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Investigating the genetic material sources of abnormal prokaryotes formed by different in vitro fertilization methods

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Abstract

Objective

Abnormal fertilization is relatively common in human assisted reproductive technology (ART), resulting in a decrease in the number of available embryos. This study aims to explore the genetic material of abnormal fertilized eggs from different artificial insemination methods and their clinical application value.

Methods

A retrospective analysis was conducted on 2979 patients from our center from June 2016 to December 2025. 2117 patients received short-term in vitro fertilization (IVF), and 862 patients received conventional IVF treatment. The laboratory and clinical results of the two groups were compared. The fertilization status of oocytes was observed 16–18 hours after fertilization, and biopsy was performed on 3PN fertilized eggs. The source of parental DNA in the biopsy cells was traced using NGS combined with SNP sequencing technology.

Results

The 2PN rate (73.3% vs. 63.7%, $P < 0.001$) and blastocyst formation rate (78.3% vs. 75.8%, $P < 0.001$) in the conventional IVF group were significantly higher than those in the short-term IVF group. The ≥3PN rate in the conventional IVF group was lower than that in the short-term IVF group (4.6% vs. 5.2%, $P = 0.018$). We collected 76 3PN fertilized eggs, of which 9 failed to be detected. Among the remaining 67, 26 were normal diploid fertilized eggs (38.8%), 1 was a monozygotic diploid fertilized egg (1.5%), and the rest 40 were parental triploid fertilized eggs (59.7%): 34 of the abnormal genetic material came from the father (85%), and 6 came from the mother (15%). In short-term IVF, 16 cases (84.2%) were paternal triploid, and 3 cases (15.8%) were maternal triploid; while in conventional IVF, 18 cases (85.7%) were paternal triploid, and 3 cases (14.3%) were maternal triploid.

Conclusion

There was no significant difference in clinical pregnancy rates between short-term IVF and conventional IVF. However, the 2PN rate and blastocyst formation rate in short-term IVF were significantly lower than those in conventional IVF, and the incidence of 3PN fertilized eggs increased. Multiple sperm fertilization remains the main cause of 3PN formation. Interestingly, the study found that not all 3PN embryos have chromosomal abnormalities, and the mechanism of abnormal pre-implantation formation needs further research.

Introduction

Fertilization represents a fundamental biological prerequisite for successful human pregnancy[1]. Despite decades of investigation, the molecular and cellular mechanisms governing this highly orchestrated process remain incompletely understood. During natural conception, thousands of spermatozoa traverse the female reproductive tract to reach the oocyte; however, stringent physiological barriers-including the cumulus-oocyte complex (COC) and the zona pellucida (ZP)-ensure that typically only a single sperm achieves zona penetration, oolemma fusion, and subsequent oocyte activation[2]. Successful fertilization is morphologically confirmed by extrusion of the second polar body (2PB) and the synchronous formation of two distinct pronuclei (2PN)-one paternal and one maternal, within the ooplasm. This cascade entails tightly regulated biochemical events, including calcium oscillations, cortical granule exocytosis, and resumption of meiosis, culminating in syngamy and initiation of embryonic development [1].

In clinical assisted reproductive technology (ART), total fertilization failure (TFF) remains an unpredictable and distressing complication, occurring in approximately 1–5% of conventional IVF cycles[3–5]. Although late-rescue intracytoplasmic sperm injection performed 18–22 hours post-insemination can salvage unfertilized oocytes, resulting embryos exhibit markedly reduced developmental competence and poor clinical outcomes-including significantly lower blastocyst formation, implantation, and live birth rates[3, 6–12]. Evidence suggests that short-term insemination followed by early rescue ICSI performed 4–6 hours post-insemination may mitigate oocyte aging and improve fertilization outcomes[13, 14]. The impact of early cumulus removal on fertilization fidelity-particularly the incidence of abnormal fertilization remains controversial. Some studies have reported an increased incidence of tri-pronuclear (3PN) zygote formation following cumulus cell removal at 4 hours post-insemination, suggesting that premature oocyte exposure to sperm may predispose to polyspermy[15–17]. Conversely, Xiong et al.[18] observed a reduction in polyspermy with early denudation, while several other studies found no significant difference in ≥3PN rates between protocols[19–21]. These inconsistencies underscore the need for mechanistic clarification beyond morphological assessment alone.

Critically, conventional fertilization scoring relies exclusively on pronuclear morphology and cannot distinguish chromosomal ploidy status or parental origin of genetic material. Consequently, abnormally fertilized oocytes-especially those exhibiting ≥3PN-have historically been discarded without cytogenetic validation, despite emerging evidence that a subset may harbor euploid, biparental, or even viable genomic configurations. Therefore, this study applied next-generation sequencing (NGS) coupled with single-nucleotide polymorphism (SNP)-based haplotyping-using matched parental saliva-derived DNA-to systematically characterize chromosomal ploidy status and parental genomic origin in abnormally fertilized zygotes.

This study aimed to evaluate the clinical relevance of abnormally fertilized oocytes and to investigate whether the duration of sperm-oocyte co-incubation influences fertilization outcomes, and subsequent

clinical efficacy.

MATERIALS AND METHODS

Research subjects

A single-center retrospective cohort study was conducted at the Center for Assisted Reproduction, Second Affiliated Hospital of Fujian Medical University. A total of 2,979 patients undergoing IVF treatment between November 2016 and October 2025. Inclusion criteria were: (1) female age < 38 years; (2) first fresh IVF cycle. The study design is illustrated in Fig. 1.

Samples collection

A total of 76 3PN zygotes derived from patients undergoing IVF procedure were collected between 2023 and 2024. All patients provided voluntary, written informed consent for the inclusion of their 3PN zygotes in this study. The study protocol was reviewed and approved by the Second Affiliated Hospital of Fujian Medical University.

Pronuclear assessment and biopsy of the 3PN zygotes

All oocytes underwent in vitro culture for 2–4 h prior to insemination. Cumulus cells were removed either at 4–6 h (short-term group) or at 16–18 h (conventional group) post-insemination. Extrusion of the second polar body was used as an early morphological indicator of fertilization. Specifically, 4 hours post-insemination, cumulus–oocyte complexes were denuded, and oocytes were immediately transferred to fresh cleavage medium for assessment of fertilization based on the presence of a fully extruded second polar body. Pronuclear morphology assessment and documentation were performed 18–20 hours post-IVF using an inverted microscope. Biopsy was carried out in 10 μL drops of G-MOPS-PLUS medium (Vitrolife, Sweden), For 3PN zygotes designated for biopsy, the zona pellucida was opened using laser-mediated drilling(OCTAX NaviLase). Subsequently, the two polar bodies were aspirated and discarded via a biopsy pipette, followed by gentle cytoplasmic aspiration to achieve complete detachment of the cytoplasm from the zona pellucida (Fig. 2). Biopsied samples were washed and transferred into sample protective tubes (Yikon, Shanghai, China). Each tube was individually labeled with the corresponding patient's name. Concurrently, parental saliva samples were collected for genomic DNA extraction to enable parental origin tracing. All samples were stored at − 20°C until processing.

Copy number variation haplotype analysis

This study employed a robust preimplantation genetic testing for aneuploidy combined with haplotype analysis protocol, adapted for MGI high-throughput sequencing platforms. Genomic DNA extracted from buccal epithelial cells and biopsy samples was subjected to whole-genome amplification using the

ChromSwift method (Yikon Genomics Co., Ltd., Suzhou, China). Sequencing libraries were constructed through enzymatic fragmentation, end-repair, adapter ligation, and PCR enrichment, followed by conversion to fulfill MGI platform requirements for DNA nanoball generation and paired-end 150-bp (PE150) sequencing. Raw sequencing data were processed using the ChromGo bioinformatics pipeline for quality filtering, copy number variation identification, and SNP genotyping. For copy number variation (CNV) analysis, the reporting criteria included whole-chromosome-level abnormalities and segmental abnormalities greater than 4 Mb. For mosaic abnormalities, reporting required both a minimum segment size of ≥ 10 Mb and an estimated mosaicism level > 30% (Fig. 3).

Parental origin analysis of chromosomal aberrations in tripronuclear zygotes

Based on Mendelian inheritance principles, a customized analytical strategy was established to interpret embryo SNP genotypes and detect chromosomal abnormalities. Within this framework, the maternal B-allele frequency was defined to assess parental allele contributions, with characteristic deviations indicating specific karyotypic anomalies—monosomies exhibited mBAF values of 0 (maternal loss) or 1 (paternal loss) accompanied by loss of heterozygosity (LOH), while trisomies showed mBAF values around 0.33 (paternal gain) or 0.67 (maternal gain) (Fig. 4). Additionally, informative SNP sites were utilized to distinguish between mitotic and meiotic error origins based on heterozygosity patterns. Kinship assessment was performed using SNP-derived kinship coefficients, with quality control monitored through allele dropout rates and Mendelian inconsistency events (Fig. 5). Haplotype phasing was further applied to trace the parental origin of chromosomal aberrations.

Statistical analysis

Quantitative variables were presented as mean ± standard deviation (SD) and compared by Student's t test. Categorical variables were presented as % (n) and compared by the Chi-squared test or Fisher's exact test as appropriate. All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS, Version 27.0, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Compare the effects of different insemination methods on embryo fertilization, development and implantation rate

In total, 2979 ovarian stimulation cycles were analyzed: conventional IVF (n = 862), short-term IVF (n = 2117).

Table 1 presents the baseline demographicof patients undergoing conventional IVF versus short-term IVF. There were no significant differences were observed between groups in terms of age, BMI, basal

FSH level, serum estradiol levels on the day of hCG administration, total FSH dosage and duration of stimulation. In the overall cohort, the duration of infertility was significantly longer in short-term IVF group than conventional IVF group (3.8 ± 2.5 vs. 2.7 ± 3.2, *P*<0.001). The proportion of primary infertility in the short-term IVF group was significantly higher compared to the conventional IVF group (65.9% vs. 9.7, *P*<0.001).

Table 1
Baseline characteristics of the patients.

Parameter	**Short-time insemination (group A)**	**Conventional insemination (group B)**	***P* value**
No. of patients	2117	862	
Female age (years)	31.1 ± 3.4	31.3 ± 3.5	0.127
Female BMI (kg/m^2)	22.4 ± 3.5	22.4 ± 3.2	0.761
Duration of infertility(years)	3.8 ± 2.5	2.7 ± 3.2	**<0.001**
Basal FSH (mIU/ml)	7.7 ± 3.6	7.6 ± 3.2	0.457
Type of infertility %(n)			**<0.001**
Primary	65.9(1396/2117)	9.7(84/862)	
Secondary	34.1(721/2117)	90.3(778/862)	
Total FSH dosage (IU)	2705.6 ± 1005.2	2715.8 ± 975.1	0.797
Duration of stimulation (days)	11.1 ± 2.2	11.1 ± 2.3	0.641
Estradiol level on the hCG day(pg/ml)	3604.6 ± 2552.8	3572.3 ± 2453.7	0.747

Across the entire cohort, the 2PN rate (73.3% vs. 63.7%, *P*<0.001) and blastocyst formation rate (78.3% vs. 75.8%, *P*<0.001) in the conventional IVF group were both significantly higher than those in the short-term IVF group (Table 2). At the same time, there was difference between the two groups in ≥3PN rate. The conventional IVF group had lower ≥3PN rate than that in the short-term IVF group (4.6% vs. 5.2%, *P* = 0.018). No significant differences were observed among the groups in terms of the number of retrieved oocytes, number of embryos transferred, the rate of 1PN, the embryo cleavage rate and the rate of high-quality embryos (Table 2). Table 3 presents clinical outcomes following fresh embryo transfer in the conventional IVF and short-term IVF groups. A total of 325 and 734 fresh embryo transfer cycles were performed in the conventional IVF and short-term IVF groups, respectively. No significant differences were observed between groups in biochemical pregnancy rate, clinical pregnancy rate, implantation rate, miscarriage rate, ongoing pregnancy rate, and multiple pregnancy rate.

Table 2
Comparison between the two different types of insemination

parameter	Short-time insemination (group A)	Conventional insemination (group B)	*P* value
No. of oocyte retrieved	12.1 ± 6.8	12.2 ± 7.0	0.651
No. of embryo transferred	1.3 ± 0.5	1.3 ± 0.5	0.779
Two pronuclei rate %(n)	63.7(16255/25523)	73.3(7702/10502)	**<0.001**
≥ Three pronuclei rate %(n)	5.2(1329/25523)	4.6(484/10502)	**0.018**
One pronuclei rate %(n)	3.8(963/25523)	4.1(431/10502)	0.139
Cleavage rate %(n)	98.4(15992/16255)	98.6(7597/7702)	0.180
High-quality embryo rate %(n)	44.1(7169/16255)	45.4(3494/7702)	0.067
Blastocyst rate %(n)	75.8(8202/10827)	78.3(4092/5224)	**<0.001**

Table 3
Pregnancy outcomes of fresh embryo transfer

parameter	Short-time insemination (4 h group)	Conventional insemination (16–18 h group)	*P* value
No. of patients	734	325	
Clinical pregnancy rate %(n)	59.1(434/734)	57.8(188/325)	0.696
Biochemical pregnancy rate %(n)	61.7(484/784)	59.9(205/342)	0.570
Ongoing pregnancy rate %(n)	53.8(422/784)	54.4(186/342)	0.862
Miscarriage rate %(n)	14.7(64/434)	12.8(24/188)	0.515
Implantation rate %(n)	47.4(497/1048)	47.1(217/461)	0.900
Multiple pregnancy rate %(n)	14.5(63/434)	16.0(30/188)	0.643

Quantitative Analysis of WGA DNA in biopsied cells

To explore the genetic material of abnormal fertilization oocytes after routine in vitro fertilization, we successful analyzed 67 samples of biopsied cells that were derived from Day 1 of abnormal fertilization oocytes. The DNA concentration was assessed in each sample after WGA. Regular protocols for single-cell analysis would be efficient for analyses.

Detection of parental DNA contamination in biopsied cells

In all, 67 biopsied cell samples with successful WGA results were sequenced for aneuploidy analysis. Therefore, in order to examine the origin DNA of abnormal fertilization oocytes, SNP sequencing was performed on sample triads. qPCT has been shown to be an accurate and repeatable method for verifying parental contamination of biopsy cells [22].

Seventy-six ≥3PN zygotes were collected from our center, of which 9 failed to be detected. Interestingly, among the remaining 67 zygotes, 26(38.8%) were diploid and biparental, 1 (1.5%) was diploid and biparental, and 40 (59.7%) were parental triploid (Fig. 6A). Of the triploid cases, paternal excess accounted for 34 (85.0%) and maternal excess for 6 (15.0%), confirming polyspermy as the predominant mechanism of 3PN formation. Stratified by insemination timing: among short-term IVF-derived 3PN zygotes (n = 19), 16 (84.2%) were paternal triploid and 3 (15.8%) maternal triploid; among conventional IVF-derived 3PN zygotes (n = 21), 18 (85.7%) were paternal triploid and 3 (14.3%) maternal triploid (Fig. 6B).

Discussion

In the conventional IVF procedure, sperm and oocytes were co-cultured for 16–18 h, and the cumulus cells were then removed to assess fertilization status[23]. Prolonged oocyte exposure to sperm may, however, subject them to adverse effects of sperm metabolites-such as reactive oxygen species (ROS)-which can impair fertilization and embryonic development[24–27]. Findings regarding early cumulus cell removal are inconsistent. Some studies have reported comparable rates of normal (2PN) fertilization, 3PN formation, and clinical pregnancy between early cumulus cell removal and standard removal at 16–18 hours[28–30]. Lundqvist et al. observed a significantly lower 3PN rate in the early (2-hour) cumulus cell removal group compared to the 20-hour removal group[31]. A meta-analysis, however, found no significant differences in normal fertilization rates or 3PN rates between short-term and conventional IVF[32]. In the present study, the 2PN rate(63.7% vs. 73.3%, $P < 0.001$), blastocyst formation rate（75.8% vs. 78.3%, $P < 0.001$）of the short-term IVF group was found to be significantly lower than that of the conventional IVF group.

Our data clearly show that the short-term group was composed predominantly of patients with primary infertility (65.9% vs. 9.7%, $P < 0.001$) and a significantly longer duration of infertility (3.8 vs. 2.7 years, $P < 0.001$). Primary infertility is frequently associated with more profound, often unexplained gamete dysfunction, including oocyte activation deficiencies or subtle sperm-oocyte interaction defects that are not captured by standard semen analysis[33]. These intrinsic biological impairments confer a lower baseline fertilization potential and reduced embryonic developmental competence, irrespective of the insemination method employed. Consequently, the observed reductions in 2PN formation and blastocyst development in the short-term IVF group may reflect, at least in part, the greater biological complexity of this patient cohort-not merely a direct technical limitation of abbreviated co-incubation. This constitutes a classic confounding-by-indication scenario: short-term IVF was selectively applied to patients with prior fertilization failure, introducing systematic baseline differences that compromise the comparability of outcomes with the conventional IVF. Moreover, early fertilization assessment-relying on visualization

of the second polar body 4–6 hours post-insemination-is methodologically inadequate. Therefore, fragmentation of a single polar body may be mistaken for two separate polar bodies, resulting in an inaccurate determination of fertilization status. As a result, such oocytes may be erroneously classified as fertilized despite the absence of actual fertilization.

In this study, we conducted a comprehensive analysis of the chromosomal constitution of 3PN zygotes produced through conventional IVF and short-term IVF. Using whole-zygote next-generation sequencing (NGS) and SNP genotyping, we determined the ploidy status and parental origin of chromosomes in 3PN zygotes, enabling unambiguous discrimination between diploid, triploid, and mosaic, as well as diandric versus digynic triploidy. Our findings revealed that 38.8% of 3PN zygotes generated through IVF were diploid. Kenji Ezoe et al. observed that 33.3% of analyzed 3PN embryos were chromosomally normal (euploid), and all such embryos successfully reached the blastocyst stage[34]. Kattera et al. demonstrated that diploidization can occur in 3PN zygotes when one pronucleus—typically the supernumerary paternal pronucleus—is extruded or undergoes degeneration prior to the first mitotic cleavage [35]. Given the heterogeneous origins of 3PN zygotes, the developmental outcome critically depends on the parental origin and identity of the eliminated pronucleus: selective loss of the extra pronucleus restores diploidy and genomic integrity. This mechanistic insight accounts for the observed capacity of a subset of 3PN zygotes to generate viable, euploid blastocysts. Extended blastocyst culture may thus function as a biologically informed selection tool, enabling the identification of 3PN-derived embryos with euploid karyotypes.

Previous studies have indicated that approximately 80% of triploid embryos possess an extra haploid set of paternal origin[36–38], typically arising from fertilization of a mature oocyte by two haploid spermatozoa. In the present study, 85% of triploid zygotes exhibited an extra paternal chromosome set, indicating that most 3PN zygotes result from polyspermy, whereas fertilization by a single diploid sperm is extremely rare. The remaining 15% displayed digynic triploidy, attributable to failure of second polar body extrusion-a mechanism further supported by observations of transient third polar body-like structure reabsorbed into the ooplasm during early culture, leading to extra pronucleus formation[39]. 3PN zygotes may also arise from oocyte-derived meiotic errors. Numerous studies investigating abnormal fertilization patterns have employed cytogenetic analysis, cellular morphology, and time-lapse cinematography (TLC) to assess morphokinetic parameters[36, 40–42]. Nevertheless, the underlying mechanisms of these abnormal fertilization patterns remain poorly understood.

Compared with conventional IVF, short-term insemination is more difficult to remove cumulus cells around oocytes. Repeated aspiration and mechanical manipulation increase the risk of ooplasmic disruption, cytoskeletal damage, and mitochondrial perturbation, thereby compromising oocyte developmental competence. Crucially, effective block to polyspermy depends on timely cortical granule exocytosis and zona pellucida hardening-processes requiring a fully mature, metabolically coordinated cytoplasm[43]. Premature cumulus removal may delay or attenuate these responses, weakening the polyspermy block and providing a biologically plausible explanation for the significantly higher ≥3PN rate

in the short-term group (5.2% vs. 4.6%, $p < 0.05$). The cumulus matrix itself also acts as a physical and biochemical filter for sperm, and its early removal further exacerbates this risk.

Notably, despite these adverse effects on early developmental parameters, no significant differences were observed in clinical pregnancy rate, implantation rate, between groups, which is consistent with the findings of Zhang et al[44]. We attribute this equivalence in clinical outcomes mainly to rigorous morphology-guided embryo selection before transfer. This approach serves as an effective biological filter, eliminating embryos with sublethal damage caused by mechanical stress or incomplete ooplasmic maturation, and ensuring that only embryos with evident developmental competence are transferred.

Conclusion

In conclusion, short-term insemination was associated with a significantly reduced rate of normal fertilization (2PN formation) and blastocyst development, alongside an elevated incidence of 3PN zygotes. However, no significant difference in clinical pregnancy rate was observed between short-term insemination and conventional insemination. Polyspermy remains the predominant etiology of 3PN formation and different insemination protocols did not influence the parental origin of genomic content in abnormally fertilized oocytes. Crucially, not all 3PN embryos harbor chromosomal abnormalities. These findings require validation in larger, well-powered embryo cohort studies.

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.

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Author Contribution

J.C. and H.C. performed assisted reproductive technology; J.W. designed the study; J.C. analyzed the data and wrote the paper. Z.Z. and C.W. prepared the original draft. Z.W. collected samples and clinical data of the 3PN zygotes; J.C. prepared the figure 1, 2, 3, 4, 5 and 6. H.C. prepared the table 1, 2 and 3. All authors read and reviewed the final manuscript.

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Figures

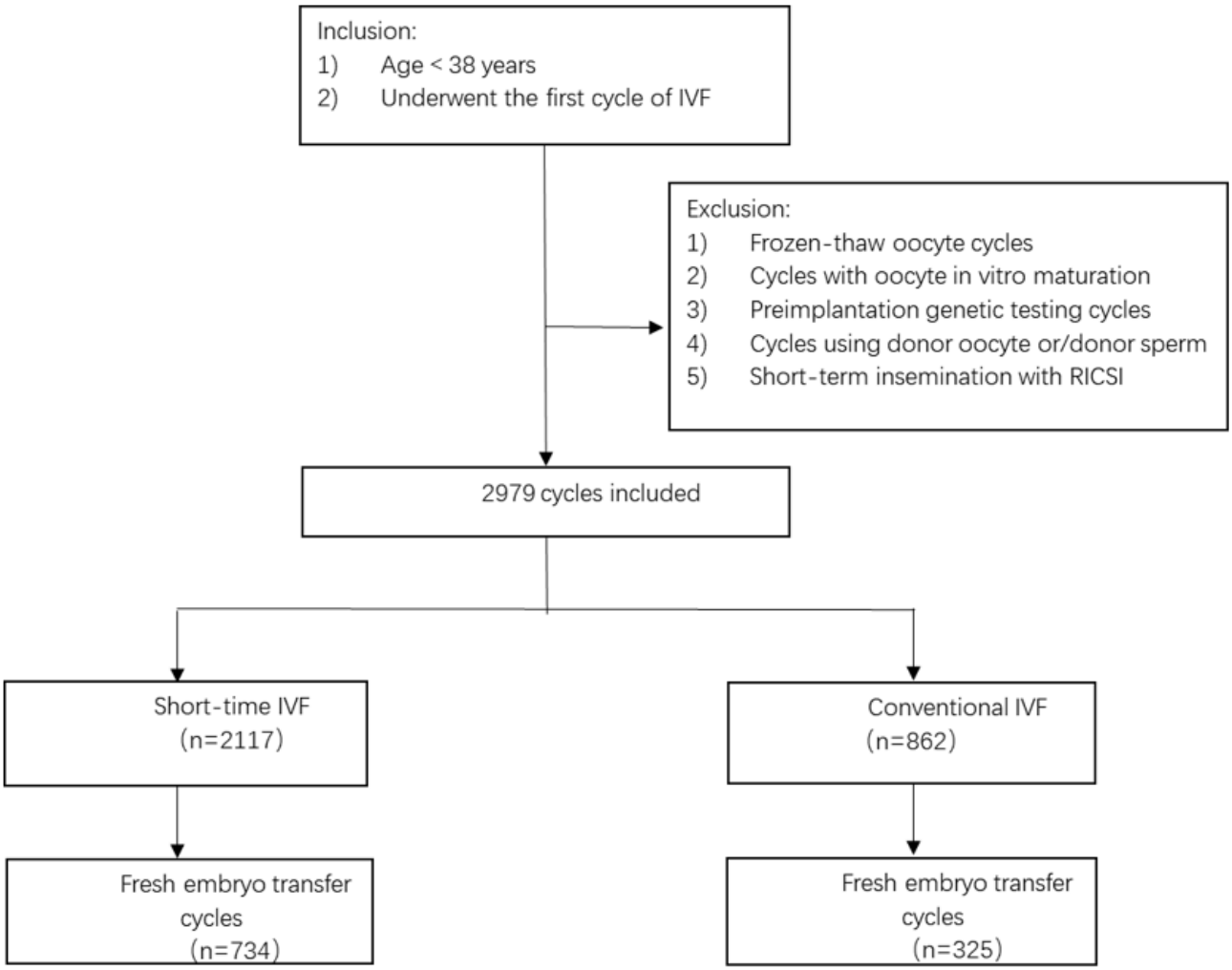


Figure 1

Flow chart of the study design. IVF: In Vitro Fertilization; ICSI: Intracytoplasmic Sperm Injection; R-ICSI: Rescue Intracytoplasmic Sperm Injection

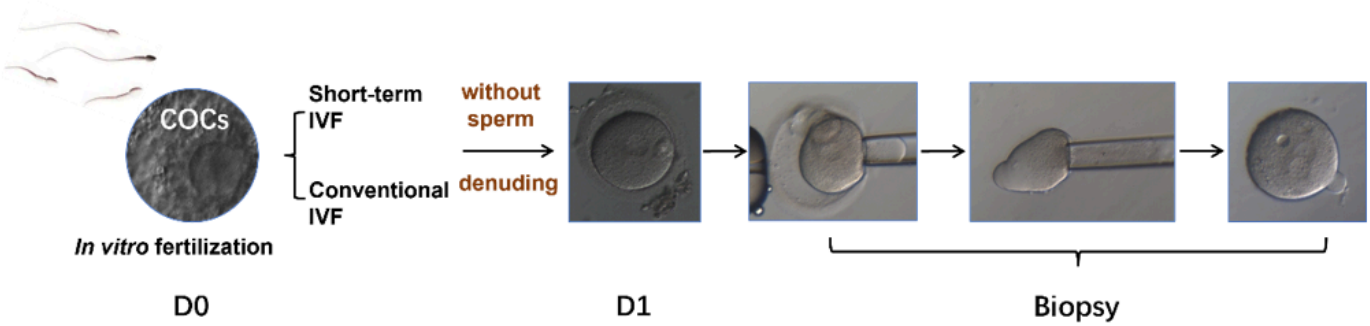


Figure 2

Pattern diagram of the biopsy. COCs: Cumulus-Oocyte Complexes. D0: Oocytes Retrieval Day. D1: 17-20h after insemination.

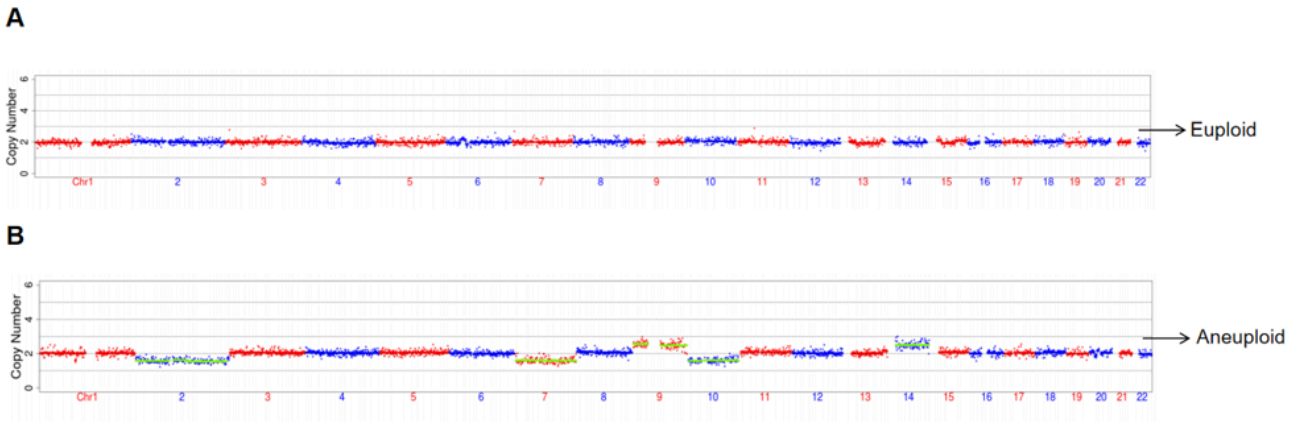


Figure 3

CNV pattern plot via NGS. (A) CNV plot of euploid embryos. (B) CNV plot of aneuploid embryos. Copy Number: Copy number variations pattern plot via NGS.

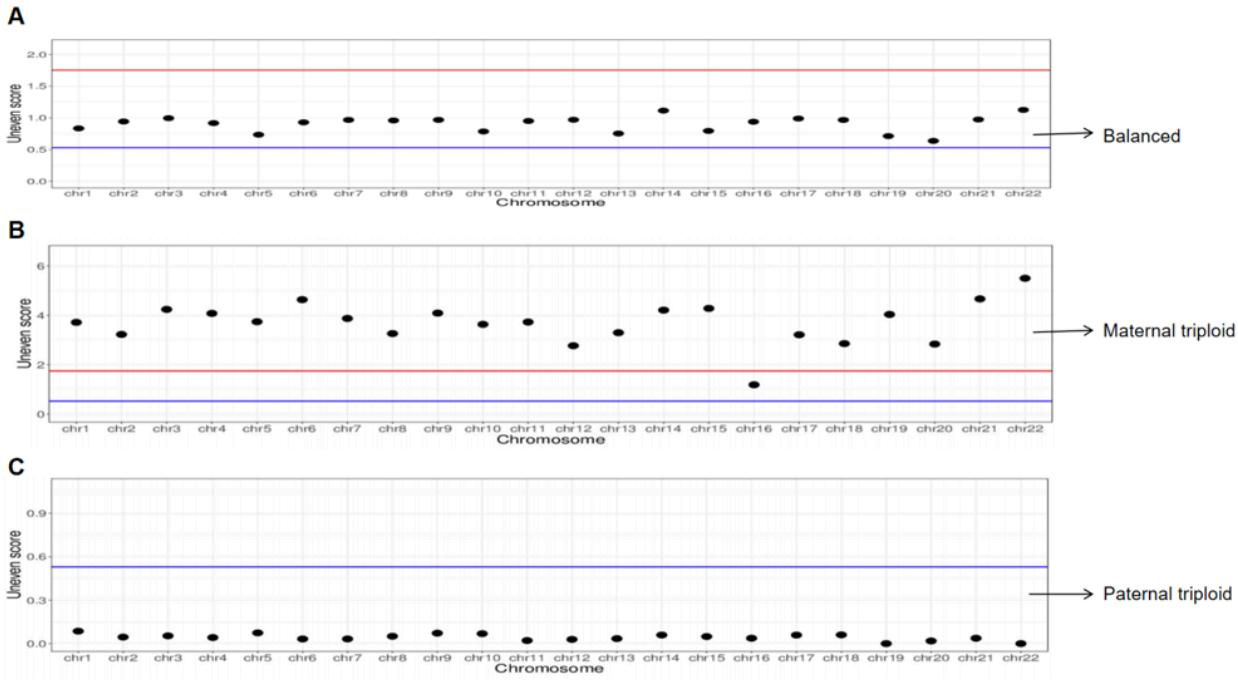


Figure 4

Maternal or paternal aneuploidy signal plot. (A) The normal diploid sample. (B) The threshold of maternal preference. (C) The threshold of paternal preference. 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.

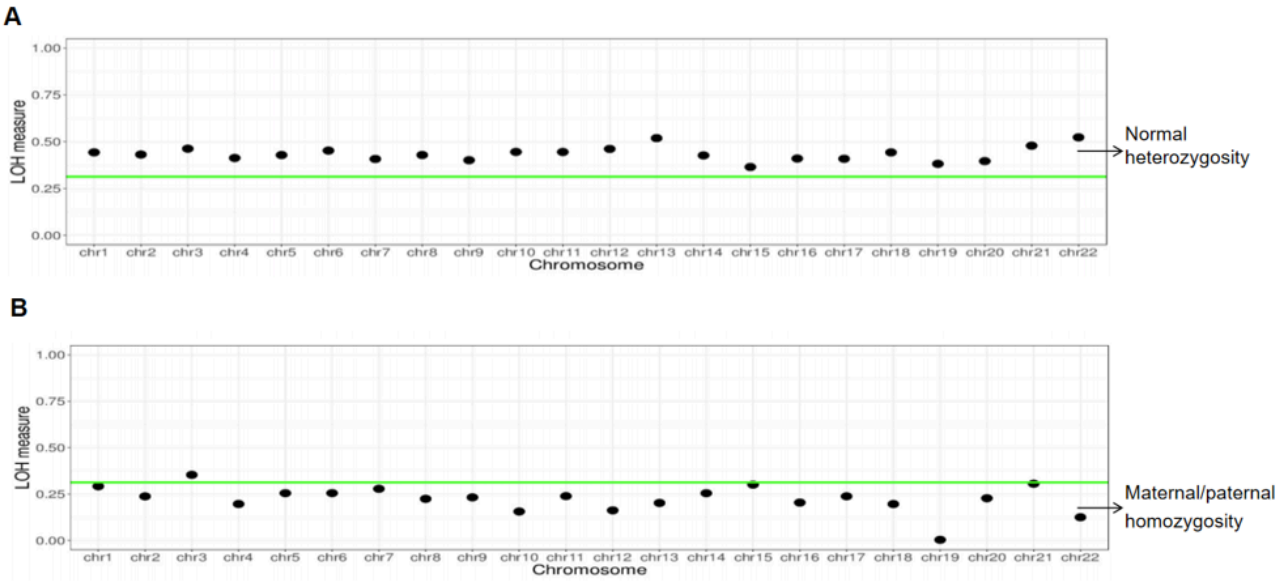


Figure 5

Maternal or paternal allele signal plot. (A) Normal heterozygosity allele signal plot. (B) Maternal/paternal homozygosity allele signal plot. LOH score: When the aternal 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.

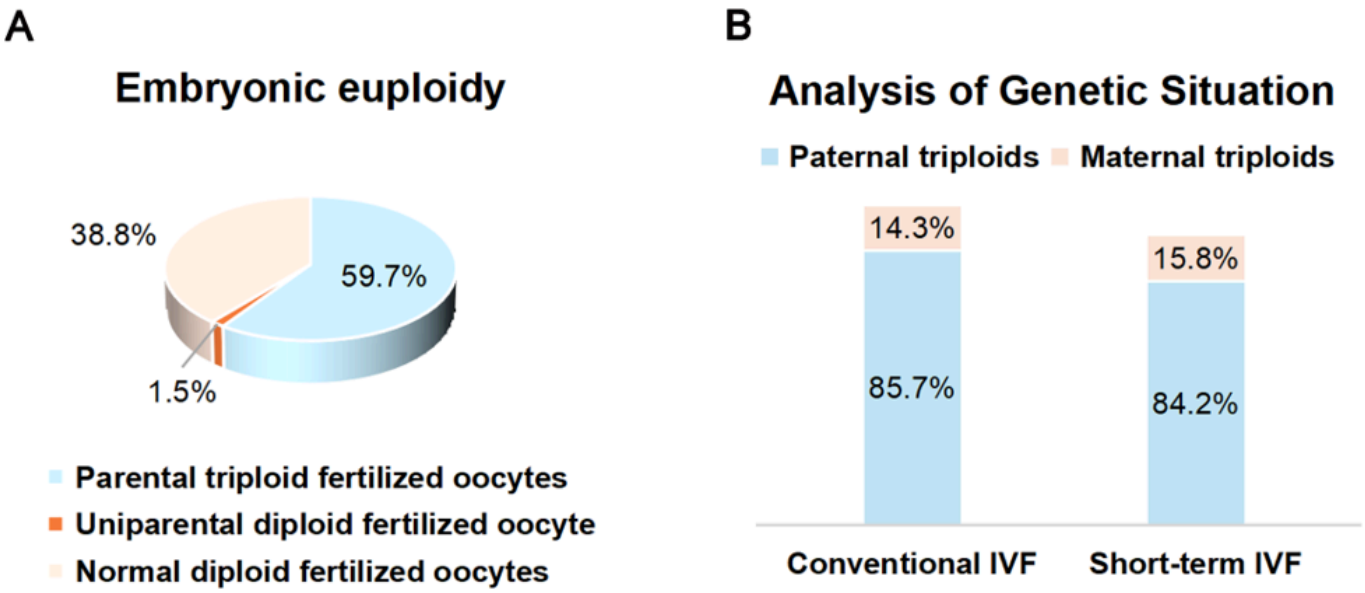


Figure 6

Euploidy status and genetic origin of tripronuclear zygotes. (A) The proportion of embryonic euploidy. (B) Analysis of genetic situation within conventional IVF and short-term IVF. IVF: In Vitro Fertilization.