructions.

Determination of immature oocyte embryos ploidy status by NGS

To analyse the ploidy status of the immature oocyte embryos, the amplified DNA of the biopsied samples was sequenced using a NextSeq 550 sequencer (Cat No. SY-415-1002, Illumina, Inc., USA) with a single-ended read length of 55 bp. Approximately 2 million raw reads were generated for each sample. Genome-wide CNVs were analysed to determine the euploid or aneuploid status of each embryo. Embryos were diagnosed as aneuploid when the size of the CNVs was greater than 4 Mb and the extent of mosaicism was above 30%.

Genotyping assay of immature oocyte embryos and parental genomes

To determine the genotype of each immature oocyte embryos and parental genome, the Infinium Asian Screening Array (ASA) bead chip (Cat No. 20016317, Illumina, Inc., USA) and iScan system (Cat No. SY101-1001, Illumina, Inc., USA) were utilized. Parental genomic DNA (gDNA) was extracted from peripheral oral exfoliating cells. A commercial genomic DNA extraction kit (DNeasy Blood & Tissue Kit, Cat. No. 69504, Qiagen, USA) was used according to the manufacturer's instructions. Signal scanning was performed with the iScan system. The Iaap-cli gencall algorithm (Version 1.1.0, Illumina, Inc., USA) was applied to analyse the genotype of each sample, and the B allele frequency (BAF) and log R ratio (LRR) values of all SNPs were generated simultaneously.

Determination of parental contamination by BAF analysis

As shown in (19), parental genetic contamination can be measured by BAF analysis. For an SNP with paternal AA and maternal BB genotypes or paternal BB and maternal AA genotypes, the genotype of the embryo is genetically expected to be heterozygous AB, with a BAF of approximately 0.5. Deviation from a BAF of 0.5 in an embryo may be caused by parental contamination, DNA copy number aberrations or allelic amplification bias and errors (e.g., allele dropout or the preferential amplification of one allele) resulting from single-cell WGA technology. For detailed methods, we refer to reference (19).

Statistical analyses

This study involves descriptive analysis and standard statistical methods of percentage. Statistical analyses comparing groups were performed using GraphPad Prism version 9.0.0 (GraphPad Software, Inc., San Diego, CA, USA). Trends in the proportion of embryos formation were evaluated using Chi-square test for trend. Error bars indicate SEM where presented, and P-values of < 0.05 were considered significant.

RESULTS

Development of embryos derived from immature oocytes in vitro fertilization

By retrospective analysis of 5957 embryos developed to blastocyst stage, we found that 35 of the blastocysts were derived from immature oocytes (GV or MI), accounting for 0.59% (35/5957). On day 3, the high-quality embryo (include good quality and fair quality) formation rate was 42.86% (15/35), which was significantly lower than that of mature oocyte derived embryos 72.49% (4293/5922) (42.86% *vs* 72.49%, $P < 0.001$). Blastocysts derived from these immature oocytes in vitro insemination, the blastocyst formation rate of Day5 was 88.57% (31/35), which showed no statistical difference compared with that of mature oocytes 89.4% (5294/5922). In addition, there was no difference in the rate of good quality blastocyst (include top quality, good quality and average quality) formation and available blastocyst compared with the mature oocyte group 34.28% (12/35) *vs* 36.0%(2132/5922) and 82.86% (29/35) *vs* 79.2% (4690/5922), respectively. These results suggested that in vitro fertilization of immature oocytes could develop into blastocyst stage with low incidence. However, once a blastocyst was formed, the rate of blastocyst formation and the quality of blastocyst were no different from those of mature oocytes. A list of these embryos derived from immature oocytes and associated results is presented in Supplementary Table 1.

Scoring and grading of immature oocyte after fertilization

To analyze the in vitro fertilization potential of immature oocytes and reveal the possibility of parthenogenesis of these embryos, we collected embryos that developed further after insemination of immature oocytes. Oocytes denudation was performed immediately after 4–6 hours insemination. Immature oocytes for GV or MI were incubated in cleavage medium for 3 days. Further development of 14 immature oocytes was observed, 2 of which were developed into the 2 cells and 4 cells stage, while 7 arrested at the 2 pronucleus(2PN), 5 had multiple pronucleus. The scoring of zygotes and embryos derived from immature oocytes were provided in Table 1 and Table 3.

Quantitative Analysis of WGA DNA in biopsied cells

To explore the genetic material of immature oocyte derived embryos after routine in vitro fertilization, we analyzed 9 samples of biopsied cells that were derived from Day 3 of immature oocyte embryo. The DNA concentration was assessed in each sample after WGA and was found to range from 11.36 to 36.25 ng/ul (Table 1). Regular protocols for single-cell analysis would be efficient for analyses.

Detection of parental DNA contamination in biopsied cells

In total, 8 biopsied cell samples that yielded successful WGA results were sequenced for aneuploidy analysis. Thus, to examine the origin DNA of immature derived embryos, SNP sequencing was performed on sample triads. qPCT has been proved to be an accurate and reproducible method for verifying parental contamination of biopsy cells(19). In the present study, qPCT was used to detect parental genetic material of immature oocyte embryos. Interestingly, all immature oocyte embryos with further development had maternal alloploidy except for one embryo with aneuploidy (Fig. 2 and Table 2). Moreover, the Y chromosome was detected in 4 embryos (Fig. 2 and Table 2). This result illustrated that these immature oocytes could receive spermatogenic signals for

further development. The chromosomal abnormalities in immature oocyte derived embryos were more likely to be due to maternal heteroploidy than polyspermia. This may be due to incomplete meiosis and immature nucleus of immature oocyte.

FISH staining with a CEPX/CEPY probes for biopsied cells

The application of the FISH technique helped to reassess the fertilization status of immature oocytes and highlighted the differences between the initial diagnosis obtained by cell sequencing and the application of specific probes. FISH staining with a CEPX/CEPY probes were performed for biopsied cells to preliminarily confirm the sperm into the immature oocyte. Using FISH techniques for 5 immature oocyte derived embryos 2 among three showed evidence of sex chromosomal abnormalities. Y chromosome was detected in 3 of them, which further proved that sperm can participate in the embryonic development of immature oocytes (Fig. 3). One biopsy cell failed with no results.

DISCUSSION

About 15 ~ 20% of retrieved oocytes from controlled ovarian stimulation cycles are immature, either at metaphase I (MI) or germinal-vesicle (GV) stage, in human IVF(20, 21). These oocytes are usually discarded due to the possibility of abnormal embryonic development. However, the use of immature oocytes for IVF is important in order to increase the number of embryos obtained in each cycle. The present of varying in vitro oocyte maturation (IVM) culture techniques and procedure can save some immature oocytes. Some of these oocytes may extrude the first polar body during in vitro culture, and may be used as a source of oocytes for sperm injection in ICSI cycles. Oocyte maturation in vitro is a relatively fast event that can occur shortly after oocyte retrieval(Balakier, Sojecki et al. 2004). There seems to be no agreement regarding the optimal incubation period for maturation. There have been many reports of successful pregnancy through ICSI after in vitro maturation of GV or MI oocytes obtained from stimulation cycles(Kim, Lee et al. 2000). ICSI of germinal vesicles (GVS) and metaphase I (MI) oocytes has been attempted in mammals and humans. Although most embryos develop with impaired function, ICSI MI still develops blastocysts(Strassburger, Friedler et al. 2004, Shu, Gebhardt et al. 2007).

However, in the clinical work of ART, we have occasionally observed the development of immature oocytes into blastocysts after IVF. Some immature oocytes observed after denudation also developed into embryos after routine in vitro insemination. In this study, we retrospectively analyzed 5957 embryos developed to blastocyst stage, and found that 35 blastocysts were derived from immature oocytes (GV or MI). Compared with mature oocyte-derived blastocysts, we found that immature oocytes can develop into blastocysts in vitro fertilization with a low incidence, accounting for 0.59% (35/5957). However, once a blastocyst is formed, the rate of blastocyst formation and the quality of the blastocyst are not different from those of mature oocytes, supplementary Table 1. Immature oocytes can develop further, such as pronucleus formation and cleavage. It is not clear whether the embryos derived from this part are normally fertilized or parthenogenetic. The utilization value of embryos formed from immature oocytes during routine IVF was reported rarely. The clinical use of embryos derived from GV or MI oocytes still mystery.

Some studies have shown that some immature oocyte parthenogenesis can be activated by promoting ovulation and in vitro culture(Rola, Zanetti et al. 2021, Akbari, Foruozandeh et al. 2023). To further analyze the IVF potential of immature oocytes and reveal the possibility of parthenogenesis of these embryos, we collected embryos that developed further after fertilization of immature oocytes, hoping to explain part of the problem through genetic sequencing. We found a high rate of abnormal pronucleus formation in these embryos, suggesting that these oocyte nucleus maturation disorder. The genetic material of these pronuclear zygotes was analyzed by gene amplification and NGS. Interestingly, five of the 2PN pronuclear zygotes were maternal alloploidy. One 3PN embryo was a normal ploidy. This suggests that the pronuclear zygote of this part of embryo does not correspond to the ploidy of genetic material. In addition, the Y chromosome was detected in four embryos, suggesting that fertilization was occurring in these immature oocytes rather than parthenogenesis. These results suggest that embryos derived from immature oocytes might have clinical application value during routine in vitro fertilization.

It has been suggested that cortical particles may appear during maturation of immature oocytes in vitro, preventing subsequent sperm fusion. During IVM, the pericortical particle density decreased with the increase of meiosis ability(Kulus, Kranc et al. 2020). Our results showed that in immature oocyte embryos, except for one embryo with aneuploidy, all embryos had maternal heteroploidy (Fig. 2 and Table 2), and FISH results confirmed this conclusion (Fig. 2 and Table 2). Chromosomal abnormalities in immature oocyte derived embryos were more likely to be due to maternal heteroploidy than polyspermia. This might be due to incomplete meiosis and nuclear immaturity of immature oocytes.

In conclusion, our results suggest that: First, abnormal pronucleus formation does not represent abnormal genetic material. Second, immature oocytes can undergo normal in vitro fertilization, which may depend on the mature state of cytoplasm and nucleus. Third, routine in vitro fertilization of immature oocytes into normal embryos may have clinical application value and is worth further discussion. Fourth, sperm can enter immature oocyte after short-term insemination in vitro, and the physiological process of fertilization needs to be further studied.

Declarations

Ethics approval

Approval for this study was obtained from the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University ([2022] Fuyi Medical Ethics Review Number (430)). All samples were obtained from the department of Reproductive Medicine of the Second Affiliated Hospital of Fujian Medical University, and all the patients signed the informed consent form before any study-specific procedures were performed.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

H. performed assisted reproductive technology; W. designed the study; J. analyzed the data and wrote the paper. Z. prepared the original draft. S. collected samples and clinical data of the immature oocyte embryos; H. prepared the figure 1 and 2. J. prepared the table 1 and 2. All authors read and reviewed the final manuscript.

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Availability of data and materials

The data that support the study are available upon reasonable request to the corresponding author.

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Figures

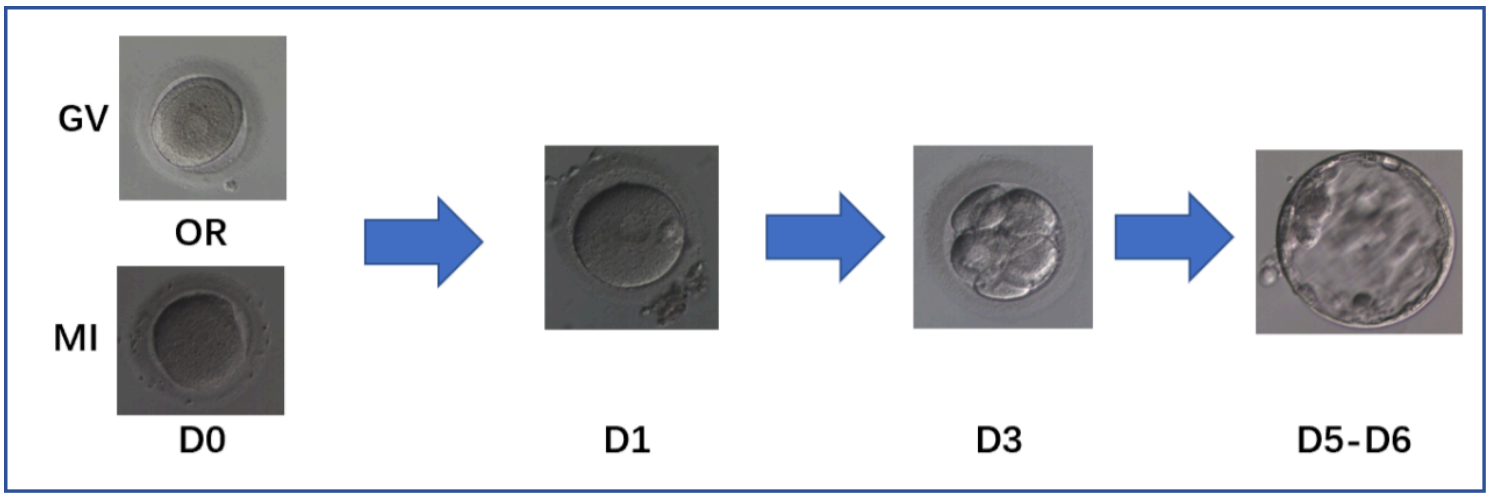


Figure 1

Pattern of embryo development after insemination of immature oocyte.

D0: 4-6 h after short insemination. D1: 17-20h after short insemination. D3: 68-72h after short insemination. D5-D6: 116-140h after short insemination.

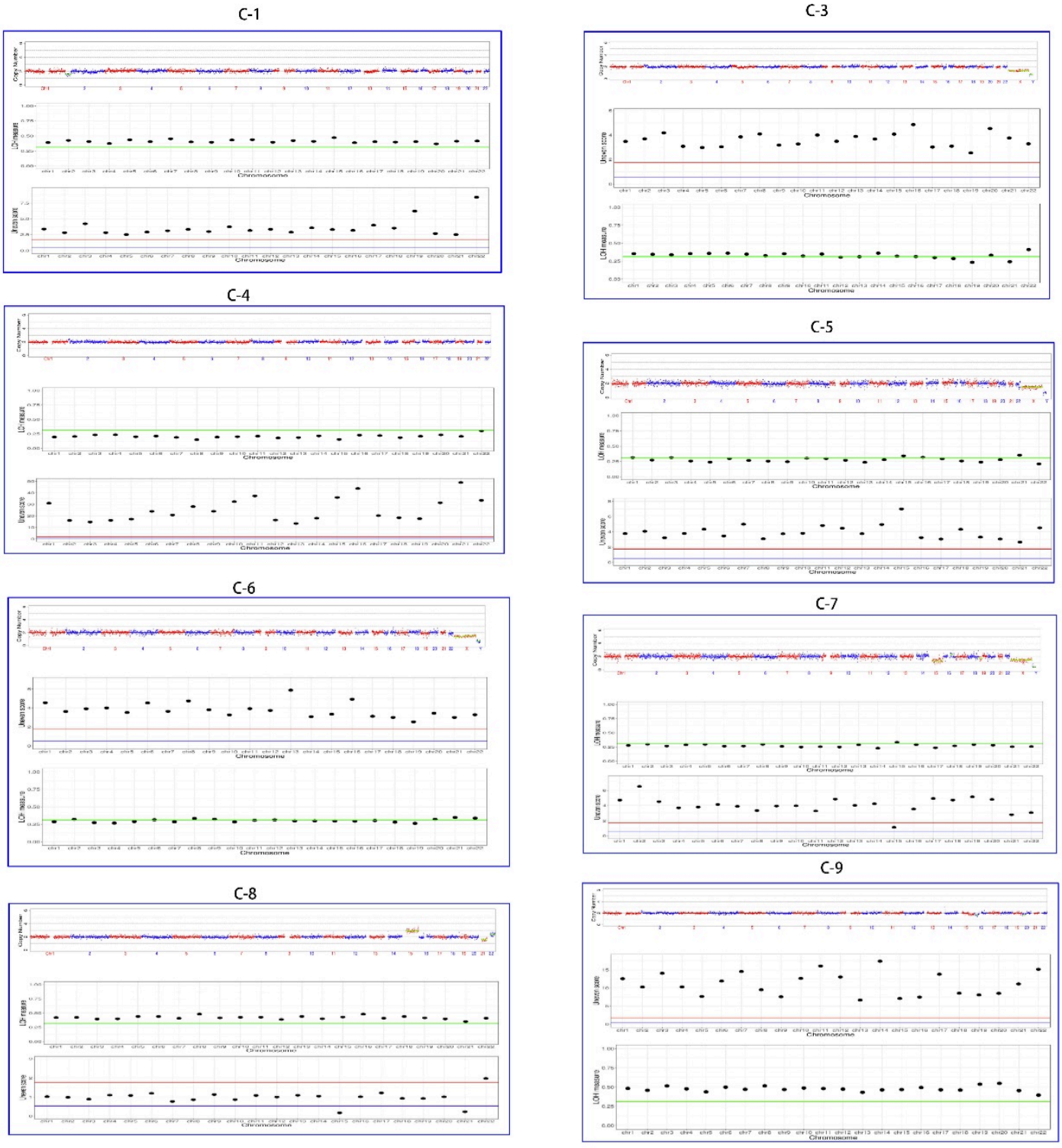


Figure 2

Molecular genetic analysis (including isoploid analysis) of 8 immature oocyte derived embryos. Mos(mosaic) indicates suspected chimerism, while CNV indicates possible biological reasons for chimerism: a. The sample itself is chimeric; b. Chimerism caused by sampling operations; c. granulosa cell contamination (common in X and Y chromosome copy number abnormalities); d. Cells are in the replication phase, and the replication of different chromosomes is not synchronous; e. Other unknown causes; Uneven score: Based on the normal diploid sample, the number of maternal preference sites/number of paternal preference sites, with the red and blue lines representing the threshold of maternal preference and paternal preference, respectively. LOH score: When the maternal source and the paternal source are homozygous sites of different types, the proportion of heterozygous sites in the sample, and the green line represents the threshold of heterozygous degree.

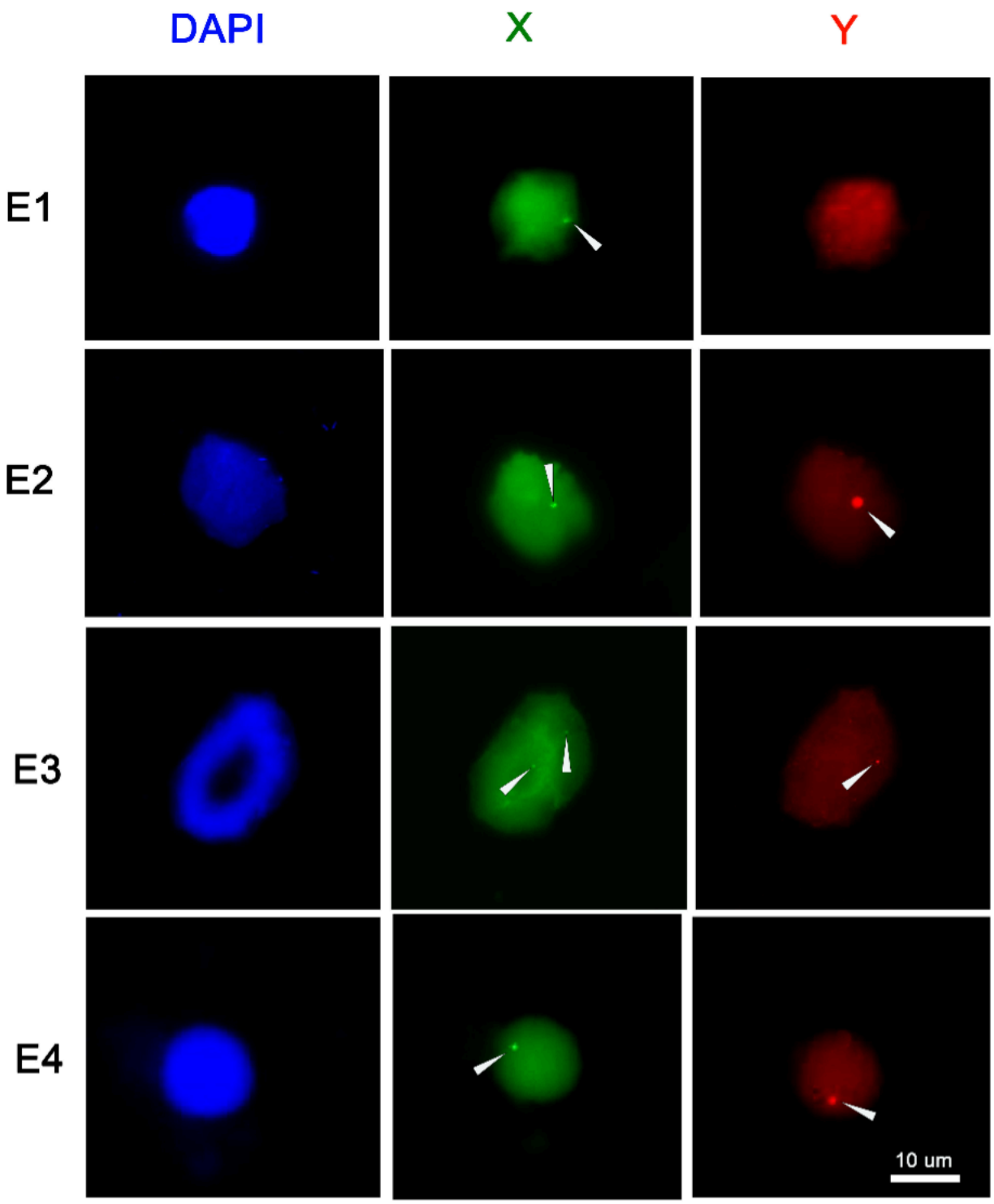


Figure 3

Karyotype analysis of sex chromosomes in immature oocyte derived embryos by FISH. DAPI was the nucleus, and the fluorescent highlights were X and Y chromosomes respectively, in which green represented X chromosome and red represented Y chromosome. Scale bars, 10 μm.