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Molecular and cytogenetic research advances in human reproduction, volume II

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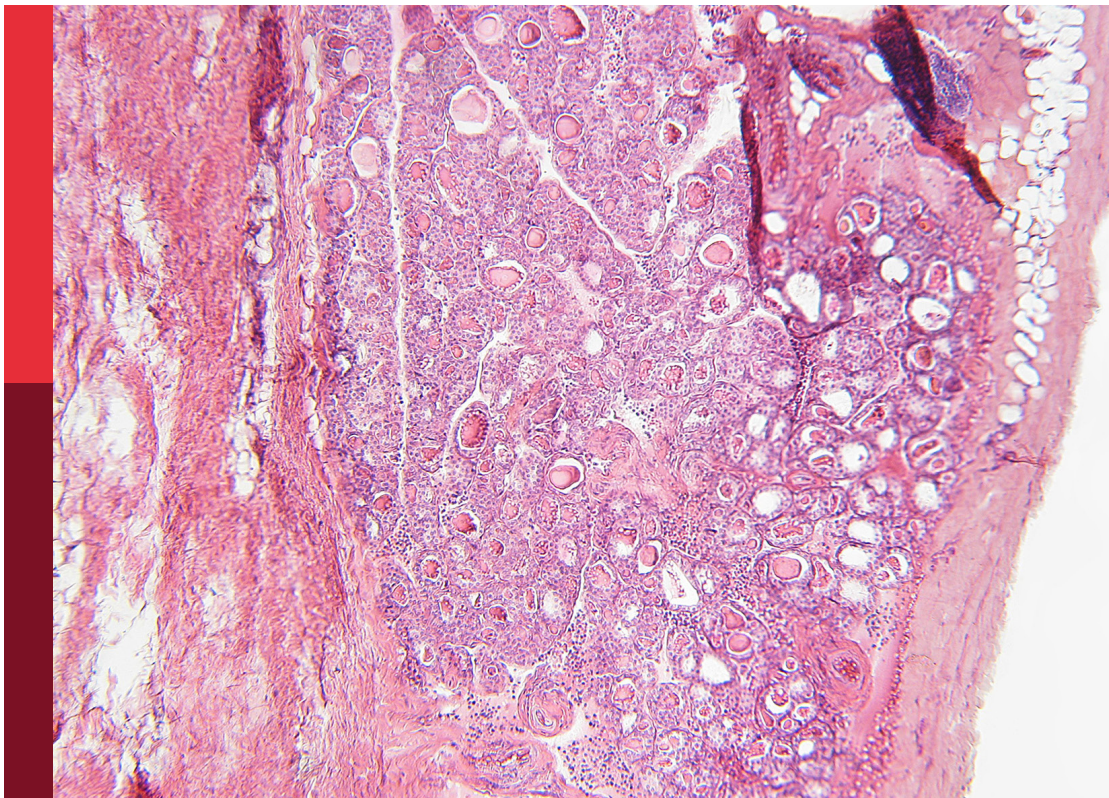

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Molecular and cytogenetic research advances in human reproduction - Volume II

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Editorial: Molecular and cytogenetic research advances in human reproduction - volume II

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Editorial on the Research Topic
Molecular and cytogenetic research advances in human reproduction - volume II

Infertility is a prevalent disorder that affects over 15% of couples worldwide, with various causes such as meiotic arrest due to cytogenetic shuffling, toxins, and other physical factors (1–4). Among the genetic causes of male infertility, chromosomal abnormalities have been reported in approximately 6% of cases, and microdeletions in the Y chromosome account for spermatogenic failure in 5-20% of men with azoospermia (3, 5–8). The most common structural Y chromosome aberrations in infertile men with azoospermia have been reported to be an isodicentric or isochromosome Y, with duplications in the Yp region and deletions in the Yq region, or vice versa (9–12). The human X and Y chromosomes pair at their distal short arms during male meiosis, with the telomeres first approaching each other during the zygotene stage of meiotic prophase (13, 14). As pachytene progresses, a paired segment is formed, and a synaptonemal complex (SC) which, on average, extends to about one-third of the total length of the Y chromosome (15–17).

Recently, pathogenic genetic mutations have been identified in individuals suffering from reproductive disorders; these mutations are generally detected in the genes mainly involved in germ cell development and other reproductive processes (18–21). Nevertheless, thousands of functionally relevant genes are expressed in human testes and ovaries, and malfunctioning of even a single gene can potentially cause infertility (22–24). Although the overall mechanistic contribution of specific genes has been studied in animal models, more attention is now being paid to clinically detected alterations in DNA, which include mutations potentially affecting gene regulatory sequences such as the 3'UTR (25, 26). However, the impact of mutations in gene 3'UTR sequences may not be adequately recognized in current sequencing analyses. This provides a potentially massive gap between

fertility genetics and clinical applications, a problem which needs to be addressed to ensure proper diagnosis and treatment of reproductive disorders.

The objective of this Research Topic was to gather clinical and basic research studies that report on cytogenetic, molecular, and clinical aspects of human reproduction. After rigorously reviewing submitted articles, the current volume presents an authoritative collection of eleven articles exploring new dimensions of human reproduction, providing valuable insights and advancing our understanding in this field.

Bourdon et al. conducted a study to evaluate the putative role of FGF21 on spermatozoan function. The results showed that *in vitro* treatment by FGF21 significantly increased sperm motility and ATP levels, indicating that the metabolic factor FGF21 positively modifies the activity and quality of human spermatozoa parameters. This finding has implications for developing therapeutic strategies for male infertility.

Li et al. conducted a comprehensive investigation into the association between blastocyst morphology, developmental rate, euploidy and live birth rates (LBRs) in single euploid frozen-thawed embryo transfer (FET) cycles. Their results demonstrated a positive association between the quality of embryos, blastocyst developmental rate, and euploidy rate in middle-aged women, which was positively associated with LBRs. These findings provide insights into the understanding of embryonic development and potentially have significant implications for improving assisted reproductive technology (ART) outcomes.

Liu et al. determined the association between antinuclear antibodies (ANAs) and recurrent pregnancy loss (RPL) and the effects of immunotherapy on pregnancy outcomes in women with positive ANAs and history of RPL. Their findings suggest that the presence of ANAs is strongly correlated with RPL and has prognostic value for subsequent pregnancy outcomes in women with a history of recurrent pregnancy loss. This study suggests ANAs levels could be considered in the diagnostic workup of RPL and the potential benefit of immunotherapy in improving pregnancy outcomes for these patients.

Ma et al. conducted a comprehensive review of the factors associated with recurrent implantation failure (RIF), which can be caused by a range of factors such as immunology, thrombophilias, endometrial receptivity, microbiome, anatomical abnormalities, male characteristics, and embryo aneuploidy. The authors suggest that targeted and precision therapy can improve the chances of successful implantation in RIF patients, indicating the need for personalized treatment strategies for this patient population. This study sheds light on the complex nature of RIF and highlights the importance of understanding its underlying mechanisms for developing practical treatment approaches.

Chuang et al. conducted a study that analyzed the correlation of mitochondrial DNA (mtDNA) content of a single biopsy at trophoblast with the developmental potential and reproductive outcomes of the blastocyst. The authors concluded that the mtDNA ratio depends on the period after blastocyst formation. Lower mtDNA ratios were observed at day 6 of euploid single embryo transfers, suggesting that the timeline of the embryos is an important covariate of mtDNA content. These findings have implications for optimizing the selection of embryos for transfer and improving outcomes in assisted reproductive technology (ART).

Wu et al. conducted a retrospective study investigating the correlation between transferred embryos and multiple pregnancy/live birth rates in frozen embryo transfer cycles. Their findings suggest that single-good-quality blastocyst transfer is an appropriate strategy for women under 40 years old. In contrast, double high-quality embryo transfer may be more suitable for women over 40. This study provides valuable information for clinicians in selecting embryos for transfer and improving the chances of successful pregnancy outcomes in women undergoing frozen embryo transfer.

Yang et al. conducted a study that found that the Sperm DNA Fragmentation Index was positively correlated with blastocyst aneuploidy rate, indicating that sperm DNA damage may contribute to chromosomal abnormalities in embryos. On the other hand, sperm motility and morphology rate were negatively correlated with blastocyst aneuploidy rate, suggesting that better sperm quality is associated with improved embryonic chromosomal integrity. These findings highlight the importance of assessing sperm quality when selecting embryo transfer in ART procedures.

Liu et al. found that the rate of *de novo* chromosomal abnormalities increased with maternal or paternal age. However, controlled ovarian hyperstimulation parameters did not influence the incidence of *de novo* chromosomal abnormalities or clinical pregnancy outcomes. These findings suggest that while advanced maternal or paternal age poses a risk for chromosomal abnormalities, factors related to ovarian stimulation protocols may not significantly impact the incidence of chromosomal abnormalities or pregnancy outcomes. These findings have implications for patient counseling and treatment planning in ART procedures.

Yuan et al. demonstrated that sperm telomere length has diagnostic and predictive value for male fertility and clinical pregnancy and may be used as a biomarker for the diagnosis of male infertility and predicting embryonic development. This finding has implications for improving the accuracy of male infertility diagnosis and developing personalized treatment plans for couples undergoing assisted reproduction.

Racca et al. aimed to determine the ideal progesterone (P4) levels for the day of embryo transfer and whether supplementing progesterone (P4) on the day of human chorionic gonadotropin (hCG) release can improve the success rate of frozen embryo transfer (FET) cycles. They retrospectively analyzed 664 females who had vaginal 600 mg/day of P4 as hormone replacement therapy (HRT)-FET cycles starting 6 days before the FET. The study found that the likelihood of detecting P4-hCG < 10.6 ng/ml decreased as the level of serum P4 the day before ET increased, indicating the importance of adequate progesterone supplementation in ART cycles. Additionally, the study showed that low P4 levels if not supported by supplementation were associated with significantly lower live birth rates (LBR), suggesting the need for careful monitoring of P4 levels and timely intervention in ART cycles.

Ruan et al. identified biallelic mutations of CEP70 in two unrelated infertile male individuals with oligoasthenoteratozoospermia that followed a recessive inheritance pattern. Furthermore, the study

found morphological and ultrastructural defects in the acrosome and flagellum of sperm from the patient, which were similar to those seen in Cep70-/- male mice. These findings provide insights into the genetic basis of male infertility and highlight the importance of investigating rare genetic variants in diagnosing and managing infertility.

Conclusion

Recent advances in next-generation sequencing (NGS) technologies have transformed the landscape of investigating rare and common human disorders. The ability to generate genome-wide sequencing data with in-depth coverage in a short time frame represents a cost-effective replacement for conventional approaches that primarily focus on specific regions for gene discovery and clinical testing. The articles included in the current Research Topic provide valuable insights into various aspects of human reproduction, including the roles of different genes, hormones, and technologies. These findings have significant implications for improving our understanding of reproductive biology and developing more effective diagnostic and therapeutic strategies for individuals affected by infertility.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Hepatokine FGF21 Increases the Human Spermatozoa Motility

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Lifestyle, environment and excess body weight are not only associated with an increased risk of metabolic disorders, such as type 2 diabetes, but also to other pathological processes, such as infertility. A hormone produced mainly by the liver called fibroblast growth factor 21 (FGF21) is closely linked to the energy status and is increased in patients suffering from obesity or insulin resistance. Recently, FGF21 has been shown to be associated with female fertility disorders, but no or few data about the role of FGF21 on human male fertility has been described. In the present study, FGF21 was measured in the seminal fluid at a lower level in comparison to the blood level. Thus, in the present *in vitro* study, we aimed to decipher the FGF21 system in human semen. To evaluate the putative role of FGF21 on spermatozoa function, we incubated human spermatozoa with increasing concentrations of recombinant human FGF21. The FGF21 in seminal fluid is potentially produced by male reproductive tract tissues. In spermatozoa, the FGF21 signal was transduced by the two main receptors FGFR1-c and FGFR3 and the cofactor β-klotho, which are colocalized in the middle piece of spermatozoa and stimulated the PI3K/Akt and MAPK pathways. Finally, *in vitro* treatment by FGF21 significantly increased sperm motility and ATP levels. Concomitantly, exposure to FGF21 improved the oxidative stress, as a lower ROS level was observed. Overall, these results seem to indicate that the metabolic factor, FGF21, positively modifies the activity and quality of the parameters of human spermatozoa.

Keywords: human, spermatozoa, FGF21 (fibroblast growth factor 21), sperm moTility, metabolism diseases, fertility

INTRODUCTION

Infertility is a public health problem that affects about 15% of couples of childbearing age. In almost 50% of cases, the male factor is involved (1). Furthermore, public health authorities note an increase in the use of conception centers by patients with metabolic disorders, such as obesity/insulin resistance. Because of the current prevalence of obesity (up to 20% of adults), the question of improving the fertility rate score is of great interest. It is now well established that links exist

between energy metabolism and reproductive activity, involving both nutritional and hormonal regulations. Changes in metabolic status result in alterations in hormonal signals (insulin, IGF-1, and hormones produced by adipocytes called adipokines) and nutrient flow (fatty acids, glucose, and amino acids), all acting directly or indirectly on the hypothalamic-pituitary-gonadal axis (2). Interestingly, recent data support that a hormone produced mainly by the liver called fibroblast growth factor 21 (FGF21), is involved in female fertility disorders such as polycystic ovary syndrome (3–6), suggesting its contribution to the control of female fertility and raises the question about its involvement in male fertility.

FGF21 is a metabolic hormone mainly produced by the liver and described in the 2000s. This peptide belongs to the fibroblast growth factor (FGF) family, which is constituted of 22 members divided into seven subfamilies depending on phylogeny and functions (7, 8). All FGFs have paracrine activity, except the FGF19/21/23 subfamily, which present the specificity to be endocrine factors in contrast to others FGFs. FGFs bind specific FGF receptors (FGFRs) associated with a cofactor. FGFRs include FGFR1, FGFR2, FGFR3 and FGFR4, and all these receptors participate in heparan sulfate (HS)-dependent signaling. However, only the FGF19/21/23 subfamily has been shown to require cofactors from the Klotho family (α-klotho and β-klotho) of transmembrane proteins. Thereby, FGF21 needs to bind to FGFR1c, FGFR3c or FGFR4 associated with the presence of the β-klotho (KLB) cofactor, conferring the specific FGF21 activity and cell signaling. In humans, FGF21 plasma levels are detected between 0.05 and 5 $ng.ml^{-1}$ (8) and between 0.1 and 1 $ng.ml^{-1}$ in mice (9). However, pronounced interindividual variations exist in both species. Thus, in patients with type II diabetes or obesity, plasma concentrations of FGF21 are significantly increased compared to control healthy subjects (10). It has also been shown that the expression of FGF21 is induced by the nuclear receptor, peroxisome proliferator-activated receptor α, known to be a major regulator of energy homeostasis (11). The binding of FGF21 to its receptor and the cofactor β-klotho, causes phosphorylation of ERK1/2 kinases, or leads to phosphorylation of AMPK. Through these pathways, FGF21 modulates the activity of several metabolic organs, including adipose tissue, the pancreas, muscle and brain. FGF21 is upregulated in both cases (lack or excess of energy) and regulates glucose and lipid homeostasis by promoting lipid catabolism, including lipolysis; fatty acid oxidation; and mitochondrial oxidative activity, resulting in the improvement of insulin sensitivity (12, 13). For example, in adipose tissue, after binding of FGF21 to its receptors, it induces an increase in metabolic protein such as SIRT1, PGC1-a as well as UCP1 ultimately leading to heat dissipation, a sign of lipolysis.

Several FGFs (FGF1, 2, 4, 5, 8 and 9) and all FGFRs have already been localized in the mouse testis, including Sertoli, Leydig, and germ cells (14–16), and also in human testis (17). The paracrine and local functions of these FGFs have been demonstrated during testis development to stimulate proliferation, survival or to contribute to the formation of the interstitial compartment of the testis (16, 18). However, published data are scarce regarding the endocrine FGF19/21/23 subfamily. A mouse model overexpressing FGF21 in the liver has shown a strong decrease in pituitary LH levels, leading to a delay in puberty associated with female infertility. In addition, the deletion of the *fgf21* gene in mice led to elevated levels of apoptotic germ cells in the testis, which could be rescued after administration of recombinant FGF21, leading to sperm production (19). These recent data also support a regulatory role of the FGF21 hormone on testis and male fertility. However, no data about the role of FGF21 on human semen have been described.

The current work aimed to evaluate the presence of FGF21 in semen samples raising the question about the role of FGF21 on the sperm function. We aimed to decipher the FGF21 system in human semen, by determining the localization of FGF21 receptors and their activities in human sperm cells. To evaluate the role of FGF21 on spermatozoa function, we incubated human spermatozoa with increasing concentrations of recombinant human FGF21.

MATERIALS AND METHODS

Patients

Human blood and semen are issued from the following cohort "Fertiprotect", including healthy men and their respective normal semen quality, according to the WHO (2010) guidelines. Exclusion criteria include seropositivity for HIV, HBV or HCV, smoking and male explained infertility (chemotherapy, varicocele or genital surgery). Patients were enrolled into the Assisted Reproductive Centers (Tours, FERTIPROTECT protocol) for couple infertility exploration during a medical consultation. Forty participants gave full-informed written consent to participate in the study, and ethical approval was obtained from the Ethics Committee of the Vinci Clinic and CHRU Bretonneau. Patients (n = 40, 29–53 years old) (**Supplementary Data Sheet 1**) were separated in two groups depending on the body mass index (BMI), with 18.5-25 kg/m^2 considered as normal BMI (and noted BMI ≤ 25 kg/m^2) and BMI ≥ 30 kg/m^2 considered as obese (**Table 1**). However, blood and seminal fluid were recovered from the same individual in a group of 20 patients (BMI ≤ 25, n=10; BMI ≥ 30, n=10). Blood and seminal fluid analyses were obtained in fasted patients, and semen collection was obtained after a recommendation of 2-5 days of abstinence. For *in vitro* analysis, recombinant human FGF21 from Sigma-Aldrich was prepared in water (100 µg/mL, stock solution). The selective FGFR1 and FGFR3 inhibitor PD173074 was obtained from Tocris Bioscience (Minneapolis, MN, USA) and used at the concentration of 100 nM (20, 21). Analysis of FGF21 was performed on fresh washed human spermatozoa from others patients. Spermatozoa were exposed for 15 min (for Western blot analysis) or 30 min to 0–10 ng/mL recombinant human FGF21, as described in the legends.

Hormone and Metabolites Assay

ATP concentrations was measured by using the CellTiter-Glo™ ATP Assay Kit (Promega, France), and total-cholesterol

TABLE 1 | Biological and semen parameters of samples from the BMI ≤ 25 kg/m^2 group and the BMI ≥ 30 kg/m^2 group.

	Patients with BMI<25 (n=12)	Patients with BMI>30 (n=12)	Significance
Age (years)	34.91 ± 1.28 (29 - 43)	37.08 ± 1.83 (30 - 53)	**NS**
BMI (kg/m2)	23.97 ± 0.50 (22 - 25)	34.96 ± 1.36 (30 - 44)	****** p<0.0001**
fasting glucose (mmol/L)	5.55 ± 0.06 (5 - 6)	5.27 ± 0.28 (5 - 6)	**NS**
cholesterol (mmol/L)	5.40 ± 0.54 (5 - 7)	4.57 ± 0.69 (3 - 6)	**NS**
triglyceride (mmol/L)	1.47 ± 0.80 (0 - 4)	1.72 ± 0.30 (1 - 2)	**NS**
FSH (UI/L)	4.32 ± 0.92 (3 - 8)	3.88 ± 0.87 (2 - 8)	**NS**
TSH (mUI/L)	1.92 ± 0.36 (1 - 3)	2.46 ± 0.61 (2 - 4)	**NS**
LH (UI/L)	4.58 ± 0.41 (4 - 6)	5.30 ± 0.93 (3 - 9)	**NS**
prolactin (mUI/L)	185.56 ± 71.18 (11 - 331)	109.24 ± 97.22 (4 - 498)	**NS**
testosterone (nmol/L)	20.43 ± 1.96 (15 - 29)	13.50 ± 1.28 (10 - 18)	****p<0.01**
estradiol (pmol/L)	72.00 ± 8.08 (62 - 88)	121.00 ± 36.87 (77 - 231)	**NS**
semen volume (mL)	4.80 ± 0.48 (3 - 7)	3.42 ± 0.46 (2 - 8)	***p<0.05**
semen concentration (million/mL)	39.78 ± 9.06 (11 - 95)	68.57 ± 15.75 (2 - 200)	**NS**
vitality (after 1h)	69.20 ± 5.42 (31 - 88)	70.85 ± 2.98 (45 - 84)	**NS**
motility (rapid progression) (after 1h)	35.00 ± 4.09 (20 - 57)	42.00 ± 3.84 (15 - 66)	**NS**

Data are expressed as mean ± SEM (range).
*NS, non-significant; *, p < 0.05; **, p < 0.01; ****, p < 0.0001.*

concentration was measured by using the spectrophotometric assays (Biolabo, France), according to the manufacturer's instructions. Plasma and seminal fluid levels of FGF21 were measured using the commercial Human FGF-21 Quantikine ELISA Kit (Bio-Techne, France).

Western Immunoblotting and Immunoprecipitation

Pellets of human spermatozoa were lysed [Tris 1 M (pH 7.4), NaCl 0.15 M, EDTA 1.3 mM, EGTA 1 mM, VO43–23 mM, NaF 0.1 M, NH2PO41%, Triton 0.5%] and the protein concentration of samples was measured using a kit bicinchoninic acid (BCA) protein assay (Interchim, Montluçon, France) and equal protein concentrations were electrophoresed (40µg). Saturation of membrane was done with Tris-Buffered Saline Tween buffer (0.05% of Tween 20 and 5% of milk) for 30 min at room temperature. Then, the membranes were incubated at 4°C overnight with the following antibodies (all diluted at 1/1000): phospho-Akt (ser473), Akt, phospho-ERK (Thr202/Tyr204), and ERK (Cell Signalling Technologies, USA). Experiments were performed on five different patients.

Immunoprecipitation of FGF21 in seminal fluid was performed on a pool of human seminal fluid devoid of spermatozoa (2 mL), which was incubated overnight with rabbit polyclonal anti-FGF21 antibody (Thermo-Fisher Scientific, USA) and immunoprecipitated by 100 mg protein G agarose beads. After several washes, the immunoprecipitated proteins and depleted seminal fluid extract were analyzed by Western blot. Experiments were performed on five pools of patients. Detection of proteins was done by using chemiluminescence (Western Lightning Plus-ECL, Perkin Elmer, Villebon-sur-Yvette, France) with a G-box SynGene (Ozyme, St Quentin en Yvelines, France).

Immunohistochemistry

Paraffin-embedded testis, epididymis, prostate and seminal vesicle samples were retrieved from autopsy specimens from the Department of Histopathology and Urology/Andrology of the CHRU Bretonneau Tours Hospital and Hospices Civils de Lyon, France, following approval by the ethical committees of these institutions. Sections (7 µm) of the following human tissues (testis, epididymis, prostate and seminal vesicle) were deparaffinized and rehydrated in xylene and in various baths containing decreasing concentrations of alcohol (100, 90, 75%) for 10 minutes for each step. Immunohistochemical slides were washed in a PBS bath and microwaved for 2–3 min in antigen unmasking solution (Vector Laboratories, Inc., AbCys, Paris, France). An incubation with PBS 1X/0.1 M Glycine for 15 minutes at room temperature, has been performed to ensure saturation of aldehyde groups. To permeabilize cells on sections, an incubation for 15 min with a solution of 0.1% Triton X-100 (w/v) in PBS has been done. Finally, all nonspecific binding sites have been obstructed in 2% BSA solution for 15 min. For FGF21 immunostaining, sections were incubated overnight at 4°C with PBS/1% bovine serum albumin (BSA) containing primary antibody against FGF21 (Sigma-Aldrich, USA) at a 1:100 final dilution. Then, the sections were incubated with a "ready to use" labelled polymer-HRP anti-rabbit for 30 min (DAKO Cytomation Envision Plus HRP System, Dako, Ely, UK). Visualization was achieved by incubation in a DAB peroxidase substrate solution (Invitrogen, Cergy-Pontoise, USA).

Fresh human spermatozoa were fixed in 4% paraformaldehyde (PFA)/PBS for 15 min, then washed in a PBS bath. Spermatozoa were permeabilized with PBS-Triton 0.1%, and nonspecific binding sites were blocked in 2% BSA for 15 min, then incubated for 60 min at room temperature with FGFR3 (Thermo-Fisher, USA), FGFR1, FGFR4 and FGF21 (Sigma-Aldrich, USA) and β-klotho (Thermo-Fisher, USA) antibodies at a 1:100 final dilution. Rabbit or mouse IgG (Sigma-Aldrich, USA) antibodies (Sigma-Aldrich, USA) were used as negative controls. Analyses were performed in five different patients.

Computer-Assisted Semen Analysis

Before FGF21 incubation, fresh semen samples were washed and centrifuged at 1200 rpm), resuspended in DMEM (Ref D6546, Sigma-Aldrich, USA) (4500 mg/L glucose, sodium pyruvate, and sodium bicarbonate, without L-glutamine, without albumin and with 5% serum) and counted. From each patient, 5 million spermatozoa were incubated at 37°C in a water bath with increasing concentrations of recombinant FGF21 (0, 0.01, 0.1, 1, 10, and 100 ng/mL) for 30 min, with or without preincubation with the specific FGFR inhibitor PD173074 for 15 min. Sperm motility, as a percentage of motile spermatozoa, was evaluated by using a computer-assisted semen analyzer (CASA) (Hamilton-Thorne Sperm Analyser IVOS version 12.2l, Hamilton Thorne Biosciences, USA) with a Makler Counting Chamber (0.01 sq.mm/0.01 mm Deep). Three microscopic fields were analyzed, and a minimum of 200 spermatozoa per field were evaluated. The following parameters were measured: percentage of motile sperm, percentage of progressively motile spermatozoa, average path velocity (VAP, average velocity/smoothed average position of the spermatozoa), progressive velocity (VSL, straight line distance between the beginning and the end of the track), curvilinear line velocity (VCL, average velocity measured over the actual point-to-point track followed by the cell), straightness (STR, a measure of side-to-side movement of the VCL determined by the ratio VSL/VAP × 100), linearity (LIN, a measure of the departure of the cell track from a straight line), amplitude of lateral head (μm) (ALH), and beat cross frequency (BCF) (Hz). All results are presented in **Table 2**.

Viability and Mitotracker Analysis Using Flow Cytometry

Sperm membrane integrity was assessed with dual fluorescent probes, SYBR-14 and propidium iodide (PI) (Live/Dead Sperm Viability Kit, InvitrogenTM, Eugene, OR, USA) and semen were analyzed by using flow cytometry (MoFlo Astrios^{EQ}, USA). Mitochondrial activity was determined using a 200 nM mitotracker (Orange CM-H2TMRos, Invitrogen, Fisher Scientific, France) and samples were analyzed by using flow cytometry. Twenty thousand events were collected per sample. Only sperm emitting orange fluorescence (R1, R2) were classified with a high mitochondrial membrane potential (HMMP), which is associated with mitochondrial activity. Orange fluorescence is in y axis and SSC in x axis. We have separated a R1 population with a very high orange fluorescence and a mild fluorescence population R2, from the negative control (without mitotracker).

All results are presented in **Supplementary Data Sheet 4**.

Measurement of ROS [Hydrogen Peroxide (H_2O_2)]

The contents of the ROS hydrogen peroxide (H2O2) in human sperm (n=4 patients per condition) was measured by Ros-Glo H2O2 assay kit (Promega, Charbonnières-les-Bains, France). Two million spermatozoa previously stimulated by FGF21 were incubated with H2O2 substrate solution during 4 hours. H2O2 present in samples degradate the H2O2 substrate into Luciferin Precursor and produce luminescence which is measured with a luminometer Luminoskan Ascent (Thermo-Fisher, USA). Luminescence is correlated with the concentration of H2O2 and the ROS activity as detailed in the Ros-Glo H2O2 assay kit.

Intracellular Calcium Measurements

A total of 2 million spermatozoa were loaded in a 96 microwell plate for fluorescent plate reader analysis, and the kinetics of intracellular calcium measurement were performed after a 2 μM Fluo-4 AM incubation. Fluorescence was measured every 30 s. The intracellular calcium intensity was plotted as the percentage change in fluorescence (ΔF/F0, %) compared with baseline (F0).

Acrosome Integrity

After a 30 minutes exposition with/without FGF21, spermatozoa were incubated with 10 μM calcium ionophore A23187 to induce acrosome reaction and stained with 25 μg/mL FITC-conjugated pisum sativum agglutinin (FITC-PSA; Sigma-Aldrich) for another 30 min at room temperature. The same experiment has been performed without calcium ionophore A23187. The percentage of acrosome reaction was estimated by counting 200 spermatozoa per patient. Only spermatozoa without FITC-PSA staining or FITC-PSA staining at the equatorial segment were identified as those with acrosome reactions.

Statistical Analysis

Data were tested for homogeneity of variance by Bartlett's test and for normal distribution by the Shapiro-Wilk test. One-way

TABLE 2 | Kinematic parameters of human spermatozoa exposed to FGF21.

	0 ng/mL rFGF21	0.01 ng/mL rFGF21	0.1 ng/mL rFGF21	1 ng/mL rFGF21	10 ng/mL rFGF21	PD173074	PD173074 + 10 ng/mL rFGF21
VSL (μm/sec)	35.5 ± 2.1	27.1 ± 0.7	43.1 ± 2.9	43.6 ± 2.9	45.41 ± 4.0*	37.9 ± 1.6	36.0 ± 2.6
VCL (μm/sec)	62.2 ± 2.6	71.6 ± 1.4	78.14 ± 2.6**	73.98 ± 2.3*	73.8 ± 4.6*	68.1 ± 2.0	67.9 ± 3.9
VAP (μm/sec)	40.8 ± 1.9	37.1 ± 0.8	51.5 ± 2.3*	51.1 ± 2.9*	56.0 ± 6*	46.4 ± 2.0	44.1 ± 2.7
ALH (μm)	2.8 ± 0.3	3.0 ± 0.1	3.2 ± 0.2	3.0 ± 0.1	3.0 ± 0.2	2.6 ± 0.1	3.0 ± 0.2
LIN (%)	56.4 ± 2.5	54.9 ± 0.7	56.5 ± 3.0	59.4 ± 2.4	60.1 ± 1.5	59.3 ± 1.6	55.8 ± 1.8
BCF (beats/sec)	20.3 ± 1.4	16.3 ± 0.4	19.7 ± 1.2	15.5 ± 1.1	17.2 ± 0.8	18.6 ± 1.2	18.7 ± 1.3
STR (%)	84.3 ± 1.2	82.5 ± 0.8	83.0 ± 2.0	84.2 ± 1.2	83.6 ± 1.1	83.8 ± 0.9	83.6 ± 1.7

Computer-assisted sperm analysis (CASA) of spermatozoa were performed after 30 min of recombinant human FGF21 exposition with or without preincubation with the selective FGFR1-3 inhibitor PD173074 for 15 min. The following kinematic parameters were measured : VSL, Straight-Line Velocity; VCL, Curvilinear Velocity; VAP, Average Path Velocity; ALH, Amplitude of Lateral Head; LIN, Linearity; BCF, Beat Cross Frequency; STR: VAP, Straightness. All results are expressed as Mean ± SEM, n=9.

**p < 0.05, **p < 0.01, compared with 0 ng/mL rFGF21.*

ANOVAs were performed with Tukey's multiple comparisons tests or Dunnett's multiple comparisons tests as appropriate. Data from the CASA system were compared by the Friedman test and Dunn's multiple comparison test. All statistical analyses were performed using GraphPad Prism 6 (La Jolla, CA, USA). The results are expressed as mean ± SEM. Values were determined to be significant when * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, **** $p < 0.0001$, indicating a significant difference between the groups and control ($p < 0.05$).

RESULTS

FGF21 Concentrations in Seminal Fluid

Firstly, we have investigated the presence of FGF21 in human seminal fluid by an ELISA and immunoprecipitation assay. We have analyzed the FGF21 levels in fasting conditions in blood and seminal fluid from two groups of patients using the BMI, a normal BMI in the range 18.5–25 kg/m^2 and with obese patients with a BMI ≥ 30 kg/m^2 (**Figure 1** and **Table 1**). FGF21 plasma levels were 2.4-fold higher in obese patients in comparison to control patients (**Figure 1A**). However, a similar FGF21 level was measured in the seminal fluid of both groups (**Figure 1A**). We observed that the FGF21 concentration was nearly 12 to 28 times lower in the seminal fluid as compared to that in the plasma, and the FGF21 seminal fluid/FGF21 plasma ratio was lower in obese patients (**Figure 1B**). Due to the lower levels in seminal fluid, we had confirmed the presence of FGF21 in seminal fluid after immunoprecipitation and its absence in the depleted protein extract (**Figure 1C**). No significant relationship between plasma or seminal FGF21 levels and semen parameters (semen volume, sperm concentration, motility, or sperm abnormality) was observed (**Table 1** and **Supplementary Data Sheets 1**, **2**).

Because the majority of seminal fluid proteins are produced by the epididymis, seminal vesicles and prostate, immunochemistry against FGF21 has been performed on the male reproductive tract (**Figure 2**). The expression of FGF21 was reported in Leydig cells in the testis, in the epithelium of the epididymis, and in the seminal vesicles, with weaker staining in the epithelium of the prostate gland (**Figure 2**). However, no staining was observed in human spermatozoa (**Figure 3B**.1).

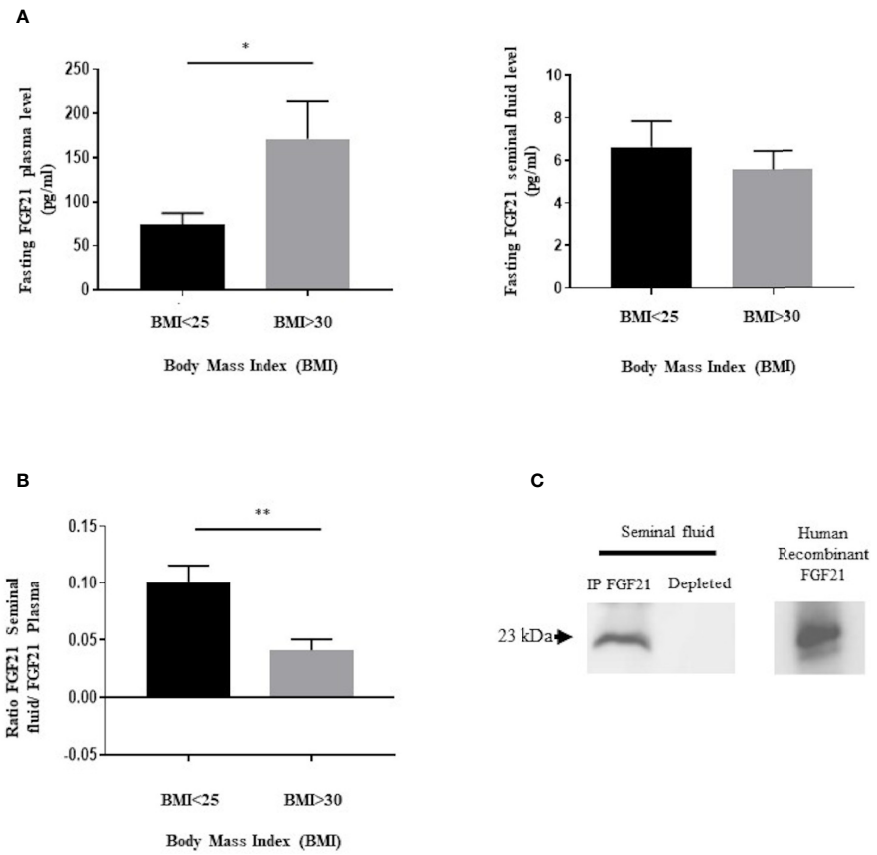

FIGURE 1 | Presence of FGF21 in human plasma and seminal fluid. **(A)** FGF21 was determined, from two groups of fasting patients, in plasma (n = 10/BMI group) and in seminal fluid (n = 12/BMI group), in function of their body mass index (BMI). Control corresponding to a BMI ≤ 25 kg/m^2, and obesity corresponding to a BMI ≥ 30 kg/m^2. **(B)** Ratio of fasting FGF21 level between seminal fluid and plasma in the two groups of patients. **(C)** Immunoblot of a pool of human seminal fluid protein extracts (400 µg protein per sample) after immunoprecipitation with the FGF21 polyclonal antibody. Human recombinant FGF21 was loaded as the control to confirm the molecular weight of the immunoprecipitated FGF21 protein. *$p < 0.05$, **$p < 0.01$.

FGF21 Receptors and Cofactor Are Localized in Spermatozoa

As reported in the literature, FGF21 signaling is transduced by activating the β-klotho-FGFR1c/FGFR3 complex, which stimulates the components of the MAPK and PI3K pathways, and the calcium-dependent protein. In our condition, we have confirmed, by Western blot, the presence of FGFR1, FGFR3, FGFR4 and KLB proteins in human spermatozoa (**Figure 3A**). In order to localize the FGFR complex, a confocal microscopic analysis was performed and demonstrated the colocalization of the two FGF21 receptors (FGFR1 and FGFR3) with the cofactor KLB (**Figure 3B**; 5, 8) in the middle piece of the spermatozoa, behind the head and weakly in the tail. Weak staining of the FGFR4 receptor was reported in the head and in the neck of the spermatozoa (**Figure 3B**; 9).

After 15 min of stimulation of fresh human spermatozoa with increasing concentrations of recombinant human FGF21 (0.01 ng/mL-10 ng/mL), we observed a dose-dependent increase in the phosphorylation of both Akt and ERK, which was significant at the 10 ng/mL FGF21 concentration (in comparison to control, phospho-Akt had a 4.3-fold increase and phospho-ERK had a 2-fold increase) (**Figures 4A, B**). We observed that, at the low dose of 0.1 ng/mL, phospho-Akt increased 2.6-fold, but this increase was statistically insignificant. In addition, we observed that 15 min after exposure to recombinant human FGF21, the intracellular flux of Ca^{2+} was increased in a dose-dependent manner and significantly at the 10 ng/mL concentration of FGF21 (**Figure 4C**).

FGF21 Increased Sperm Motility

To determine the effect of FGF21 on spermatozoa, we incubated fresh human spermatozoa with recombinant FGF21 for 30 min. The investigation of time effect was performed on spermatozoa with progressive motility, as presented in **Supplementary Data**

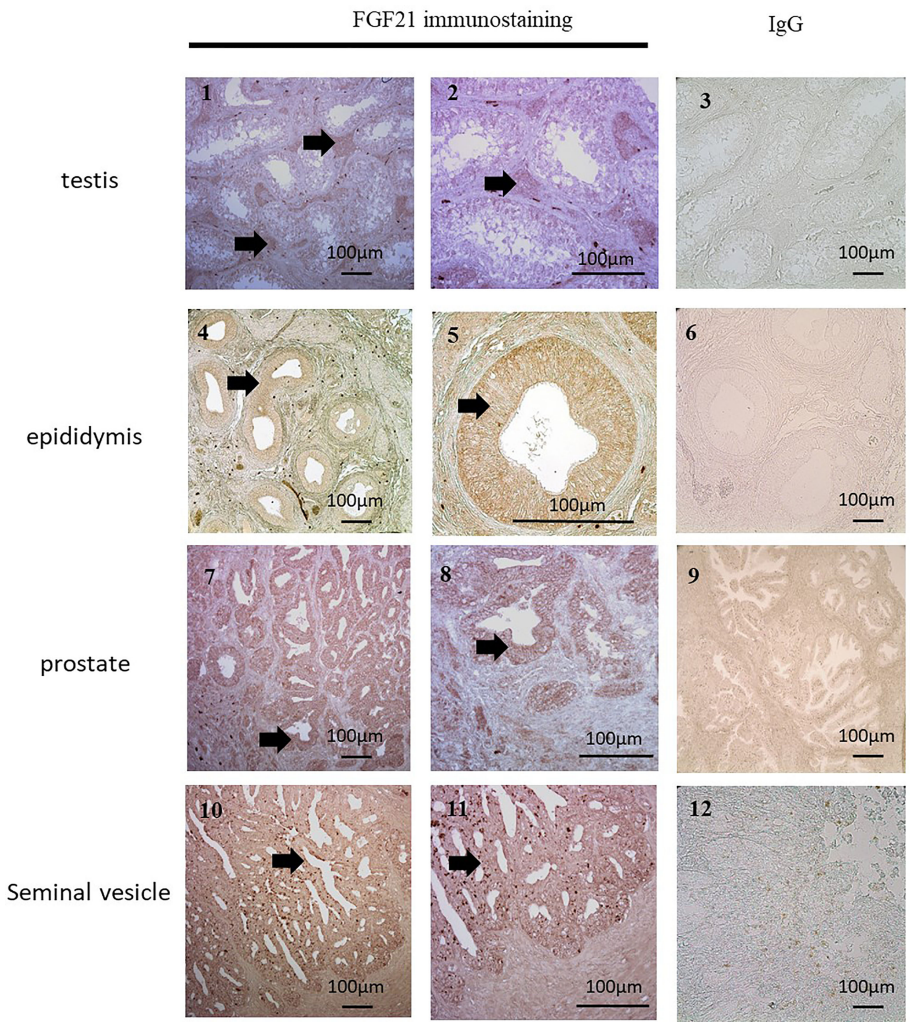

FIGURE 2 | Localization of FGF21 in reproductive organs. FGF21 was localized by immunohistochemical staining in human testis (1 and 2), epididymis (4 and 5), seminal vesicles (7 and 8) and prostate (10 and 11). Negative controls (3, 6, 9, and 12) were sections incubated with IgG. We observed FGF21 expression in Leydig cells and the epithelia of the epididymis, prostate and seminal vesicles (see arrow), which contained all secretory cells.

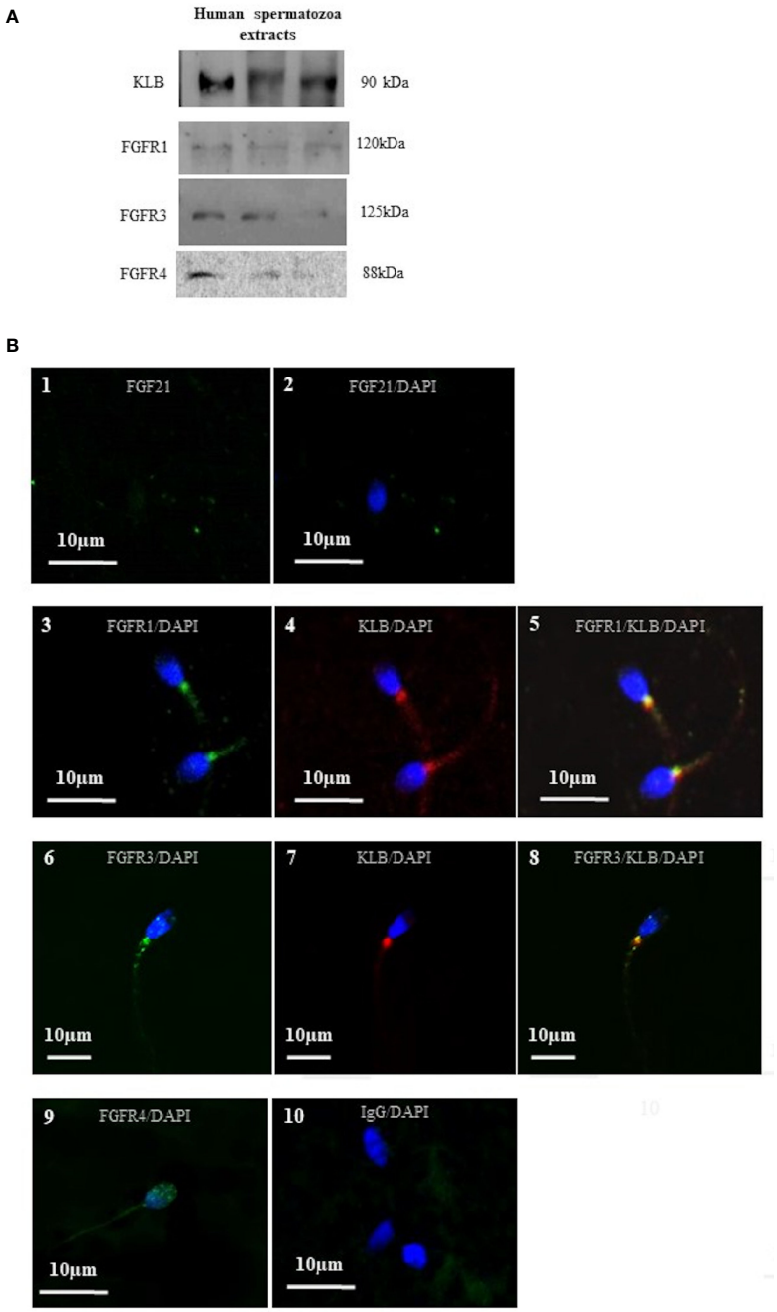

FIGURE 3 | Localization of FGF21 receptors on human spermatozoa. **(A)** Detection of sperm cofactor KLB and the FGFR1, FGFR3, and FGFR4 using Western immunoblotting. Red ponceau was used to check protein deposition on the membrane. Each lane represents extracts from different donors. Receptors and cofactor were tested on different gels. **(B)** FGF21 (1 and 2), FGFR1, FGFR3, FGFR4 (3, 6, and 9), and KLB (4 and 7) localization in human spermatozoa were analyzed by confocal microscopy after immunofluorescence. FGFR1, FGFR3 and FGFR4 were stained by Alexa Fluor 488 goat anti-rabbit IgG (green) and KLB (4 and 7) by Alexa Fluor 633 rabbit anti-mouse IgG (red). Merged picture of FGFR1 and FGFR3 with KLB showing the colocalization in the mid-piece of spermatozoa (5 and 8). Negative control was incubated with IgG (10). DNA was counterstained with DAPI.

Sheet 3A, showing that 30 min is the optimal time. No consequence on the viability of sperm after FGF21 stimulation was measured by SYBR-14 or propidium iodide staining (**Supplementary Data Sheet 3B**).

We have analyzed the effect of FGF21 on the motility of sperm motility after exposing human spermatozoa to FGF21 for 30 min at 37°C. Compared to the control condition, FGF21 was able to significantly increase progressive motility at the 0.1 ng/mL

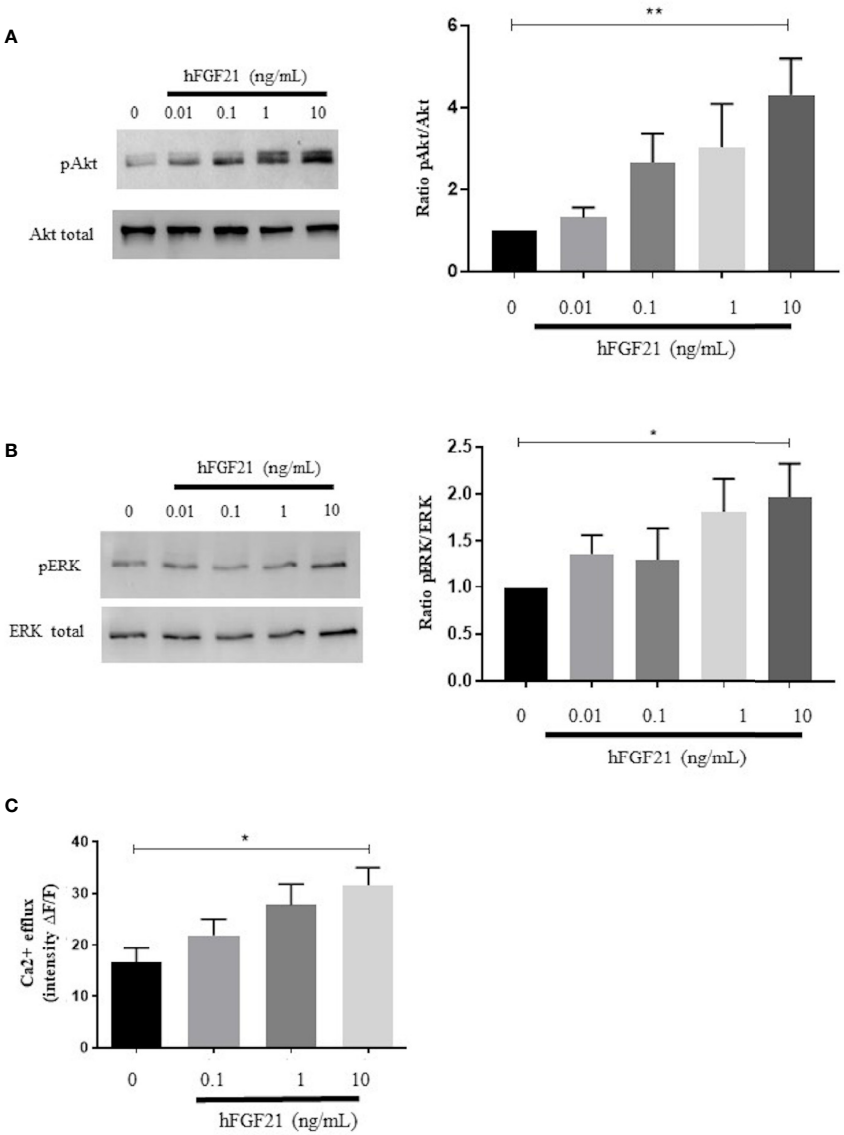

FIGURE 4 | Signaling pathways activated by the exposure of human spermatozoa to FGF21. **(A, B)** Western blot and analysis of phosphorylated (ser473) Akt and phosphorylated (Thr202/Tyr204) ERK in human spermatozoa exposed to recombinant FGF21 (0.01-10 ng/mL), as described in Materials and Methods section. Results are representative of at least four independent experiments. **(C)** Intracellular calcium responsiveness of sperm to recombinant FGF21 stimulation determined by fluorescence intensity relative to baseline (ΔF/F) at 30 min. *p < 0.05, **p < 0.01.

and higher concentrations of FGF21 (**Figure 5A**). Preincubation with the FGFR inhibitor PD173074 was able to eliminate the stimulatory effect induced by 10 ng/mL FGF21 (**Figure 5A**). Moreover, the average velocity and curvilinear line velocity of the spermatozoa determined by VCL and VAP were also improved by about 20% to 27% (0.1-10 ng/mL FGF21) and were returned to control values if a preincubation with PD173074 was performed (**Table 2**). Because motility is highly associated with mitochondria activity, the ATP level and mitochondrial membrane potential were analyzed. Despite spermatozoa still having a high mitochondrial membrane potential (control: 45.71% ± 1.89; FGF21: 0.1 ng/mL: 46.33% ± 2.12; FGF21: 1 ng/mL: 47.19% ± 2.19; FGF21: 10 ng/mL: 46.72% ± 2.15) (**Supplementary Data Sheet 4**), a significant increase of both the ATP and cAMP levels in the spermatozoa was measured at the 10 ng/mL FGF21 concentration as compared to those in the control (**Figures 5B, C**).

Furthermore, we did not observe any consequence of FGF21 exposure to the already high percentage of acrosome-reacted sperm induced with by calcium ionophore [**Figure 5D**-(2)] or without [**Figure 5D**-(1)]. But, FGF21 improved oxidative stress

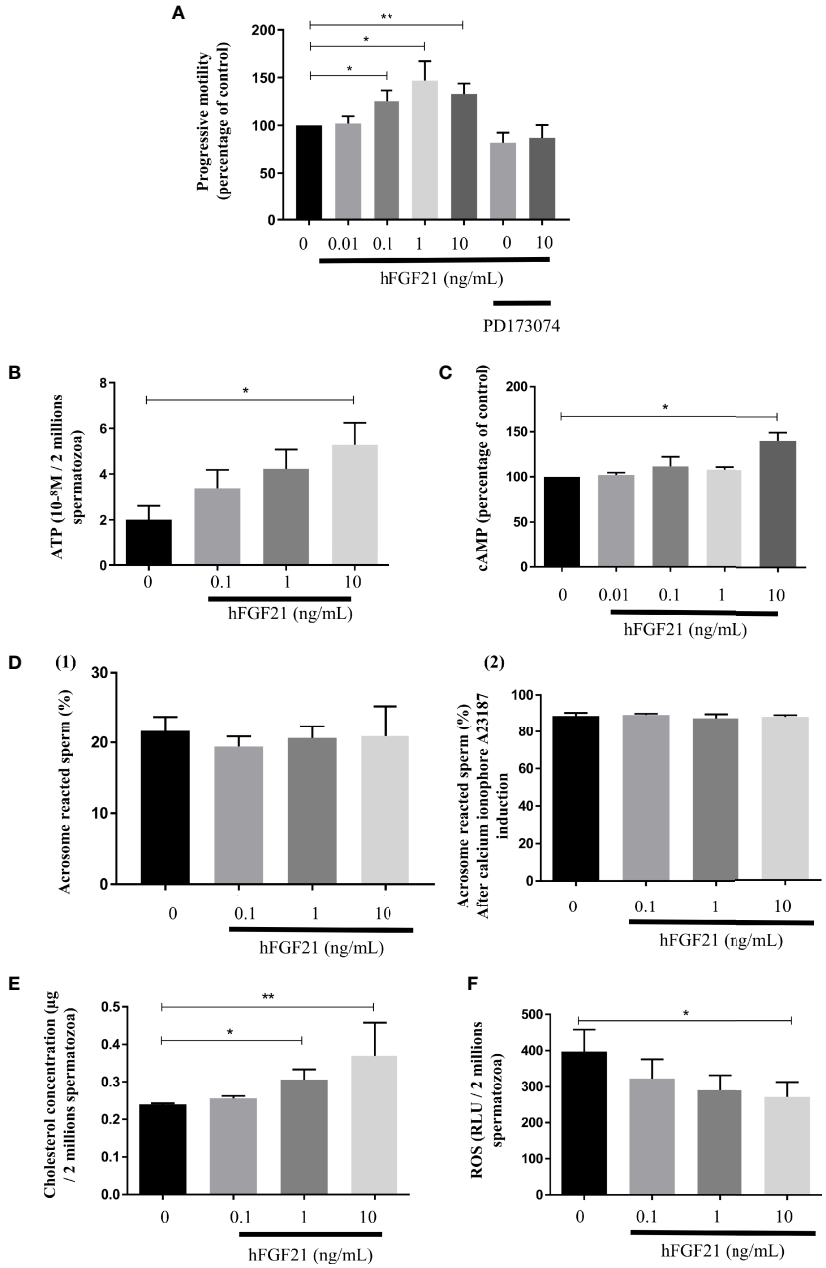

FIGURE 5 | FGF21 treatments increase spermatozoa motility. **(A)** Sperm progressive motility analyzed by computer-assisted sperm analysis (CASA) after 30 min of stimulation with recombinant FGF21, was presented in percentage of control. A preincubation with the FGFR inhibitor PD173074 was also used in presence of absence of a 30 min of stimulation with recombinant 10 ng/mL FGF21, (n = 8 patients). **(B, C)** Concentration of ATP in sperm stimulated for 30 min with recombinant FGF21 (10^{-8}M per 2.10^6 cells) and cAMP production in percentage of control, (n = 7 patients). **(D)** Percentage of acrosome-reacted sperm with (2) or without (1) calcium ionophore A23187 was quantified after PSA staining (percentage of PSA negative cells). Acrosome reaction of spermatozoa after 30 min of stimulation with recombinant FGF21 (n = 5 patients). **(E)** Total cholesterol concentration quantified in human spermatozoa after 30 min of stimulation with recombinant FGF21 (µg per 2.10^6 cells, n = 7 patients). **(F)** ROS levels in human spermatozoa incubated with increasing concentrations of recombinant FGF21 for 30 min (relative luminescence units per 2.10^6 cells, n = 7 patients). *$p < 0.05$, **$p < 0.01$.

by reducing the levels of reactive oxygen species (ROS) in a dose-dependent manner in human sperm (**Figure 5F**). Moreover, the stimulation of sperm by FGF21 induced a significant increase in cholesterol levels (**Figure 5E**).

DISCUSSION

We demonstrated the presence of FGF21 in human seminal fluid and argued that seminal FGF21 could be produced by the

different tissues of the male reproductive tract. Our immunohistochemical studies on human sperm revealed that FGF21 can transduce signaling by activating the β-klotho-FGFR1c or FGFR3 complex in the mid-piece of spermatozoa. The *in vitro* sperm stimulation by FGF21 leads to increased mobility by boosting the production of ATP in spermatozoa, and reduced oxidative stress. FGF21 activated in a dose-dependent manner the Akt and ERK phosphorylation and modification of the calcium efflux (**Figure 6**).

As in the literature, significant variations in plasma concentrations of FGF21 were measured: between 50 and 5 000 pg/mL in humans (9) and between 100 and 1 000 pg/mL in mice (22). We showed the presence of FGF21 in seminal fluid with concentrations 20-fold lower than that in the plasma. Interestingly, we observed that the levels of FGF21 in seminal fluid are independent of the BMI, semen volume, sperm concentration or sperm abnormalities. Changes in FGF21 plasma levels due to BMI were not recovered in seminal fluid. This raises two hypotheses regarding its origin. Firstly, FGF21 crosses (passively or actively) the testicular barrier from blood or secondly, seminal FGF21 is produced by local cells. Because the seminal vesicles secrete up to 75% (23) of the total volume of seminal fluid and are also completed by the prostate gland, we performed immunohistochemical studies of FGF21 in these human glands. The strong staining of FGF21 in human epididymis and seminal vesicle samples is coherent with the physiological role of these tissues. In hepatocytes, FGF21 is known to be regulated by starvation and PPARα activity. Interestingly, PPARα has already been described to be expressed specifically in epithelial cells of the prostate, which, in our study, also expressed the FGF21 protein (24, 25). To elucidate the origin of FGF21 in seminal fluid, the use of transgenic mouse lines with conditional knockouts could better determine if FGF21 is produced locally or not.

FGF21 preferentially activates FGFR1 and FGFR3, with the recruitment of the specific β-klotho cofactor (8). In our conditions, in human spermatozoa, we colocalized the FGFR1, FGFR3, and β-klotho in the mid-piece of the spermatozoa. Then, the machinery for signal transmission of FGF21 is in place in the part of spermatozoa that contain the mitochondria. These results are similar with previous immunocytochemical studies that allowed the localization of FGFR1, 2, 3 and 4 in ejaculated human sperm (17). In adipocytes, activation of receptors by FGF21 leads to phosphorylation of MAPK (ERK) and Akt (26, 27). As in adipocytes, we report, in human semen, an increase in phosphorylated (ser473) Akt and phospho-(Thr202/Tyr204) ERK. Some components of these pathways, such as ERK, PI3K and Akt, have been described to play an essential role in the maintenance of sperm function in mammalian sperm (28, 29). It has been shown that inhibition of Akt decreases sperm motility in mice, and activation of Akt stimulates sperm motility in humans (17, 30, 31). In somatic cells, the FGF/FGFR1 system has been shown to facilitate cell motility and migration by activation of the PI3K and ERK pathways (15, 32). From these data, it is not surprising to observe an enhancement of ATP levels in spermatozoa, as well as an increasing percentage of motile sperm. Sperm requires exceptionally high amounts of ATP when compared to somatic cells (33). Interestingly, an autocrine FGF (FGF2) has been reported to be present directly in human spermatozoa and 10 ng/mL recombinant FGF2 is able to enhance the motility of sperm (34). We notice that FGF2 needs a different receptor complex for FGF21, using the HS cofactor.

In the case of metabolic syndrome, these observations raise questions about the expression and the role of the endocrine FGF21 factor in sperm. Currently, a protective role for FGF21 is advanced, as well as multiple positive actions, and FGF21 could lead to the activation antioxidant pathways in targeted cells (35).

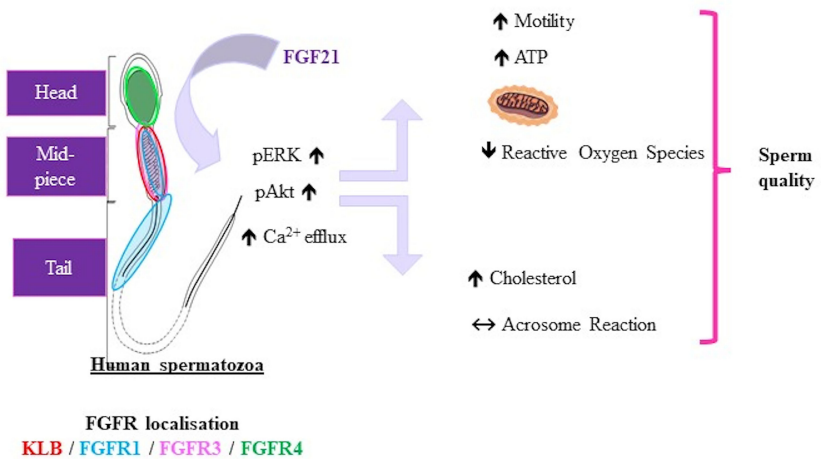

FIGURE 6 | Representative schema of FGF21 effects on human spermatozoa. Representative schema of the localization of FGFRs and KLB in spermatozoa and effects of FGF21 on human sperm. FGF21 induced phosphorylation of the Akt and ERK pathways and calcium levels, then increased the sperm motility associated with ATP content in a dose-dependent manner, and reduced oxidative stress.

In our study, we investigated the levels of ROS in human semen and describe a decrease in the ROS level 30 min after sperm stimulation by FGF21, suggesting a putative role in the observed improvement in motility. Indeed, the high susceptibility of sperm cells to ROS results from the composition of its membranes, which is rich in polyunsaturated fatty acids (PUFA) and thus highly susceptible to attack by ROS (36, 37). The low ROS levels after FGF21 treatment suggests a possibility to improve the quality and/or motility of human spermatozoa (38).

Events that are associated with capacitation include elevation of intracellular Ca^{2+}, higher levels of intracellular cAMP, and cholesterol efflux from the membranes of sperm that increases membrane fluidity (39). In sperm, Ca^{2+} plays a central role in the events preceding fertilization, such as motility, chemotaxis, and the acrosome reaction. In our conditions, we observed a FGF21 dose-dependent increase in Ca^{2+} efflux in spermatozoa; however, because the acrosome reaction was already elevated, no change in the acrosome reaction was noted. In the same way, the membranes of sperm and cholesterol efflux contribute to mechanisms that control sperm capacitation (40, 41). We observed a significant increase in cholesterol levels after FGF21 stimulation. In somatic cells, FGF21 promotes the efflux of cholesterol (42). If we transpose this knowledge to spermatozoa, we can hypothesize that FGF21 promotes cholesterol efflux, which can occur to the capacitation process.

The hepatokine FGF21 can be associated with other similar metabolic signals called adipokines, which have recently been shown to be involved in male fertility. Over the last decade, several adipokines have been detected in seminal fluid (43, 44) and have been shown to have a role in sperm functions, such as leptin (which is found to enhance sperm capacitation). Similarly to FGF21, differences in the concentrations of adipokines between seminal fluid and plasma have been reported with lower or enhanced concentrations in seminal fluid (45–47). It would be interesting to see if the concentrations of FGF21 in seminal fluid are associated with metabolic markers in blood and could be used as a biomarker related to fertility. Likewise, a better identification of the origin of FGF21 (between local secretion and peripheral) would make it possible to not only use the circulating rates as a predictor of the quality of male fertility but also to know whether the use of an FGF21 agonist would impact the spermatozoon.

In conclusion, we propose the endocrine factor FGF21 as a novel regulator of male reproductive function with direct actions on germ cells. FGF21 is able to improve sperm motility, oxidative stress, and markers of capacitation. FGF21 is then involved in the crosstalk between human metabolism and spermatogenesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Patients were enrolled into the Assisted Reproductive Centers (Tours, FERTIPROTECT protocol) for couple infertility exploration during a medical consultation. Forty participants gave full-informed written consent to participate in the study, and ethical approval was obtained from the Ethics Committee of the Vinci Clinic and CHRU Bretonneau. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GB and AE performed the study. CC and CR were technical support. FG, J-SB, CV, IP, EC-S are in charge of patients included in the study. GF and DDR provided human histological sections. JD participated in the review. PF and PHD designed the study and performed experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.775650/full#supplementary-material

Supplementary Data Sheet 1 | Biological and semen parameters of samples from all patients (n=40). Data are expressed as mean ± SEM (range).

Supplementary Data Sheet 2 | Correlations between FGF21 levels (plasma or seminal fluid) and sperm parameters (n=20).

Supplementary Data Sheet 3 | (A) Investigation of time effect on spermatozoa progressive motility. The optimal time to observe a change in sperm motility, in FGF21 (1ng/ml) condition was after 30 min of stimulation. All results are expressed as mean ± SEM (n = 4). *p < 0.05, **p < 0.01, Mann-Whitney Test. **(B)** Effect of FGF21 on semen viability was determined by a double-fluorescent labeling technique (SYBR-14 and propidium iodide). The PI negative and SYBR-14 positive population showing green fluorescence was considered alive. Samples were analyzed using flow cytometry (MoFlo AstriosEQ, USA).

Supplementary Data Sheet 4 | Mitochondrial activity was determined using a 200 nM mitotracker (Orange CM-H2TMRos, Invitrogen, Fisher Scientific, France). Samples were analyzed using flow cytometry (MoFlo AstriosEQ, USA). Twenty thousand events were collected per sample. Only sperm emitting red fluorescence were classified with a high mitochondrial membrane potential (HMMP), which is associated with high mitochondrial activity. The 'R2' window is Mitotracker negative cells and the 'R1' window is Mitotracker positive cells.

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Effect of Blastocyst Morphology and Developmental Rate on Euploidy and Live Birth Rates in Preimplantation Genetic Testing for Aneuploidy Cycles With Single-Embryo Transfer

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Objective: The aim of this study was to investigate whether blastocyst morphology and developmental rate are associated with euploidy and live birth rates (LBRs) in single euploid frozen–thawed embryo transfer (FET) cycles.

Design: Retrospective cohort study.

Methods: This study included 431 preimplantation genetic testing for aneuploidy (PGT-A) cycles followed by 393 FET cycles performed at our center from June 2017 to March 2021. All cycles were analyzed for euploidy based on blastocyst morphology (good, average and poor), developmental stage (day 5 and 6) and maternal age (< 35 and ≥ 35 years old). Multivariate logistic analysis models were used to identify the independent effects of conventional blastocyst morphology, developmental rate and morphological parameters (degree of blastocoele expansion, and grade of inner cell mass and trophectoderm (TE)) on LBRs.

Results: In the group of women aged < 35 years, compared with poor-quality blastocysts, good-quality blastocysts (62.90% vs. 32.46%; odds ratio (OR) 3.163, 95% confidence interval (CI) 2.247–4.451; $P < 0.001$) and average-quality blastocysts (46.70% vs. 32.46%; OR 1.665, 95% CI 1.287–2.154; $P < 0.001$) had significantly higher euploidy rates. Additionally, day 5 blastocysts were associated with higher euploidy rates than day 6 blastocysts (49.28% vs. 35.02%; OR 1.506, 95% CI 1.191–1.903; $P= 0.001$). In the group of women aged ≥35 years, euploidy rates were also associated with blastocyst morphology, with 41.86%, 45.65% and 24.39% of good, average and poor-quality embryos, respectively, exhibiting euploidy. However, no relationship was seen between euploidy and blastocyst developmental rate. Multiple logistic regression analysis show that overall blastocyst morphology of euploid embryos was not associated with LBR, only embryos with A-grade TE had significantly higher LBRs than those with C-grade TE (62.71% vs. 45.40%; OR 2.189, 95% CI 1.166–4.109; P=0.015). Similarly, LBRs were

significantly higher when day 5 blastocysts were transferred than when day 6 blastocysts were transferred (57.75% vs. 41.67%; OR 2.132, 95% CI 1.370–3.318; $P = 0.001$).

Conclusion: Poor-quality embryos have reduced rates of euploidy. However, blastocyst developmental rate only significantly associates with euploidy rates in women aged younger than 35. Furthermore, only TE grade and blastocyst developmental rate are significantly associated with LBRs following FET cycles.

Keywords: preimplantation genetic testing for aneuploidy, blastocyst morphology, developmental rate, euploid rates, live birth rates

INTRODUCTION

Although the development of assisted reproductive technologies (ARTs) has helped many couples to conceive, early embryo arrest and recurrent pregnancy loss remain significant challenges in the treatment of infertility (1). Recent studies have shown that more than half of human preimplantation embryos produced using *in vitro* fertilization (IVF) possess some degree of chromosomal abnormality (2). Furthermore, the proportion of aneuploid embryos increases significantly with advancing maternal age (3). Therefore, a major goal of reproductive medicine is to identify the best embryos, thereby maximizing the rate of success of IVF-derived embryo transfer.

The traditional system of embryo grading is based on the evaluation of morphological parameters, including the degree of blastocoel expansion, and the grades of inner cell mass (ICM) and trophectoderm (TE), and is widely used to select embryos with optimal development potential. However, this system cannot accurately evaluate the ploidy status of embryos, as more than half of embryos of good morphological grade are later determined as being aneuploid (4). With advances in molecular genetic techniques and improved embryo culture methods, preimplantation genetic testing for aneuploidy (PGT-A) with next-generation sequencing (NGS) may be a reliable method to improve embryo selection by identifying euploid embryos with normal chromosomes. However, controversy remains regarding the clinical effectiveness of PGT-A (5–7). A randomized controlled study suggested that PGT-A reduced the early pregnancy loss rate after the first embryo transfer (6), while a recent trial showed that PGT-A did not improve the frequency of cumulative live-birth (7). Preimplantation Genetic Diagnosis (PGD) European Consortium recommended PGT-A for advanced maternal age (AMA), recurrent implantation failure, recurrent pregnancy loss (RPL) and severe male factor (SMF) (8).

However, it remains unclear whether conventional parameters of embryo assessment, such as blastocyst morphology and developmental rate, correlate with the ploidy status of embryos. In an observational study, blastocyst morphology was found to be predictive of chromosomal status (9). Similarly, another study reported that blastocyst morphology was weakly associated with ploidy status (10). Additionally, Majumdar et al. (11) concluded that day 5 blastocysts had higher euploidy rates than day 6 blastocysts (70% vs. 34.1%). However, the relationship between blastocyst morphology, developmental rate, morphological parameters and pregnancy outcomes after the transfer of euploid blastocysts is not well understood. In 2017, Irani et al. reported that blastocyst morphologic grading, particularly ICM grade, is a valid predictor of pregnancy rate subsequent to frozen embryo transfer (FET) (12). Similarly, in another study, the live birth rates (LBRs) of day 5 euploid blastocysts were significantly higher than those of day 6 blastocysts (13). In contrast, Anderson et al. suggested that clinical outcomes were not significantly influenced by embryo morphology, provided a single euploid embryo is transferred (14).

In view of these contradictory findings, the aim of the present study was to investigate whether blastocyst morphology and developmental rate correlate with embryo euploidy or live birth rates (LBRs) following an FET cycle and the transfer of euploid embryos. Such knowledge would improve our understanding of euploid embryo selection, and assist physicians in clinical practice and patients undergoing PGT-A for conception.

MATERIALS AND METHODS

Study Design and Population

This was a retrospective cohort study conducted at the Reproductive Center of the Third Affiliated Hospital of Zhengzhou University from June 2017 to March 2021. We only included patients who underwent first PGT-A cycles and subsequently received first single euploid FET cycles. We excluded cycles if the embryos underwent preimplantation genetic diagnosis, were donated or involved women with uterine malformation. This study was performed in accordance with the Code of Ethics in the Declaration of Helsinki and was approved by the Ethics Review Committee of our hospital (protocol number 2021-WZ-010).

Ovarian Stimulation Protocol

Ovarian stimulation was performed using a gonadotropin-releasing hormone (GnRH) antagonist protocol in patients

Abbreviations: LBRs, live birth rates; FET, frozen–thawed embryo transfer; NGS, next-generation sequencing; PGT-A, preimplantation genetic testing for aneuploidy; ICSI, intracytoplasmic sperm injection; ICM, inner cell mass; TE, trophectoderm; AMA, advanced maternal age; RIF, recurrent implantation failure, RSA, recurrent spontaneous abortion; BMI, body mass index; FSH, follicle stimulating hormone; LH, luteinizing hormone; HRT, hormone replacement therapy; 2PN, two pronuclear.

undergoing PGT-A cycles. Briefly, recombinant follicle-stimulating hormone (FSH; Gonal-F, Merck Serono, Switzerland) was administered on day 2 or 3 of the menstrual period, and the ovarian response was monitored by transvaginal ultrasound and determination of serum estradiol concentrations. When the diameter of the dominant follicle reached 12–14 mm, 0.25 mg of a GnRH antagonist (Cetrotide, Merck Serono, Switzerland) was injected to cause pituitary suppression. Ovulation was triggered by administration of 0.2 mg Dophereline (Ipsen Pharma Biotech, France) or 250 μg recombinant human chorionic gonadotropin (hCG; Ovidrel, Merck Serono, Switzerland). Ultrasound-guided oocyte retrieval was performed 33–36 h after the triggering of ovulation.

Laboratory Protocols

Intracytoplasmic sperm injection (ICSI) was used for all cycles included in this study, and was carried out 4–6 h after oocyte retrieval. The presence of two equally sized pronuclei was assessed 16–18 h after insemination. All embryos were cultured to the blastocyst stage in Vitrolife sequential medium (Goteborg, Sweden). Blastocyst evaluation was performed prior to embryo biopsy. Embryologists graded the blastocysts based on the degree of expansion and the morphology of ICM and TE (15). The degree of expansion included the following six grades: (1) a nonexpanded embryo with the blastocele filling <50%; (2) the blastocele filling >50% of the embryo; (3) a full blastocyst with a blastocoele filling the embryo; (4) an expanded blastocyst with a blastocoele volume larger than that of the full blastocyst, with a thinning zona; (5) a hatching blastocyst with the TE starting to herniate through the zona; (6) a hatched blastocyst, with the blastocyst completely escaping from the zona. The ICM was graded as follows: (A) tightly packed with many cells, (B) loosely gathered with several cells or (C) very few cells. The TE was assigned one of the following grades: (A) many cells forming a cohesive epithelium, (B) few cells establishing a loose epithelium or (C) very few large cells. In our center, only blastocysts with ICM grade of at least B were biopsied. We divided blastocysts into three quality groups based mainly on ICM and TE grades: good (4AA, 5AA, 6AA, 4AB, 5AB, 6AB, 4BA, 5BA and 6BA), average (4BB, 5BB and 6BB) and poor (4AC, 5AC, 6AC, 4BC, 5BC and 6BC). Next, the embryos were biopsied on day 5 or 6, depending on the time of blastulation. Day 5 blastocysts were defined as "faster growing" and day 6 blastocysts as "slower growing." All embryo grading was carried out by two experienced embryologists to ensure consistency. TE biopsies were performed as previously described (16). If patients obtained more than 6 blastocysts, only 6 blastocysts are generally biopsied for economic reasons. NGS on the NextSeq550 platform (Illumina Inc., San Diego, CA), a widely used technology for 24-chromosome aneuploidy screening based on quantitative polymerase chain reaction (17), was used to diagnose embryos as euploid, aneuploid or mosaic. Finally, the biopsied blastocysts were cryopreserved using the Kitazato-based vitrification method (18).

Endometrial Preparation

Endometrial preparation FET included natural cycles or hormone replacement therapy (HRT) cycles. In general, women with regular menstrual cycles underwent natural FET cycles in which transvaginal ultrasound was used from day 10 of the menstrual cycle to monitor the development of the dominant follicle and endometrial thickness. Embryo transfer was performed on day 5 after a positive urinary urine luteinizing hormone test. Subsequently, 10 mg oral dydrogesterone (Duphaston; Solvay Pharmaceuticals BV) was administered three times daily from the day after ovulation to support the luteal phase. Patients who underwent HRT FET cycles took 4–8 mg estradiol valerate (ValieraVR; Laboratories Recalcine) daily for 12 days from day 3 of the menstrual cycle. When the endometrium measured ≥ 7 mm in thickness, as monitored by transvaginal ultrasound, twice-daily 10 mg oral dydrogesterone (Duphaston; Solvay Pharmaceuticals BV) and daily 90 mg progesterone sustained-release vaginal gel (Xenotong, Merck Sherano, Switzerland) daily was initiated. On day 6 of progesterone administration, a single euploid embryo was transferred. If pregnancy occurred, luteal support was continued until 7 weeks of gestation.

Outcome Measures

Primary outcomes were euploidy rates and LBRs. Women receiving PGT-A cycles were divided into two age groups: < 35 and ≥ 35 years old. Euploidy rates were compared for different blastocyst morphology (good, average and poor) and developmental rates (days 5 and 6) within the same age group. Euploidy rates were calculated as the number of euploid embryos with 46 chromosomes divided by the total number of biopsied embryos with genetic results. The primary endpoint was LBRs after single-euploid FET. The LBRs were defined as the number of live births divided by the sum of embryo transfer cycles included in the cohort.

Statistical Analysis

All statistical analyses were performed using SPSS 25.0 statistical software (IBM, United States) and the figures were produced using GraphPad Prism 9 software. Continuous variables were tested for normality and are expressed as means ± standard deviations, and were compared using Student's *t*-tests or Mann–Whitney *U* tests. Categorical variables are shown as percentage frequencies, and chi-squared tests were performed to identify statistically significant differences. The association between blastocyst morphology and developmental rate and the occurrence of euploidy was studied using generalized estimating equations analysis to adjust for the embryos derived from the same patient. Multivariate logistic analysis was used to investigate the effect of blastocyst morphology, developmental rate and morphological parameters on LBRs. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated to control for confounding factors. $P < 0.05$ was considered statistically significant.

RESULTS

Four hundred and thirty-one PGT-A cycles, during which 1,872 available blastocysts were generated and 1,647 embryos were biopsied for ploidy status and which were followed by 393 first FET cycles, were included in the data analysis. The mean maternal age of the patients was 32.04 ± 5.18 years. The embryological characteristics and clinical outcomes are listed in **Table 1**. Forty-seven of the biopsied blastocysts gave no genetic result (2.85%) due to failed amplification. The overall euploidy, aneuploidy and mosaic rates of the embryos were 40.44%, 47.13% and 12.44%, respectively.

Euploidy rates for all age groups are shown in **Supplementary Figure**. The generalized estimating equations analysis revealed that compared with poor-quality blastocysts, good-quality blastocysts (60.00% vs. 31.64%; OR 2.900, 95% CI 2.130–3.948; $P < 0.001$) and average-quality blastocysts (47.98% vs. 31.64%; OR 1.828, 95% CI 1.445–2.312; $P < 0.001$) had significantly higher euploidy rates after adjusting for blastocyst developmental rate, maternal age, maternal BMI, duration of infertility, type of infertility, infertility diagnosis, number of prior pregnancies, indication for PGT-A and basal FSH. Besides, day 5 blastocysts were associated with higher euploidy rates than day 6 blastocysts (48.49% vs. 34.72%; OR 1.431, 95% CI 1.155–1.772; P= 0.001) after adjusting for blastocyst morphology, maternal age, maternal BMI, duration of infertility, type of infertility, infertility diagnosis, number of prior pregnancies, indication for PGT-A and basal FSH. Meanwhile, in order to eliminate the embryos derived from the same patient, we performed generalized estimating equations analysis to further assess the association between blastocyst morphology/developmental rate and euploidy rate. After adjusted for blastocyst morphology, developmental rate, maternal age, maternal BMI, duration of infertility, type of infertility, infertility diagnosis, number of prior pregnancies, indication for PGT-A and basal FSH, blastocyst morphology and developmental rate are still associated with euploidy rate (**Supplemental Table**).

The association between women's age and euploidy rate was evaluated in **Figure 1**. Euploidy rate was start to gradually decline in women older than 35 years of age.

Euploidy rates for the two different age groups are shown in **Figure 2**. In the < 35 years age group, blastocyst morphology was not associated with the age of patients, and the corresponding age of good, average and poor-quality embryos were 29.40±2.86, 29.27±2.83 and 29.09±3.01, respectively (P=0.349). Compared with poor-quality blastocysts (236/727), good-quality blastocysts (117/186) (62.90% vs. 32.46%; OR 2.818, 95% CI 1.401–5.665; P=0.004) and average-quality blastocysts (184/394) (46.70% vs. 32.46%; OR 1.669, 95% CI 1.286–2.166; P< 0.001) yielded significantly higher euploidy rates after adjusting for blastocyst developmental rate, maternal age, maternal BMI, duration of infertility, type of infertility, infertility diagnosis, number of prior pregnancies, indication for PGT-A and basal FSH. Additionally, day 5 blastocysts (274/556) were associated with higher rates of euploidy than day 6 blastocysts (263/751) (49.28% vs. 35.02%; OR 1. 496, 95% CI 1.181–1.894; P = 0.001) after adjusting for blastocyst morphology, maternal age, maternal BMI, duration of infertility, type of infertility, infertility diagnosis, number of prior pregnancies, indication for PGT-A and basal FSH.

Similarly, in the ≥ 35 years age group, compared with poor-quality blastocysts (50/205), good-quality blastocysts (18/43) (41.86% vs. 24.39%; OR 2.131, 95% CI 1.071–4.242; P = 0.048) and average-quality blastocysts (42/92) (45.65% vs. 24.39%; OR 2.722, 95% CI 1.566–4.732; P = 0.003) had significantly higher euploidy rates after adjusting for developmental rate, maternal age, maternal BMI, duration of infertility, type of infertility, infertility diagnosis, number of prior pregnancies, indication for PGT-A and basal FSH. However, the difference in euploidy rates between the day 5 (48/125) and day 6 (62/215) blastocysts was not statistically significant (38.40% vs. 28.84%; OR = 1.135, 95% CI 0.677–1.903; P = 0.630) after adjusting for blastocyst morphology, maternal age, maternal BMI, duration of

TABLE 1 | Embryological and clinical outcomes in patients who underwent PGT-A cycles.

Characteristic	Description
Number of PGT-A cycles (n)	431
Maternal age (years)	32.04 ± 5.18
Maternal BMI (kg/m^2)	23.81 ± 3.05
Duration of infertility (years)	2.90 ± 1.99
Type of infertility, n (%)	
Primary	108 (25.06)
Secondary	323 (74.94)
Infertility diagnosis, n (%)	
Tubal factor	155 (35.96)
Male factor	126 (29.23)
Diminished ovarian reserve	61 (14.15)
Combined factor	64 (25.06)
Unexplained factor	25 (5.80)
Number of prior pregnancies (n)	2.07 ± 1.82
Number of prior embryos transfer (n)	1.28 ± 0.65
Number of prior successful transfer (n)	0.67 ± 0.50
Indication for PGT-A, n (%)	
AMA	45 (10.44)
RIF	89 (20.65)
RPL	111 (25.75)
Combined indication	186 (43.16)
Basal FSH	6.57 ± 2.16
Number of oocytes retrieved (n)	6,481
Number of mature oocytes (n)	4,885
Number of 2PN cleavages (n)	3,997
Number of available blastocysts generated	1872
Number of blastocysts biopsied (n)	1,647
Blastocysts with no genetic results (n,%)	47 (2.85)
Results of 1,600 blastocysts biopsied (n, %)	
Euploid	647 (40.44)
Aneuploid	754 (47.13)
Mosaic	199 (12.44)
Number of euploid FETs (n,%)	393
Implantation rate	234/393 (59.54)
Miscarriage rate	36/234 (15.38)
Live birth rate	198/393 (50.38)

Values are presented as means ± standard deviations or n (%). BMI, body mass index; PGT-A, preimplantation genetic testing for aneuploidy; FSH, follicle-stimulating hormone; AMA, advanced maternal age; RIF, recurrent implantation failure; RPL, recurrent pregnancy loss.

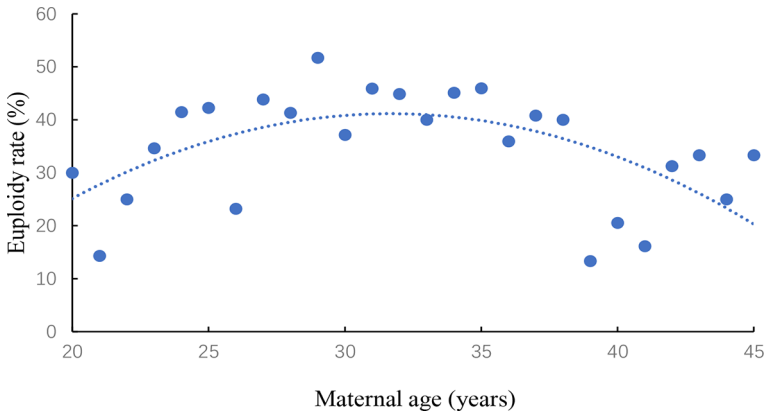

FIGURE 1 | Embryo euploidy according to women's age.

infertility, type of infertility, infertility diagnosis, number of prior pregnancies, indication for PGT-A and basal FSH.

Only 393 cycles progressed to first single-euploid FET; 198 of these ended with live birth and 195 did not achieve live birth. The characteristics of patients who underwent FET cycles are summarized in **Table 2**. The possibility of live birth was not significantly influenced by maternal age, duration of infertility, type of infertility, infertility diagnosis, number of prior pregnancies, number of prior embryos transfer, number of prior successful transfer, indications for PGT-A or basal FSH ($P > 0.05$). The proportions of natural FET cycles and maternal body mass index (BMI) were significantly higher in the no live birth group than in the live birth group ($P < 0.05$). Endometrial thickness on transfer day was also found to be significantly lower in the no live birth group than in the live birth group ($P < 0.05$).

Finally, to determine whether conventional blastocyst morphology, developmental rate and morphological parameters influenced LBRs, a multivariate logistic regression was performed after adjusting for confounding factors (maternal age, maternal BMI, type of infertility, infertility diagnosis, number of prior pregnancies, endometrial preparation protocols and endometrial thickness on transfer day), as shown in **Table 3**. We found that the odds of a live birth were significantly higher for A-grade TE blastocysts than for C-grade TE blastocysts (62.71% vs. 45.40%; OR 2.212, 95% CI 1.164–4.201; $P = 0.015$). Moreover, the likelihood of live birth was significantly higher for day 5 euploid

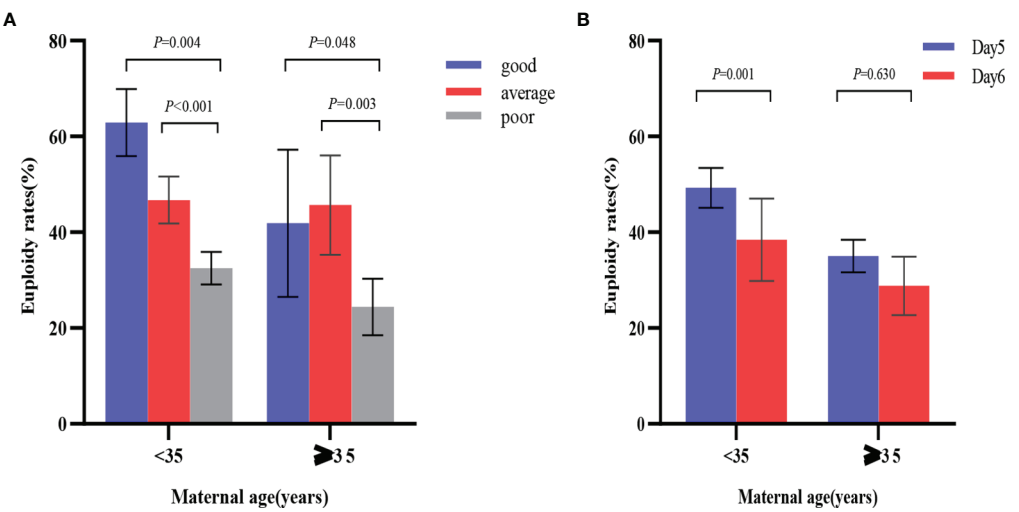

FIGURE 2 | Comparison of euploidy rates by blastocyst morphology and developmental rates in the two age groups. **(A)** odds ratio was adjusted for blastocyst developmental rate, maternal age, maternal BMI, duration of infertility, type of infertility, infertility diagnosis, number of prior pregnancies, indication for PGT-A and basal FSH; **(B)** odds ratio was adjusted for blastocyst morphology, maternal age, maternal BMI, duration of infertility, type of infertility, infertility diagnosis, number of prior pregnancies, indication for PGT-A and basal FSH. *P* values are adjusted *p* values.

TABLE 2 | Patient characteristics of transferred frozen-thawed euploid blastocysts in terms of birth outcome.

Parameters	Live birth (*n* = 198)	No live birth (*n* = 195)	*P* value
Maternal age (years)	31.12 ± 4.76	32.05 ± 4.70	0.052
Maternal BMI (kg/m^2)	23.77 ± 3.06	24.64 ± 3.00	0.005
Duration of infertility (years)	2.78 ± 1.89	2.94 ± 2.09	0.425
Type of infertility, n (%)			0.245
Primary	58 (29.29)	47 (24.10)	
Secondary	140 (70.71)	148 (75.90)	
Infertility diagnosis, n (%)			0.979
Tubal factor	74 (37.37)	70 (35.90)	
Male factor	57 (28.79)	60 (30.77)	
Diminished ovarian reserve	27 (13.64)	29 (14.87)	
Combined factor	29 (14.65)	26 (13.33)	
Unexplained factor	11 (5.56)	10 (5.13)	
Number of prior pregnancies (n)	1.87 ± 1.68	2.22 ± 1.97	0.065
Number of prior embryos transfers (n)	1.25 ± 0.63	1.23 ± 0.51	0.706
Number of prior successful transfers (n)	0.60 ± 0.55	0.56 ± 0.52	0.908
Indication for PGT-A, n (%)			0.411
AMA	15 (7.58)	23 (11.79)	
RIF	44 (22.22)	36 (18.46)	
RPL	52 (26.26)	46 (23.59)	
Combined indications	87 (43.94)	90 (47.18)	
Basal FSH (IU/L)	6.38 ± 2.34	6.28 ± 2.47	0.683
Endometrial preparation protocols, n (%)			0.022
Natural	94 (47.47)	115 (58.97)	
HRT	104 (52.53)	80 (41.03)	
Endometrial thickness on transfer day (mm)	9.32 ± 1.53	8.82 ± 1.54	0.001

Values are presented as means ± standard deviations or n (%). BMI, body mass index; PGT-A, preimplantation genetic testing for aneuploidy; FSH, follicle-stimulating hormone; AMA, advanced maternal age; RIF, recurrent implantation failure; RPL, recurrent pregnancy loss; HRT; hormone replacement therapy.

blastocysts compared to day 6 euploid blastocysts (57.75% vs. 41.67%; OR 2.247, 95% CI 1.460–3.460; *P*<0.001). However, blastocyst morphology, the degree of blastocoele expansion and ICM grade were not significantly associated with LBRs.

DISCUSSION

This study determined the effects of blastocyst morphology and developmental rate on embryo euploidy and LBRs following FET cycles. We found that compared to poor-quality embryos, both good and average-quality embryos yield significantly higher euploidy rates for patients of the same age group. We also found that faster-growing embryos (day 5) have a greater likelihood of euploidy than slower-growing embryos (day 6), especially when maternal age is less than 35 years. TE grade and developmental rate had the strongest association with live birth.

It is well known that maternal age is a significant factor affecting pregnancy outcomes, both in ART and spontaneous conceptions. The age-related decline in reproductive ability is attributed to a decline in ovarian reserve and an increase in aneuploidy rates (19). Given this, we stratified the patients by age

TABLE 3 | Multiple logistic regression analysis of the association between live birth rate, blastocyst grade and developmental rates in single-euploid embryo transfer cycles.

Variable	Value	Live birth (%)	OR (95% CI)	*P* value
Blastocyst				
	Good	55.56 (50/90)	1.689 (0.980–2.909)	0.059
	Average	53.49 (69/129)	1.423 (0.886–2.287)	0.145
	Poor	45.40 (79/174)		
ICM				
	A	56.06 (37/66)	1.540 (0.875–2.711)	0.134
	B	49.24 (161/327)		
TE				
	A	62.71 (37/59)	2.212 (1.164–4.201)	0.015
	B	51.25 (82/160)	1.341 (0.857–2.099)	0.199
	C	45.40 (79/174)		
Developmental rate				
	Day 5	57.75 (123/213)	2.247 (1.460–3.460)	<0.001
	Day 6	41.67 (75/180)		

OR, odds ratio; CI, confidence interval; ICM, inner cell mass; TE, trophectoderm. The values for LBRs are adjusted for maternal age; maternal body-mass index; type of infertility; infertility diagnosis; number of prior pregnancies; endometrial preparation protocols and endometrial thickness on embryo transfer day.

group to investigate whether blastocyst morphology and developmental rate are associated with euploidy rates. We found that poor-quality embryos had a reduced rate of euploidy compared to good and average-quality embryos from within the same age group. This finding is in agreement with a recent study that reported embryos with better morphological scores showed a higher euploidy rate than embryos of lower quality (9). Nevertheless, traditional morphology based selection should not be relied on to ensure transfer of euploid blastocysts, as a significant proportion of aneuploid embryos exhibit good morphology according to the criteria used.

Another embryological parameter analyzed in this study was the developmental rate at which each embryo reached the blastocyst stage. Embryos reaching the expanded blastocyst stage by day 5 had higher euploidy rates than those that did so by day 6. However, the timing of blastocyst formation was not linked to chromosomal ploidy status in women aged older than 35. We therefore suggest that morphology is a more reliable parameter than the speed of blastocyst formation for predicting embryo chromosomal status, especially in AMA women.

We found that the possibility of live birth was not significantly influenced by maternal age. Consistent with Irani et al. previously published study, maternal age did not influence the implantation potential of euploid embryos (20). However, Reig et al. demonstrated that increasing maternal age is associated with diminished live birth rates when analyzing only euploid embryo transfers (21). The effects of blastocyst morphology and developmental rate on LBRs can be interpreted as an indirect indicator of their effects on embryo development and implantation potential. It seems reasonable to assume that once euploid blastocysts are transferred, pregnancy outcome is independent of blastocyst morphology. A recent retrospective cohort study showed that maternal age, blastocyst morphology and day of biopsy are all associated with sustained implantation rate even after euploid embryos are transferred (22). Indeed, we found that blastocyst morphology is not associated with embryo survival: the LBRs of poor-quality euploid blastocysts were comparable to those of blastocysts scored as good or average quality. Thus, morphology does not appear to be reliable predictor of live birth if multiple euploid embryos are available for transfer.

The LBRs of embryos with different developmental rates were found to be significantly different, suggesting that embryo developmental rate correlates with survival potential. Our findings are consistent with the results of Irani et al., who reported that day 5 euploid embryos had higher LBRs than day 6 embryos (13). This may reflect a higher survival rate of faster developing (i.e., day 5) embryos or improved synchronization between the embryo and the endometrium on day 5. However, in contrast, a retrospective cohort study demonstrated that day 5 and 6 euploid embryos yielded the same LBRs (23).

It is widely known that the Gardner and Schoolcraft grading system consists of three criteria: the degree of blastocoel expansion and the qualities of the ICM and TE (24). Previous research findings conflict on which parameters are associated with blastocyst transfer outcomes. Some studies have suggested that ICM quality might be a key factor in determining the LBR (25, 26). Indeed, as the ICM itself contributes to the fetal tissue, it is theorized that ICM grade should be the most important morphological feature influencing transfer outcomes. However, a recent publication reported that blastocoele expansion is the most significant morphological predictor of live birth after single blastocyst FET (27). In the current retrospective cohort study, we found that LBRs for A-grade TE euploid blastocysts are higher than those for C-grade TE euploid blastocysts, suggesting that TE grade influences embryo developmental competence. In line with this, numerous studies have reported that TE grade can be used as an independent and accurate predictor of pregnancy outcomes (28–30). The TE differentiates into the placenta, which requires healthy TE to be capable of invading the endometrium and initiating the complex implantation process required to maintain a healthy pregnancy. Additionally, it was shown that embryos with higher TE grades secrete hCG earlier than those with lower TE grades, with such secretion being essential for embryo implantation and embryo–endometrium cross-talk (31).

As all PGT-A and FET cycles included in this study were performed at a single reproductive center, the reliability of our results is increased. Second, embryo scoring was performed by two experienced embryologists, each of whom had 5 years of experience. Third, we only included single-euploid embryo transfer cycles, which may have eliminated additional confounding factors that might influence outcomes. However, this study also has some limitations. First, a retrospective cohort study design has its inherent limitations. Second, if more than one euploid embryo is available for transfer, blastocysts with good quality are prioritized, which might have led to selection bias. Additional prospective studies of larger sample size are required to validate our current findings.

CONCLUSION

In conclusion, euploidy rates after NGS were found to be lower for poor-quality embryos than for average-quality or good-quality embryos. Increased rates of euploidy were seen for faster-developing (i.e. day 5) embryos but only in women aged younger than 35. Moreover, blastocyst developmental rate and TE grade were associated with the highest probability of live birth after transfer of a single euploid embryo.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Third Affiliated Hospital of Zhengzhou University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HL and YG proposed the study. NL, BR, and YCZ acquired and analyzed the data. YD, HK, and YJZ prepared all tables and figures. NL wrote the manuscript. HL revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.858042/full#supplementary-material

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Correlation Between the Presence of Antinuclear Antibodies and Recurrent Pregnancy Loss: A Mini Review

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In the past decade, the incidence of recurrent pregnancy loss (RPL) has increased significantly, and immunological disorders have been considered as one of the possible causes contributing to RPL. The presence of antinuclear antibodies (ANAs) is regarded as a typical antibody of autoimmunity. However, the relationship between the presence of ANAs and RPL, the underlying mechanism, and the possible role of immunotherapy is still controversial. The aim of this mini review is to assess the association between ANAs and RPL and the effects of immunotherapy on pregnancy outcomes in women with positive ANAs and a history of RPL from the available data and to provide a relevant reference basis for clinical application in this group of women.

Keywords: antinuclear antibodies (ANAs), recurrent pregnancy loss (RPL), prognostic value, pregnancy outcome, immunotherapy

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INTRODUCTION

Pregnancy loss, or the spontaneous death of a pregnancy before the fetus reaches viability, affects up to 20% of women who conceive, making it one of the commonest complications of pregnancy (1). Currently, the definitions of recurrent pregnancy loss (RPL) vary in different countries and regions internationally. Based on the European Society of Human Reproduction and Embryology (ESHRE) guideline, RPL was defined as the loss of two or more pregnancies before 24 weeks of pregnancy (1), while the Royal College of Obstetricians and Gynaecologists (RCOG) guideline used a stricter criterion, which was defined as three or more fetal losses before 24 weeks of pregnancy, including biochemical pregnancy (2). Compared with pregnancy loss, RPL is less prevalent and affects approximately 1 to 3% of women who are trying to conceive (3). RPL has a significant negative impact on the physiological and psychological health of women and brings great emotional frustration to couples.

There are several recognized causes related to RPL, namely, genetic factors, uterine abnormalities (congenital malformations, endometrial polyps, uterine fibroids, etc.), hormonal and metabolic disorders (thyroid dysfunction, diabetes, polycystic ovary syndrome, hyper-prolactinemia, etc.), thrombophilia, immunological disorders, and male factors. However, approximately half of RPL remains unexplained in etiology, which is referred to as unexplained RPL (uRPL) (4).

Antinuclear antibodies (ANAs) are a group of autoantibodies that target components of the cell nucleus and bind to proteins, nucleic acids, and protein–nucleic acid complexes (5). ANA detection may be performed by immunofluorescence (IF) on human epithelial laryngeal carcinoma type 2

cells or by solid-phase ANA screening immunoassay with at least equivalent performance (6). Indirect immunofluorescence is an extensively used laboratory test for detecting ANAs. The result is usually expressed in titers, which are used to describe the antibody concentration in peripheral blood. Positive ANAs expressed in low titers are commonly found in healthy women, whereas the presence of high titers (>1:160) is closely related to autoimmune diseases, namely, systemic lupus erythematosus, systemic sclerosis, and Sjögren's syndrome, which are related to adverse pregnancy outcomes (7, 8). A previous cross-sectional analysis including 4,754 individuals from the US showed the prevalence of positive ANAs could reach up to 13.8% and vary widely in healthy populations, ranging from 5.92% in Chinese to 30.8% in African Americans, which was higher in women (17.8%), compared with that in men (9.6%) (9).

There is evidence that autoimmunity is an important risk factor for pregnancy loss. A series of studies have tried to elucidate the association between ANAs and RPL, but the relationship between ANAs and RPL pregnancy outcomes and whether the treatments for ANA-positive affect pregnancy outcomes are still highly controversial. However, the prognostic value of ANAs for subsequent pregnancy outcomes is unclear as well. Furthermore, the underlying pathophysiological link and mechanism that the presence of ANAs plays in women with RPL has not yet been fully understood.

Therefore, given the importance of the potential association between the presence of antinuclear antibodies and pregnancy loss, the aim of this mini review was to provide evidence on the relationship between positive ANAs and recurrent pregnancy loss and the possible underlying mechanism. Given the possible role of immunotherapy in improving pregnancy outcomes in women with a history of RPL, we also reviewed the available clinical studies on the effects of different types of immunotherapy, focusing on positive ANAs.

PRESENCE OF ANAs AND ITS PROGNOSTIC VALUE IN RPL

The presence of ANAs has been regarded as a typical feature of autoimmunity. There is growing evidence suggesting that ANAs can play a role in both early pregnancy and pregnancy loss. Although how the ANAs are present in women with RPL remains unclear, it is possible that the presence of ANAs in RPL indicates that there may be an underlying autoimmune disorder in RPL women, at least in a subgroup of patients, which affects the development of the trophoblast and can lead to early pregnancy loss. Therefore, RPL women with previous autoimmune diseases are likely to have a higher prevalence of positive ANAs. One previous study showed that in women with autoimmune disorders, a history of RPL is independently associated with reactivity against three distinct Ro antigen-related reactivities (a subtype of autoantibody of ANAs), suggesting that cumulative autoimmune responses correlate with the risk of spontaneous miscarriage (10). However, even in RPL women without autoimmune disorders, ANAs still need to be screened. A recent meta-analysis, including 2,683 women with RPL without defined autoimmune diseases and 2,355 controls, found that the total positive rate of ANAs was significantly higher in the RPL group, compared with the control group (22.0% vs 8.3%, OR = 2.97, 95% CI 1.91–4.64, P <0.001) (11). Additionally, subgroup analysis demonstrated a significant association between high ANA titers (≥1:160) and RPL (OR = 45.89, 95% CI 8.44–249.45, P <0.001), while there was no significant relationship between low titers of ANAs (1:40 ≤ANA ≤1:80) and RPL (OR = 2.44, 95% CI 0.42–14.06, P = 0.32).

In the other previous studies, most of them did not provide definite information on the past history of autoimmune disorders, and the results showed that the prevalence of positive ANAs in women with a history of RPL varied (**Table 1**). Several previous studies have found a significantly increased prevalence of positive ANAs in women with a history of RPL. There was a significantly higher proportion of women with RPL who had ANAs at ≥1:80 compared with controls (21, 23, 30). A case–control study including 294 women showed that women with RPL had a three-fold higher prevalence of positive ANAs (50%) and higher serum titers of ANAs (≥1:80) when compared with women without reproductive disorders (21). Similarly, another study including 560 Iranian women showed that the ANA-positive rate in women with a history of two or more unexplained pregnancy losses (13.21%) was significantly higher than that in control women (0.9%) (24). This observation was also supported by another systematic review and meta-analysis (31). Their results showed that the prevalence of positive ANAs in the RPL women (20.6%, 288/1,400) was significantly higher than it was non-pregnant women with no history of pregnancy loss (6.7%, 72/1,080) (31).

Nevertheless, some other studies failed to find such a significant difference between women with RPL and controls. A case–control study including 72 Bangladeshi women showed that the mean serum levels of ANAs in women with RPL (1.07 ± 0.34) were similar in cases compared with controls (0.92 ± 0.15) (17). Another study including 243 Caucasian Argentine healthy women showed that the ANA-positive rate in women with a history of three or more unexplained pregnancy losses (16%) was similar to that in control women (14%), and the median titers (1:40) (16). A recent study including 114 women with RPL and 107 healthy controls found no significant differences were ascertained regarding serum levels of ANAs (0.32 vs 0.39, P = 0.2) (27).

It is also interesting to evaluate the association of ANAs with gene polymorphisms of the hemostasis system and RPL. Hereditary thrombophilia, namely, factor V Leiden mutation, prothrombin mutation, protein C, protein S, and antithrombin deficiency, could be associated with adverse obstetric outcomes. There was one previous study investigating the presence of autoimmune antibodies (antithyroid antibodies and ANAs) and polymorphism genotypes for factor V Leiden, prothrombin gene mutation, and MTHFR in women with RPL (14). The results showed that only one out of 39 subjects had a combination of hereditary thrombophilia and positive autoimmune antibodies, suggesting a weak association between ANAs and gene polymorphisms of the hemostasis system and

TABLE 1 | The prevalence of positive ANAs in women with a history of RPL in different studies.

Author	Year	Ethnic/ Country	Study subjects	Definition of RPL (the number of pregnancy loss)	ANA detection methods (cut-off dilution)	Prevalence of ANA+ (case group)	Prevalence of ANA+ (control group)
Hefler-Frischmuth et al. (12)	2017	Caucasian	Case: 114 RPL Control: 107 age-matched healthy controls	≥3	ELISA (unclear)	NA	NA
Sakthiswary et al. (13)	2015	Malaysia	Case: 68 uRPL Control: 60 non-pregnant women without pregnancy	≥2	IF (1:80)	35.3%	13.3%
Molazadeh et al. (14)	2014	Iran	Case: 560 uRPL Control: 560 healthy controls	≥2	IF (1:40)	13.2%	0.9%
Roye-Green et al. (15)	2011	Jamaica	Case: 50 RPL Control: 135 multiparous women without pregnancy loss	≥2	IF (unclear)	2%	2.2%
Ticconi et al. (16)	2010	Caucasian	Case: 194 RPL Control: 100 non-pregnant controls	≥2	IF (1:80)	50%	16%
Giasuddin (17)	2010	Bangladesh	Case: 35 RPL Control: 37 normal pregnant women	≥3	ELISA (unclear)	20%	0.54%
Bustos et al. (18)	2006	Argentina	Case: 118 RPL Control: 125 fertile control women without abortions and two children	≥3	IF (1:40)	16%	14%
Habara et al. (19)	2002	Japan	Case: 49 uRPL Control: 72 normal women with sterility caused by male factor	≥3	IF (unclear)	NA	NA
Matsubayashi et al. (20)	2001	Japan	Case: 273 RPL Control: 200 healthy non-pregnant women	≥2	IF (1:80)	23.4%	13%
Kaider et al. (21)	1999	USA	Case: 302 RPL Control: 20 healthy fertile women	≥3	ELISA (unclear)	45.7%	10%
Kovács et al. (22)	1999	Hungary	Case: 59 uRPL Control: 25 non-pregnant women without pregnancy	≥2	IF (unclear)	3.39%	8%
Stern et al. (23)	1998	New Zealand	Case: 97 RPL Control: 106 fertile controls	≥3	IF (1:80)	22.7%	9.4%
Konidaris et al. (24)	1994	Greece	Case: 44 uRPL Control: 4 non-pregnant healthy women without pregnancy loss	≥3	IF (1:40)	9.1%	2.9%
Bahar et al. (25)	1993	Kuwait	Case: 103 uRPL Control: 85 multiparous non-pregnant women without pregnancy loss	≥3	IF (1:40)	13.6%	1.2%
Kwak et al. (26)	1992	USA	Case: 153 uRPL Control: 90 normal controls	≥3	IF (1:40)	19.0%	14.0%
Harger et al. (27)	1989	USA	Case: 277 RPL Control: 199 non-pregnant/299 pregnant women	≥2	IF (1:40)	16.3%	16.8%/16.6%
Petri et al. (28)	1987	USA	Case: 44 uRPL Control: 40 Volunteers	≥3	IF (1:40)	16%	20%
Garcia-De La Torre et al. (29)	1984	Mexico	Case: 20 uRPL Control: 30 women with normal pregnancy	≥3	IF (1:20)	30%	6.6%

NA, Not Applicable.

RPL (14). Another study evaluating thrombophilia and immunological disorders in pregnancies found that the presence of ANAs was significantly elevated in pregnancies complicated by small for gestational age, while the prevalence of inherited thrombophilia did not differ significantly. However, the authors did not provide information on the history of miscarriage of the participants (12).

Regarding the prognostic value of ANAs, there are several studies reporting different results. An earlier study found a higher subsequent miscarriage rate in ANA-positive women with RPL as compared with ANA-negative subjects (36). In another previous prospective study, Ticconi et al. investigated the ANA status in a cohort of women with unexplained RPL before pregnancy, repeated the test during the first trimester of the subsequent pregnancy, and correlated the result with the pregnancy outcome (23). Interestingly, the authors found that subsequent miscarriages occurred in women who had ANAs positive before pregnancy and remained positive in the first

trimester, whereas no miscarriages were observed in women who had ANAs positive before pregnancy but turned negative in the first trimester, which suggested that the disappearance of ANA in early pregnancy could have a favorable prognostic value in the subsequent pregnancies (23).

However, some studies did not find that the presence of ANAs could predict new pregnancy losses. One study found that the live birth rate of the next pregnancy (untreated) in the RPL patients with positive ANAs at ≥1:80 (52%) was not significantly different from that in RPL patients with negative ANAs (65.6%) (30). Likewise, Ogasawara et al. observed that the ANA-positive rate in women with RPL was 17%, and the miscarriage rate in the next pregnancy was similar to that of women who had RPL and tested negative for ANAs (32). Additionally, it was reported that the occurrence of subsequent live births was not affected by ANA levels or associated thrombophilia (25).

There are several possible causes contributing to the observed difference derived from the above studies. Firstly, different definitions of RPL were used in different studies; some studies used two or more pregnancy losses (18, 21, 24, 30, 33–35) while others used three or more pregnancy losses (13, 15–17, 19, 20, 22, 26–29) to define RPL. Secondly, the subjects recruited in the above studies were from different ethnic populations, which may have led to variability. Thirdly, these studies used different methods or assays to detect ANAs; some used IF (12, 14, 16, 19–22, 24, 26, 28–30, 36) while others used ELISA (15, 17, 27). Furthermore, different criteria were applied to define positive for ANAs; some used 1:40 (16, 19, 22, 24, 26, 28, 30, 34, 35) while others used 1:80 (14, 19, 20, 36).

POSSIBLE MECHANISMS FOR THE PRESENCE OF ANAs IN RPL

Generally, autoimmune disorders may impair all stages of pregnancy, leading to implantation failure or pregnancy loss *via* different putative mechanisms (37). It has been suggested that antiphospholipid antibody (aPL) and anti-beta(2)-glycoprotein I antibody (A-β2-GPI) can lead to placental vascular thrombosis, trophoblast dysfunction, and maternal hormone imbalance (38–40) and the presence of thyroid autoantibodies may result in dysregulation of the immune system activity at the fetal-maternal interface (41–43). Although the effects of ANAs on reproductive health are widely recognized, unlike aPL, A-β2-GPI, and thyroid autoantibodies, the exact mechanism of action of ANA in RPL is not yet clear.

Previous studies have suggested several possible mechanisms that ANAs play in pregnancy failure (**Figure 1**) (44–48). Firstly, ANAs might have a direct adverse effect on the quality and development of oocytes and embryos, resulting in reduced pregnancy and implantation rates (49). Although there was no nuclear antigen on the zona pellucida, *in vitro* studies indicated that ANAs could bind to the embryos directly and it was proposed that ANAs might recognize the glycerol moiety or protein cofactor (50). An earlier study showed that the development of embryos that were co-cultured with immunoglobulins from ANA-positive women was severely impaired (51). Another study recruiting women undergoing *in vitro* fertilization-embryo transfer (IVF-ET) treatment found that the proportion of mature oocytes and number of higher embryos and pregnancy rates in the ANA-positive group was significantly lower than in the ANA-negative group, which suggested that the presence of ANAs significantly interferes with oocyte and embryo development and therefore impairs the pregnancy outcome (49).

Secondly, the precipitation of immune complex tissues in the maternal–fetal interface may be one of the possible mechanisms leading to miscarriage in ANA-positive women (51). An animal study showed that mice treated with ANA-positive IgG obtained from RPL women had a remarkably higher embryonic absorption rate, reduced complement 3 (C3) and increased C3a serum levels, compared with those treated with IgG obtained from normal healthy women (52). Interestingly, increased C3 deposition and immune complex staining in placental tissues were also found in mice treated with ANA-positive IgG fraction from women with RPL (52). Additionally, it has been shown that ANAs can also induce the activation of plasmacytoid dendritic cells *via* Toll-like receptor-9,which can result in increased production of inflammatory cytokines (such as interferon-α) that stimulate the humoral immune response and lead to further production of ANAs (53, 54).

Furthermore, the immune complex tissue may induce local complement activation with inflammatory infiltration (52). Although there was no direct evidence of the association between ANAs and complement activation, a previous study using a mouse model of the antiphospholipid syndrome induced by passive transfer of human aPL antibodies showed that mice deficient in C3 were resistant to fetal injury induced by aPL antibodies (55). Studies defining the downstream effectors of complement activation have shown a rapid increase in decidual and systemic tumor necrosis factor-a levels, which appears to be the mediator that links complement activation to fetal damage (21, 56). The recruitment of inflammatory cells accelerates the activated pathway and creates a pro-inflammatory amplification loop that enhances C3 activation and deposition, generates additional C3a and C5a, and results in a further flow of inflammatory cells into the placenta, ultimately leading to pregnancy loss (56).

POTENTIAL TREATMENTS FOR ANA-POSITIVE WOMEN WITH RPL

Interventions for ANA-positive women with a history of RPL were recommended on the basis of the possible adverse effect of ANAs on the subsequent pregnancy outcome in this group of women. However, there is no consensus on treatment regimens yet.

Aspirin has both anti-platelet and anti-inflammatory effects, and glucocorticoids exhibit a beneficial clinical effect in most autoimmune diseases. Therefore, they are considered potential therapies in ANA-positive women with RPL, which has a

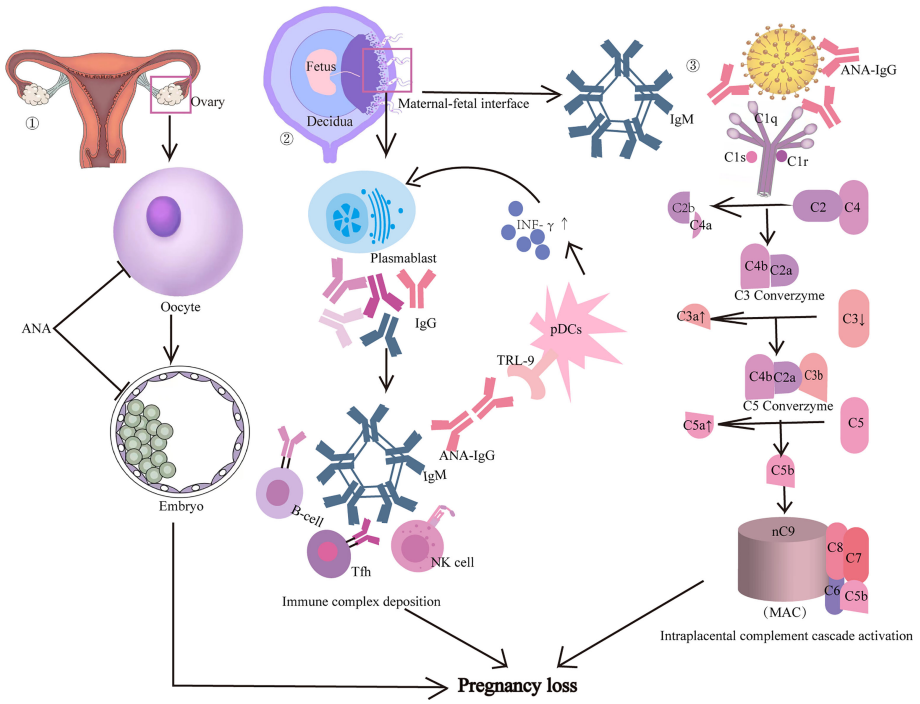

FIGURE 1 | Possible mechanisms that ANA may play in pregnancy loss. Firstly, ANA might have a direct adverse effect on the quality and development of oocytes and embryos, resulting in reduced pregnancy and implantation rates. Secondly, the precipitation of immune complex tissues at the maternal–fetal interface may be one of the possible mechanisms. Thirdly, the immune complex tissues may also induce local complement activation with inflammatory infiltration, leading to miscarriage.

suspected immune etiology. In a placebo-controlled trial, prednisolone and low-dose aspirin were used to treat women with RPL and positivity for antiphospholipid, antinuclear, anti-DNA, or anti-lymphocyte antibodies (57). Although the live birth rate was higher in the treatment group, it was not significantly different from controls (OR 1.5; 95% CI 0.8–2.6). However, the treated patients had a significantly higher risk of preterm birth (62% versus 12%, p <0.001) and higher risks for diabetes and hypertension, which are well known to be associated with high and prolonged administration of prednisolone. However, another case–control study, including more than 200 women who were diagnosed with unexplained RPL and tested positive for ANAs at a titer of 1:80 or more, showed that live birth rates were comparable in women receiving prednisone plus aspirin and women prescribed aspirin only. Additionally, no previous preterm delivery, fetal growth restriction, or placental abruption occurred in any subject (58).

In addition to glucocorticoids, there are other types of immunotherapy used to treat RPL with immunological causes, namely, intravenous immunoglobulin (IVIG), lymphocyte immunization treatment (LIT), etc. IVIG is a fractionated blood product that is used to treat certain autoimmune diseases and RPL. Two randomized controlled trials indicated that IVIG increases live birth rates in secondary RPL patients but not significantly in patients with primary RPL (59, 60). In contrast, recent meta-analyses found that IVIG did not improve the live birth rates of RPL women (61, 62). As for RPL women with positive ANAs, there is a limited study evaluating the therapeutic effect of IVIG in this group of women. One earlier study showed that low-dose IVIG therapy is beneficial for older women with immunologic abnormalities and RPL by increasing the successful pregnancy rate, in which 28% of the participants were found to have positive ANAs (63). The possible mechanisms of action of IVIG for treating RPL are multifactorial, namely, the modulation of various immune cells, the down-regulation of primary antibody production, and the modulation of complement activation (64–66).

LIT is another immunotherapy used in RPL. A review demonstrated that RPL women treated with paternal LIT had more successful outcomes (68%) than untreated women (54%, p <0.02) (67). As for those with positive ANAs, a retrospective observational study showed that the presence of ANAs is one of the risk factors for further pregnancy loss in patients with RPL treated with LIT (68). However, some previous studies have shown that patients with positive ANAs and antithyroid antibodies after receiving LIT have a higher risk of miscarriage and do not benefit from LIT (69, 70). Although the exact mechanisms of LIT have yet to be elucidated, the possible mechanisms consist of inducing the production of humoral antibodies to mask the fetal human leukocyte antigens (71), regulating Th2 cell transition (72), and decreasing NK cell activity (73).

Plasmapheresis has been used for decades for treating autoimmune disease as it is thought to have a profound modulation of the immune system, namely, the removal of

circulating immune complexes, immunoglobulins, and complement components. Plasmapheresis has also been used in pregnant women with autoimmune diseases, such as Sjoegren's Syndrome or systemic lupus erythematosus, to treat congenital fetal heart block (74, 75). Several reports have suggested that plasmapheresis may also treat pregnant women with anti-phospholipid syndrome (76, 77). For women with RPL, plasmapheresis was used to prevent future miscarriage in pregnant women immunized with anti-P or anti-PP1Pk and a history of RPL (78, 79). However, as far as we are aware, there is no study reporting the use of plasmapheresis in RPL women with positive ANAs.

Heparin is effective for its anticoagulant and anti-inflammatory properties (80). Due to the evidence from randomized controlled trials that heparin appears beneficial in treating women with RPL and other autoimmune antibodies (81, 82), heparin has been increasingly administered in clinic to RPL women with positive ANAs. However, there is a limited clinical trial investigating the therapeutic effect of heparin alone in this group of women. Overall, different results showed that the effect of different therapies on maternal and fetal pregnancy outcomes in patients with RPL is still controversial, and therefore large sample size randomized controlled trials are needed.

FUTURE DIRECTION

As discussed above, several issues should be taken into account in future studies. Firstly, due to the different definitions used for recurrent pregnancy loss among these studies, standardization of the definition is in urgent need. Secondly, since the methods for ANA detection varied as well, a standardized methodology should be proposed in the future. Thirdly, clinical pregnancy outcomes are assumed to be followed up, namely, clinical pregnancy, miscarriage, pregnancy complications (gestational hypertension, intrauterine fetal restriction, etc.), and live birth in future studies focusing on the correlation between the presence of positive ANAs and pregnancy outcomes in women with RPL. Moreover, although a series of studies have suggested the possible roles that ANAs play in pregnancy loss, the exact mechanisms are still unclear and further mechanistic investigations are needed. *In vitro* co-culture models of endometrial and trophoblast cells may provide more information in this regard.

CONCLUSION

Recurrent pregnancy loss is a challenging disease in the field of reproductive medicine that can cause great emotional frustration to the suffering couples. Although the exact mechanism that ANAs plays in women with RPL is still unclear, most studies suggest that the presence of ANAs not only correlates significantly with RPL but also has a prognostic value for the subsequent pregnancy outcome in this group of women. Interventions for ANA-positive women with a history of RPL include aspirin, glucocorticoids, and heparin. However the therapeutic effect of these regimens is still controversial and large-scale randomized controlled trials are needed.

AUTHOR CONTRIBUTIONS

TL, YZ, and XC: Substantial contribution to the conception and design of the work. TL, XG, YLia, and XC: participation in acquisition of the literature. TL, YLiu, YZ, and XC: manuscript drafting. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Reduced mitochondrial DNA content correlate with poor clinical outcomes in cryotransfers with day 6 single euploid embryos

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Objective: To investigate whether the mitochondrial DNA (mtDNA) content of a single biopsy at trophoblast correlates with the developmental potential and reproductive outcomes of blastocyst.

Methods: A retrospective analysis applied the dataset of 1,675 embryos with preimplantation genetic testing for aneuploidy (PGT-A) from 1,305 individuals, and 1,383 embryos involved cryotransfers of single euploid embryo between January 2015 and December 2019. The studied cohort was divided for algorithm establishment on the NGS platform (n=40), correlation of biological features (n=1,635), and correlation of reproductive outcomes (n=1,340). Of the algorithm derived from the NGS platform, the reliability and repeatability were validated *via* qPCR assay and inter-run controls, respectively. Of the correlation across biological features, stratification analyses were applied to evaluate the effect from a single contributor. Eventually, the correlation between the mtDNA ratios and reproductive outcomes was adjusted according to the significant effector(s).

Results: The mtDNA ratios showed statistically different between embryos with different days of blastocyst formation ([Day 5]: 1.06 vs. [Day 6]: 0.66, p=0.021), and between embryos with different expansion stages ([Expansion 5]: 1.05 vs. [Expansion 6]: 0.49, p=0.012). None or weakly correlated with the maternal age, morphology, ploidy, and gender. Analyzed by the different days of blastocyst formation with fixed expansion score as 5 in the euploid single embryo transfers (eSET), the day 6 eSET showed significantly lower reduced mtDNA ratio (n=139) in failure groups of fetal heartbeat (p=0.004), ongoing pregnancy (p=0.007), and live birth (p=0.01); however, no correlation between

mtDNA ratios and pregnancy outcomes was observed in the day 5 eSET (n=1,201).

Conclusions: The study first demonstrated that mtDNA ratio was dependent on the days of blastocyst formation while expansion stage was fixed. Lower mtDNA ratios were observed in the day 6 eSET with adverse outcomes. The present stratification analyses reveal that the timeline of embryo is an important covariate to the mtDNA content.

KEYWORDS

mitochondria, blastocyst formation day, preimplantation genetic testing for aneuploidy(PGT-A), next-generation sequencing, single embryo transfer (SET)

Introduction

Chromosome aneuploidy is important to growth arrest of embryos, implantation failure, early miscarriage, and late abortion during *in-vitro* fertilization (IVF) program (1). Since the technique of preimplantation genetic testing for aneuploidy (PGT-A) was applied in the IVF for embryo selection, the overall implantation rate per transfer has been significantly improved (2) (3). However, failure of implantation and miscarriage still occurs in a percentage of both morphologically and chromosomally normal embryos, implying that other substantial factors could determine the embryo potential (4) (5). To date, the most prevalent procedure of PGT-A is to test multi-cellular biopsies of blastocyst trophectoderm *via* the methodologies of next-generation sequencing (NGS). Additional information can be immediately obtained from these trophoblasts *via* PGT-A/NGS. Accordingly, energy supply is an issue for embryo implantation, and the correlation between mitochondria and reproductive outcome deserves to be investigated (6, 7).

Mitochondrial DNA (mtDNA) is a double-stranded circular molecule, consisting of 16,569 base pairs (8). A total of 37 genes are contained, and make mitochondria the multifunctional powerhouse, which produce adenosine triphosphate (ATP) *via* the chain of oxidative phosphorylation (9–11). Moreover, mitochondria also involve cellular regulations, such as apoptosis, calcium homeostasis, and steroidogenesis in the ovaries (12, 13). Thus the amount of mitochondria in the human germline displays a very unique and dynamic pattern. The mitochondrial copy number in the oogonia is around 10-100, while it grows to approximately 150,000-700,000 in the metaphase II (MII) oocyte at the end of folliculogenesis. High amount of mitochondria makes the mature oocyte be ready for following fertilization and development, which require sufficient energy supply. Then the proliferation of mitochondria is ceased during the post-fertilization stage, and the existing mtDNAs are subsequently distributed to the divided blastomeres, from approximately 80,000 copies per blastomere in the four-cell stage to 1,000 copies per cell in the blastocysts (14).

Since the structure of mtDNA lacks an effective repairing system as nuclear DNA, and the polymerase γ (polymerase of mtDNA) has comparatively poorer ability of proofreading, the mutation rate of mtDNA is around tenfold higher than that of nuclear DNA. Moreover, mitochondria are the major site of generating reactive oxygen species (ROS), and ROS induce several DNA oxidative damages. Attributed to these characteristics, the observed mitochondrial dysfunction are categorized as either quantitative, including reduced mtDNA content or increased heteroplasmic mitochondria, or qualitative, such as different types of mtDNA mutations (7). In some animal models, the association between age-dependent decreased fertility and reduced mtDNA content in oocytes were reported in the older populations (15, 16). In humans, significantly lower mtDNA amounts were also found in women with advanced maternal age or diminished ovarian reserve (17).

Therefore, different approaches to detect and calculate mtDNA copies in the embryos were explored when trophectoderm biopsies were performed for PGT-A. Fragouli et al. first reported that the euploid embryos with lower mtDNA to nuclear DNA ratio (mtDNA ratio) tend to behave at a higher implantation rate in a total of 89 blastocyst transfers (2015) (18). Continuously, the same research team applied the criterion of "less is better" to mtDNA ratio in a total of 199 blastocyst transfers, and then illustrated that the ongoing pregnancy rate was lower in those with elevated mtDNA ratio (2017) (19). However, some articles did not support the mtDNA ratio as an independent parameter for implantation of embryo transfer, since the mtDNA ratios between implanted and non-implanted populations displayed more like a uniform pattern rather than a binarily classified pattern (20–22).

One of the challenges to reliability of mtDNA ratio as a selection tool in euploid embryo transfers is that the potential effects from other biological features remains unclear. Since the distribution of mitochondria from each oocyte to blastomeres of the subsequent embryo is random, the feasibility of applying mtDNA ratios across individual embryos with discrepant backgrounds must be carefully determined (23). The contribution derived from other biological features could mask the actual effect of mtDNA ratio. These features to mtDNA ratio must be comprehensively controlled before evaluating correlation between mtDNA ratio and transfer outcomes.

Furthermore, the utilized methodologies and calculation algorithms could also lead to different results (24, 25). The most common method to quantify mtDNA is qPCR assay, however, the additional experiment would be burdensome in the clinical PGT-A implementation. Thus different approaches were tested on the NGS platform that has been used in the PGT-A, such as increasing sequencing depth to obtain more reads (18) or employment of correction factors to normalize the backgrounds (20). Nevertheless, no consensus for the methodologies has been reached.

Increase of sequencing depth would raise the cost per sample, and employment of multiple correction factors must be validated in accordance with individual laboratories' conditions. In the present study, we directly applied the bam files generated from the present PGT-A/NGS protocol to develop a reasonable algorithm for calculating mtDNA ratio with validations of reliability and repeatability, and then used the established mtDNA ratio in the analyses of biological effects. Eventually, the correlation between mtDNA ratios and the reproductive outcomes of blastocysts would be evaluated with controlled biological backgrounds.

Materials and methods

Study design

This retrospective study has been approved by an independent institutional review board of National Taiwan University Hospital. The entire workframe was displayed in Figure 1: first, to develop and validate a reasonable calculation method using a smaller cohort; second, to separately estimate the effect from different biological features to the established mtDNA ratios, including maternal age, day of blastocyst formation (day of biopsy), expansion stage, morphological grading, chromosome ploidy, and embryo gender, in a larger cohort; third, to evaluate the correlation between the reproductive outcomes and mtDNA ratios in the cycles with single euploid embryo transfer (eSET).

Study subjects

A sequencing database generated from 1,675 blastocysts involving 1,305 individuals with IVF/PGT-A programs was analyzed for reproductive outcomes, collected between January 2015 to December 2019. A total of 1,389 blastocysts involving 1,201 individuals amongst the study cohort underwent eSET (Supplementary Table 1). All the involved patients underwent personalized stimulation protocols for the IVF treatment (26, 27). Written informed consents were obtained from the couples before entering the programs. The fertilized oocytes were cultured to the blastocyst stage, and then biopsied using mechanical shearing force between the biopsy and holding pipettes (Origio, Måløv, Denmark). Around 5-10 cells from

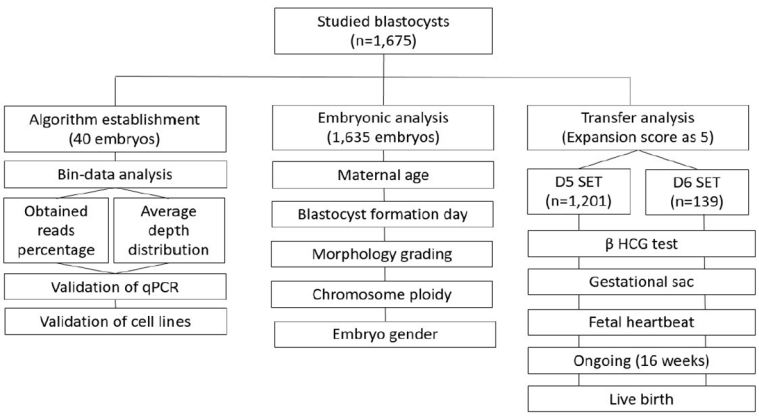

FIGURE 1
The workframe for study of mtDNA ratio is displayed. There are three stages: 1) establishment and validation of the algorithm for calculating mtDNA ratio; 2) estimation of the effect from biological features to mtDNA ratio; 3) evaluation of the correlation between reproductive outcomes and mtDNA ratios. SET, single embryo transfer; eSET, single euploid embryo transfer.

trophectoderm (TE) were removed (28). Biopsy was only conducted in the blastocysts graded above BC according to the Gardner and Schoolcraft system (1999). The biopsied samples were washed twice in sterile 1X phosphate-buffered saline (PBS) solution (Cell Signaling Technologies, Danvers, MA, US) containing 1% (w/v) polyvinylpyrrolidone (PVP; Sigma, St. Louis, MO, US). Then the sample was gently transferred into a 0.2-mL PCR tube in 2.5 µL of PBS/PVP solution and stored at −20°C for following NGS procedures.

Determination of chromosome ploidy *via* NGS

DNA extraction and amplification were applied with the Sureplex amplification system (Sureplex; Illumina, San Diego, CA, US). Initially samples were lysed with the extraction master mix. Then they were fragmented and primed using a pre-amplification cocktail. They were finally amplified using an amplification master mix. A Qubit dsDNA HS (high-sensitivity) Assay Kit (Qubit; Life Technologies, Waltham, MA, US) was used to quantify the concentration of amplified products. The products were diluted to 0.2 ng/µl for the library preparation according to the manufacturer's guidelines of VeriSeq PGS v.3 (Illumina), and then sequenced using a Miseq System (Illumina). The generated data was analyzed *via* the BlueFuse Multi Software (Illumina). For the CNV calculation, the human reference sequence of individual chromosomes was divided into several intervals with unit lengths, so-called 'bins' in the Veriseq PGS system, and reads that pass quality metrics were mapped by the intervals. The chromosomal CNV were subsequently calculated using the variation of mapped reads across these bins. If a median chromosomal CNV deviated from the default CNV represented as two, a gain or loss of chromosome would be identified (29). Internal validations of sensitivity and specificity could be referred to our previous publications (30, 31). No default setting in the sequencing parameter was changed.

Determination of mtDNA ratio *via* NGS

Bam files generated from the Veriseq PGS were utilized for calculating mtDNA ratios. Reads went through a self-developed QC metrics *via* the written commands on the pysam platform (Release 0.15.0) for filtering. Unmapped reads, duplicates, and reads with low quality scores or with poor performance of alignment were then excluded. Because normalizing the variations of biopsied cell numbers and of sequencing batches for relative mtDNA quantification were necessary, an adequate denominator derived from nucleic DNA was required. This denominator would be applied in the embryos with different backgrounds for the following analyses of biological features. Thus chromosome 6 was initially targeted due to its lowest incidence of aneuploidy across all somatic chromosomes (32). Since the ploidy identification was carried out according to the variation of bin count data, the denominator was applied with the same scale. The sequencing depth and distribution of alignments were then screened across individual bins on chromosome 6 in a total of randomly selected 40 embryos involving different sequencing runs. The particular bin(s) with the most reliable depths and stable percentage of obtained reads would be selected as the denominator. Finally, the reads of mtDNA would be normalized to that of selected bin(s) to calculate mtDNA ratio.

Validation of mtDNA ratio *via* qPCR

To validate the reliability of mtDNA ratio established from the NGS platform, a second methodology using qPCR was performed. The former selected 40 embryos were tested by NovaQUANT Human Mitochondrial to Nuclear DNA Ratio Kit (Merck, Darmstadt, Germany). All the procedures were referred to the manufacturers' user guide. Two pairs of mitochondrial gene and nuclear gene were calculated for the relative mtDNA ratios: ND1 and BECN1; ND6 and NEB. In the application, both the mitochondrial genes, ND1 and ND6, code the subunit of NADH dehydrogenase, which is the largest, highly conservative component of Complex I of mitochondrial respiratory chain; and the other two nuclear, single-copy genes, BECN1 and NEB code the highly conservative eukaryotic proteins (Beclin 1 and Nebulin), used as the factors for normalizing the biopsied cell number. Resultant *Cts* (Cycle threshold value, Ct) obtained from the qPCR assay can generate an average of $2^{\Delta Ct}$ from ND1 to BECN1, ND6 to NEB, *mean* ($2^{\Delta Ct\ of\ ND1-\ BECN1}$, $2^{\Delta Ct\ of\ ND6-\ NEB}$), for calculation of mtDNA copy number per cell (mtDNA ratio). Of manipulation, the WGA products were serially diluted to 0.1 ng/µL with DNase free water. Equal volume of 2X RT 2 Fast SYBR Green Mastermix (Thermo Fisher Scientific, Waltham, MA, USA) was mixed with a total of 1ng WGA products. Then 20µL of the mixed solution was transferred into a commercial qPCR plate separately coated with pre-aliquoted qPCR primers for individual ND1, ND6, BECN1, and NEB genes. Then qPCR assay was performed using a thermal cycler (QuantStudio 3 Real Time PCR System; Thermo Fisher Scientific), and the condition was as follows: incubation at 95°C for 1 min and then 40 cycles of 95°C for 15 sec and 64°C for 1 min. Relative mtDNA ratio was determined by an equation of $2^{\Delta Ct}$, and then the average between the two pairs of $2^{\Delta Ct}$ was calculated for the final measurement as above mentioned. Each sample was tested in triplicate.

Validation of mtDNA ratio *via* cell lines

To validate the repeatability of mtDNA ratio among different batches on the NGS platform, three cell lines with different ploidies were applied. Two self-developed amniotic stem cell lines were kindly provided by the Bioresource Collection and Research Center (Hsinchu, Taiwan): AFMSC-T (46, XY), and AFMSC-9703125 (45, X0; Turner syndrome). One cell line was purchased from the Coriell Cell Repository (Camden, NJ, USA), AG12070 (47, XX, trisomy 13). The karyotypes of these cell lines were identified by the providers previously before utilization. The cell lines were passaged according to the suppliers' guides, and then were diluted to 10-20 cells in PBS/PVP solution of 2.5 μL. A total of 10 replicates were prepared for each cell line. Samples of cell lines underwent the same procedures of amplification and library preparation as clinical biopsies. Finally, they were sequenced in 10 different NGS runs.

Correlation between mtDNA ratios and biological features

The mtDNA ratios were compared by the following features of embryos: maternal age, day of blastocyst formation, expansion stage, grading of morphology (Gardner's system), chromosome ploidy, and embryo gender. Individual comparison was performed for analyzing the possible effect from a particular feature, while the other features were controlled. Since the entire mtDNA ratios displayed a typical non-normal distribution (Supplementary Figure 1), a Kolmogorov-Smirnov test would be applied for confirming the distribution before conducting statistical comparisons.

Correlation between mtDNA ratios and reproductive outcomes

All the included patients underwent hormone replacement therapy (HRT) for endometrial preparation, and transferred with a single euploid blastocyst. Average mtDNA ratios were analyzed according to the reproductive outcomes. Five endpoints during pregnancy were recorded: serum β-HCG test after two weeks of cryotransfer, detection of gestational sac (Sac) and fetal heartbeat (FHB) after four weeks of cryotransfer, ongoing pregnancy of 16 weeks, and full-term live birth (LB) after 36 weeks of pregnancy. Embryonic parameters of the analyzed cohorts would be controlled according to the conclusions obtained from the former analyses of biological features.

Statistical methods

Before conducting any analyses, a Kolmogorov-Smirnov test was applied to confirm the normality. The Spearman's rank correlation coefficient (Spearman's ρ) was performed for evaluating correlation. For the continuous variables between two groups, Mann-Whitney test or Wilcoxon rank sum test were applied. For the continuous variables among multiple groups, Kruskal-Wallis test was used. All the statistics were accomplished using SPSS software (IBM; Armonk, NY, US).

Results

Establishment of mtDNA ratio

Detailed information of randomly selected 40 embryos derived from 37 patients was displayed in Supplementary Table 2. Mean age of the involved patients was 36.5 years (range: 26-40 years). The studied cohort included 23 euploid embryos, 16 aneuploid embryos, and 1 mosaic embryo. The sequencing depth and distribution of alignments on chromosome 6 were analyzed across individual 1 Mb bin intervals in the testing embryos (Figure 2). Figure 2A elucidated the reads with sequencing depth above five, and only a single bin on chromosome 6 obtained exclusively higher read counts, shown as a significant peak in the line chart. Then the distribution of alignment was screened in Figure 2B. The particular bin with higher read counts of depth above five also exhibited a comparatively higher percentage of obtained alignments. Both the sequencing depth and distribution of alignments of this single bin were more stable than the other intervals. Therefore, the reads of mitochondrial DNA would be normalized by that of this particular bin on chromosome 6 for calculating mtDNA ratio.

Validation of mtDNA ratio *via* qPCR

To evaluate the reliability of mtDNA ratio established from NGS methodology in the present study, a second methodology using qPCR assay was performed. Since the scales of the two methodologies are discrepant, correlation between the ratios derived from qPCR and NGS was illustrated in a log-log plot of Supplementary Figure 2. Mitochondrial DNA ratio derived from qPCR was calculated as $mean\ (2^{\Delta Ct\ of\ ND1-\ BECN1}, 2^{\Delta Ct\ of\ ND6-\ NEB})$ and that from NGS was $\frac{Read\ \ count\ \ (mtDNA)}{Read\ \ count\ \ (Chr.6\ \ target\ \ bin)}$. Strong correlation was reached between two datasets of qPCR and NGS (Spearman ρ=0.706).

Validation of mtDNA ratio *via* cell lines

To validate the repeatability of mtDNA ratio among different sequencing batches of the NGS platform, three cell lines with different ploidies were applied. Each cell line was tested in ten replicates within ten different sequencing runs, and

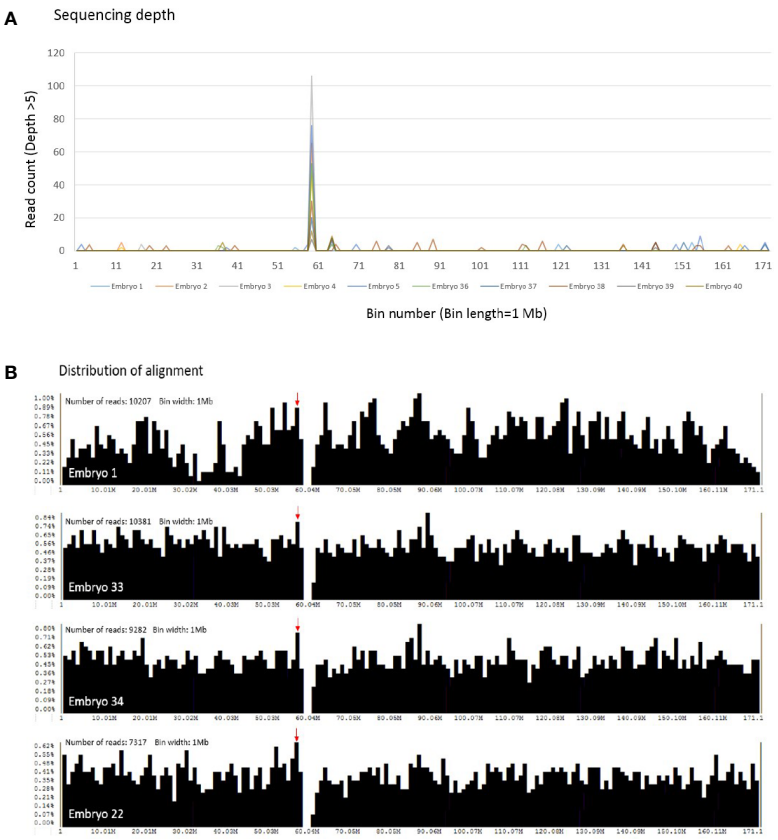

FIGURE 2
The sequencing depth and distribution of alignment across individual bin intervals on chromosome 6 are shown. **(A)** With the condition of sequencing depth above five, only a single bin contains exclusively higher read counts, and a peak is shown obviously in the randomly selected 40 embryos (10 embryos are represented in this figure). The x scale denotes the bin number on chromosome 6, and the y scale as the read counts of depth above five. **(B)** The distribution of alignment is screened in the selected embryos. A red arrow indicates the particular bin with higher read counts of depth above five, and upper left is the total read counts of chromosome 6 obtained in individual embryos. Each bar represents the percentage of obtained reads in a single bin interval. The x scale denotes the bin number on chromosome 6, and the y scale as the percentage of obtained reads.

the results were displayed as box plots (Supplementary Figure 3). Based on the calculation of precision-to-tolerance ratio (P/T), the established mtDNA ratios of ten replicates across different sequencing batches showed acceptable repeatability: [P/T of 46, XY]=15.7%; [P/T of 46,X0]=26.2%; [P/T of Trisomy 13]=17.0%.

Correlation between mtDNA ratios and biological features

In Figure 3, a stratification analysis of the mtDNA ratios derived from a total of 1,635 embryos was conducted according to the six biological features: maternal age, day of blastocyst formation, expansion stage, grading of morphology, chromosome ploidy, and embryo gender (Supplementary Table 3). To individually estimate the actual effect from a particular feature, the other features would be controlled across each stratum. In Figure 3A, the mtDNA ratios from a total of 414 euploid embryos (day 5 blastocyst, expansion score 5, graded above BB, female) were analyzed by the maternal age, and merely weak correlation was observed (Spearman ρ=0.098). In the analysis of day of blastocyst formation (Figure 3B), a total of 313 euploid embryos (maternal age<35 years, expansion score 5, graded above BB, female) showed significant difference in the mtDNA ratios between day 5 and day 6 blastocysts (p=0.021). In the analysis of expansion state (Figure 3C) of 406 euploid embryos (maternal age<38, graded above BB, female), significance also reached between the euploid embryos with expansion score 5 and 6 (p=0.012), and expansion state showed highly dependent on the days of blastocyst formation (embryos with expansion score 6 were all day 6 blastocysts, n=7). In the analysis of morphology grading of 295 euploid embryos (maternal age<35 years, day 5 blastocyst, expansion score 5, female) (Figure 3D), no significant differences in mtDNA ratios were observed among good, median,

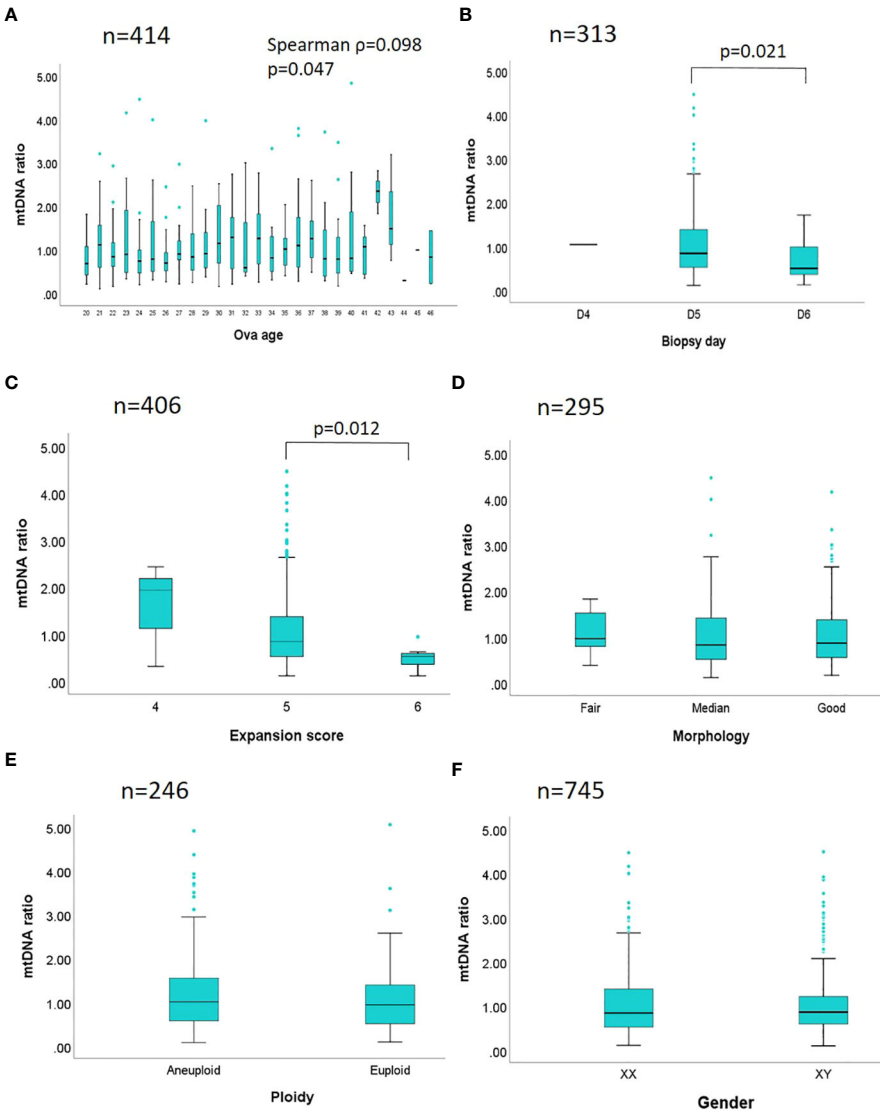

FIGURE 3
Stratification analyses between mtDNA ratio and biological features are performed. The mtDNA ratios of 1,635 embryos are compared by different biological features in six strata. **(A)** A total of 414 euploid embryos with a fixed day of blastocyst formation, expansion state, morphology grading, and gender, are analyzed by the maternal age. **(B)** A total of 313 euploid embryos with fixed age span, expansion state, morphology grading, and gender, are analyzed by the days of blastocyst formation. **(C)** A total of 406 euploid embryos with fixed age span, a fixed day of blastocyst formation, morphology grading, and gender, are analyzed by the expansion state. **(D)** A total of 295 euploid embryos with fixed age span, day of blastocyst formation, expansion state, and gender are analyzed by the morphology grading ([good]: AA, AB, BA; [median]: BB; [fair]: AC, BC; based on the Gardner's system). **(E)** Pair-matched embryos derived from a total of 246 sibling oocytes with paired maternal age, day of blastocyst formation, expansion state, morphology grading, and gender, are analyzed by the chromosome ploidy. **(F)** A total of 745 euploid embryos with fixed age span, day of blastocyst formation, expansion state, and morphology grading are analyzed by the genders. Spearman correlation is applied in the analysis of maternal age and mtDNA ratio; while Mann-Whitney test (Wilcoxon rank sum test), and Kruskal-Wallis test are applied in the comparisons of two or above two datasets respectively. The p value is labeled while the statistical significance is reached.

and fair embryos ([good]: AA, AB, BA; [median]: BB; [fair]: AC, BC; based on the Gardner's system). Of the chromosome ploidy (Figure 3E), the mtDNA ratios between pair-matched euploid and aneuploid embryos derived from 246 sibling oocytes displayed no significant difference (paired maternal age, day of blastocyst formation, expansion score, morphology grading, and embryo gender). Then no difference was found in the mtDNA ratios between two different genders in 745 euploid embryos (maternal age<35 years, day 5 blastocyst, expansion score 5, graded above BB) (Figure 3F).

Correlation between mtDNA ratios and reproductive outcomes

The profile of all the recruited eSETs was shown in Supplementary Table 1. A total of 1389 blastocysts were generated from 1201 individuals (mean age: 30.1 years), including 694 cycles using their own oocytes (49.96%) and 695 cycles using donated oocytes (50.04%). All the individuals underwent hormone replacement therapy (HRT) for endometrium preparation and euploid blastocyst cryotransfer. The overall biochemical pregnancy rate was 71.6%, implantation rate as 62.3%, clinical pregnancy rate as 57.6%, ongoing pregnancy rate as 54.6%, and live birth rate as 53.4%. Based on the former stratification analyses between mtDNA ratio and biological features, significant difference of mtDNA ratio was only observed in the embryos with different days of blastocyst formation and expansion scores. Additionally, the expansion score was highly dependent on the days of blastocyst formation, the embryos with expansion score 6 were all day-6 blastocysts while the other characteristics had been controlled. Thus the reproductive outcomes in a total of 1,340 cycles with single euploid embryo cryotransfers were divided by the days of blastocyst formation with fixed expansion score as 5, and then the reproductive outcomes of each endpoint were analyzed separately. In the stratum of day 5 blastocysts (expansion score 5), Table 1 displayed the mtDNA ratios by the outcomes of β-HCG, Sac, FHB, ongoing pregnancy (16 weeks), and LB in a total of 1,201 cycles with day 5 eSET. In the stratum of day 6 blastocysts, the same endpoints were evaluated in a total of 139 cycles with day 6 eSET with expansion score 5 (Table 2). Of the cycles with day 5 eSET, the maternal age, endometrium thickness, distribution of morphology, and mtDNA ratios between all the successful and failure groups showed no significant differences. Of the day 6 eSET, mtDNA ratios showed significant differences between the successful and failure groups in the detection of fetal heartbeat (p=0.004), ongoing pregnancy at 16 weeks (p=0.007), and live birth (p=0.01), while the other parameters were statistically similar. Additionally, a forward stratification to analyze the clinical outcomes by the quartiles of mtDNA ratios also displayed poor outcomes of fetal heartbeat, ongoing pregnancy, and live birth associated with lower mtDNA quantiles in the cohort of day 6 eSET (Supplementary Tables 4, 5). Therefore, in the day 6 eSET, reduced mtDNA ratios were observed in the failure group, and no significant correlation was found in the day 5 eSET.

Discussion

The study first demonstrated that mtDNA ratio was merely dependent on the day of blastocyst formation and the size of

TABLE 1 Reproductive outcomes of cryotransfer with day 5 single euploid embryo (expansion score 5).

Transfer outcome	Success rate	Cycle number (n=1,201)	Maternal age (years)	EM thickness (mm)	Morphology grading (Good, Median, Fair)	mtDNA ratio (Mean, SD)	mtDNA ratio (Median, IQR)	P-value* for mtDNA ratio
HCG (+)	73.77%	886	30.07	9.57	286, 540, 60 (32%, 61%, 7%)	1.06 ± 0.77	0.86, 0.75	0.26
HCG (–)		315	29.87	9.33	89, 211, 15 (28%, 67%, 5%)	1.01 ± 0.69	0.86, 0.76	
Sac (+)	64.11%	770	29.97	9.65	248, 472, 50 (32%, 61%, 6%)	1.06 ± 0.77	0.86, 0.75	0.42
Sac (–)		431	30.10	9.27	127, 279, 25 (29%, 65%, 6%)	1.03 ± 0.70	0.86, 0.76	
FHB (+)	59.70%	717	29.94	9.65	231, 438, 48 (32%, 61%, 7%)	1.07 ± 0.79	0.86, 0.75	0.29
FHB (–)		484	30.14	9.30	144, 313, 27 (30%, 65%, 6%)	1.02 ± 0.69	0.85, 0.74	
Ongoing (+)	54.62%	656	29.93	9.66	210, 400, 46 (32%, 61%, 7%)	1.08 ± 0.80	0.87, 0.75	0.11
Ongoing (–)		545	30.12	9.32	165, 351, 29 (30%, 64%, 5%)	1.01 ± 0.68	0.84, 0.76	
LB (+)	53.12%	638	29.96	9.67	202, 390, 46 (32%, 61%, 7%)	1.08 ± 0.80	0.87, 0.74	0.18
LB (–)		563	30.09	9.33	173, 361, 29 (31%, 64%, 5%)	1.02 ± 0.68	0.84, 0.78	

EM, endometrium; Sac, gestational sac; FHB, fetal heartbeat; Ongoing, pregnancy at 16 weeks; LB, live birth; IQR, interquartile range. (+), success at the pregnancy endpoint; (-), failure at the pregnancy endpoint.
*P-value is calculated from Mann-Whitney U test for the mtDNA ratios between successful and failure groups.

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TABLE 2 Reproductive outcomes of cryotransfer with day 6 single euploid embryo (expansion score 5).

Transfer outcome	Success rate	Cycle number (n=139)	Maternal age (years)	EM thickness (mm)	Morphology grading (Good, Median, Fair)	mtDNA ratio (Mean, SD)	mtDNA ratio (Median, IQR)	P-value* for mtDNA ratio
HCG (+)	60.43%	84	29.49	9.62	17, 52, 15 (20%, 62%, 18%)	0.68 ± 0.44	0.56, 0.58	0.77
HCG (-)		55	31.87	9.58	9, 39, 7 (16%, 71%, 13%)	0.66 ± 0.42	0.62, 0.49	
Sac (+)	53.24%	74	29.01	9.79	14, 47, 13 (19%, 64%, 18%)	0.71 ± 0.45	0.62, 0.62	0.18
Sac (-)		65	32.05	9.40	12, 44, 9 (18%, 68%, 14%)	0.62 ± 0.40	0.48, 0.38	
FHB (+)	46.04%	64	28.69	9.69	12, 41, 11 (19%, 64%, 17%)	0.78 ± 0.45	0.72, 0.57	0.004
FHB (-)		75	31.92	9.54	14, 50, 11 (19%, 67%, 15%)	0.58 ± 0.39	0.46, 0.31	
Ongoing (+)	38.85%	54	28.31	9.78	10, 35, 9 (19%, 65%, 17%)	0.79 ± 0.48	0.72, 0.62	0.007
Ongoing (-)		85	31.78	9.49	16, 56, 13 (19%, 66%, 15%)	0.59 ± 0.38	0.47, 0.40	
LB (+)	38.13%	53	28.36	9.74	10, 34, 9 (19%, 64%, 17%)	0.78 ± 0.48	0.71, 0.61	0.01
LB (-)		86	31.71	9.52	16, 57, 13 (19%, 66%, 15%)	0.60 ± 0.38	0.47, 0.41	

EM, endometrium; Sac, gestational sac; FHB, fetal heartbeat; Ongoing, pregnancy at 16 weeks; LB, live birth; IQR, interquartile range. (+), success at the pregnancy endpoint; (-), failure at the pregnancy endpoint.
*P-value is calculated from Mann-Whitney U test for the mtDNA ratios between successful and failure groups.

embryo. Reduced success rates were observed in the day 6 single euploid embryo cryotransfers involving reduced mtDNA ratio. Initially, the calculation algorithm was constructed from the bam files generated *via* present PGT-A/NGS procedures, validating by qPCR and cell lines assays. Then the established mtDNA ratio was stratified by different biological features for comparisons in blastocysts. The mtDNA ratios showed statistically different between embryos with different days of blastocyst formation, and the expansion stage. None or weakly correlated with the other features. Eventually, the mtDNA ratios with different days of blastocyst formation and fixed expansion stage (expansion score 5) in the cycles of eSET were analyzed by the different endpoints of pregnancy. Correlation between reduced mtDNA ratio and reduced success rates were observed in the day 6 euploid blastocysts. However, the result was opposite to the conclusion derived from the previous articles, which stated the adverse correlation between increased mtDNA ratio and reduced success rates (6, 18, 19).

Since the PGT-A technology has been used in the IVF programs, the feasibility of mtDNA ratio derived from the same biopsied sample was studied for further embryo selection. Diez-Juan et al. (2015) found that mtDNA ratios displayed differences between implanted and non-implanted populations of day 3 and day 5 embryos *via* qPCR assay (6). At the same time, Fragouli et al. (2015 and 2017) investigated mtDNA ratios between different maternal age, chromosome ploidy, and transfer outcomes *via* both qPCR and NGS assays (18, 19). They both reported that a better reproductive outcome was observed in the embryos with lower mtDNA ratios, and explained the phenomenon as an outcome of compensation to energetic stress in blastocysts. On the contrary, Victor et al. (20) analyzed mtDNA ratio by qPCR and NGS assays again according to the same variables with Fragouli et al. (19) in a comparatively large dataset, suggesting that mtDNA ratio reveals uniform between implanted and non-implanted populations (20). In the same year, Treff et al. (2017) reported that mtDNA ratios do not distinguish between sibling embryos that were implanted and not implanted through qPCR assay (22). More recently, a study involving a larger cohort conducted by Scott et al. (2020) also demonstrated that the mitochondrial DNA content is not a reliably predictive biomarker for reproductive outcomes in paired-sibling euploid blastocysts *via* qPCR measurement (33). No matter which methodologies were used to establish mtDNA ratio, the discrepant conclusions

implied that the role of mitochondria quantity in the early-stage embryos may not be a simply independent parameter, and thus made its application in the embryo selection controversial.

Unlike the analyses of biological effect to mtDNA ratio in the previous articles compared the cohorts with mixed embryonic backgrounds, especially the mixed days of blastocyst formation and expansion stage, we investigated the biological effect to mtDNA ratio through stratification analyses in a larger cohort with a size of 1,637 embryos, estimating the actual interaction between an individual feature and mtDNA quantity in the present study. Except the embryos with different days of blastocyst formation and expansion stage displayed a significant difference in mtDNA ratios, no significance was reached across individual stratum regarding the other features. Because the timeline of embryo development seemed crucial to both the embryonic size and mitochondria quantity, the subsequent analyses of reproductive outcomes and mtDNA ratios were thus separated by the days of blastocyst formation with a fixed expansion score. The final results displayed a positive correlation between mtDNA ratio and pregnancy success rates in the population of day 6 single euploid blastocyst cryotransfers while the other parameters were controlled, suggesting that the embryonic timeline and mtDNA quantity are covariate.

Mitochondrial dysfunction could be divided as qualitative and quantitative, and mtDNA ratio was one of a quantitative measurement for mitochondrial copy number. In the studies of animal models, the correlation between reproductive aging and mitochondrial copy number decline were observed in the mice and cow models (15, 16). Additionally, introductions of mutants in mitochondrial transcriptional factor A (TFAM), which regulates mitochondrial copy number, displayed significant impact on animal embryo viability (34, 35). In human embryo studies, lower mtDNA content was also found in the oocytes of women with diminished ovarian reserve (36), and it is associated with compromised fertilization and development issues (17). However, a recent article (2022) elucidated that neither the mtDNA content nor the specific mtDNA genetic variants in cumulus cells was correlated with following ART outcomes *via* analyzing a huge sequencing dataset, implying that possible tolerance existed between the oocytes and their supportive environment (37). Due to manifestation of correlation between decreased mtDNA dosage and poorer developmental competence, increasing the quantity of functional mitochondria such as mitochondrial replacement therapies (MRT) exhibited as a potential treatment to the patients with inherited or infertility problems (7, 38). Since a sufficient amount of mitochondria has been reported as essential to oocyte fertilization and zygote development, giving priority to the embryos with lower mtDNA content in blastocyst selection seemed contrary to the former knowledge, and so far no enough evidence for the benefit was proved.

According to the analysis of mtDNA ratio and transfer outcomes in the present study, mtDNA ratio may have some but not strong connection with the pregnancy success rate in euploid embryos. While the embryonic sizes were controlled, the mtDNA ratio reduced with the success rate in day 6 eSET; and it displayed no significant correlation in the reproductive outcomes of day 5 eSET. The correlation among reduced mtDNA ratio, extended developmental timeline, and larger embryonic size were also observed in the several articles (39, 40), associating the previously observed phenomenon of continuously diluting the existing mtDNA copy number during the post-fertilization stage due to ceasing of mtDNA replication (14, 35). In the eSETs of present study, the first quantile range of mtDNA ratio in day 5 blastocysts was close to the second quantile range in day 6 blastocysts. The median in each quantile range of mtDNA ratios remains approximately 1.68 times higher in day 5 to day 6 populations (Supplementary Figure 4), possibly reflecting the total cell number difference of trophectoderm between the two populations (41). On the other hand, if the expansion stage had been furtherly fixed, namely, controlling the cell number in the same day of blastocyst formation, mtDNA ratio merely displayed the correlation between the embryonic timeline and initial mtDNA content of the particular oocytes. Therefore, it could play a similar role as the time-dependent parameters of development, which have been validated as applicable biomarkers for predicting the pregnancy outcomes (42). More information could be obtained by linking the mtDNA ratio and detailed morphokinetic data *via* time-lapse culture system in the future investigations.

To date, no consensus in the calculation of mtDNA ratio has been reached. Both additional qPCR assay or increasing NGS depth to obtain more mitochondrial reads required intervention in the original PGT-A/NGS implementation. In this study, a calculation algorithm was established from a commercial Veriseq PGS procedure, and then it was validated through qPCR and cell lines for the reliability and repeatability. Applied with this algorithm, two extremely high values were obtained in a total of 1,637 studied embryos, and they were filtered out in the analyses due to the possible events of allelic drop-out (ADO). Although the established algorithm showed stable performance in the analyses of this study, bias from ADOs could not be excluded. To screen the bins with sequencing depth above five across all somatic chromosomes, and combine them as a normalization factor, is applicable for future modifications.

Nevertheless, the study is limited to its retrospective nature. Evaluation of the relative mtDNA quantity lacks the evidence of gene integrity and functional assessment. Mitochondria display uniparental inheritance to reduce heteroplasmy, exhibiting a genetic bottleneck to prevent possible deleterious mutants in offspring (43, 44). However, mtDNA has a higher mutation frequency due to the insufficient repairing system and higher oxidative stress, the effect derived from these heteroplasmic mitochondria involving *de novo* variants in early-stage embryos derived from women without inherited mitochondrial disease remained uncertain. Deep sequencing or other functional assays in blastocyst biopsies may be useful to determine the role of mitochondria in human embryos more extensively. On the

practical side, application of machine learning-based technologies to analyze the weighting of mtDNA quantity among multivariate databases of embryo selection could provide another data-driven approach before clinical implementation.

Conclusions

In conclusion, mtDNA ratio is highly dependent on the days of blastocyst formation while the expansion stage was fixed, and it could reflect the immediate cell division progress. Reduced mtDNA ratio associated with reduced reproductive outcomes in the day 6 single euploid blastocyst transfers, but showed no significant correlation in the day 5 single euploid blastocyst transfers. Analysis of mtDNA quantity and developmental timeline in early-stage embryos is worthy for further investigation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by National Taiwan University Hospital, Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

T-HC designed the study and wrote the manuscript. C-YeC conducted the experiments and statistical works. C-SK built the bioinformatics pipelines. H-HL, C-LH, M-JL, and Y-TL, recruited the patients. Y-JC prepared the cell lines. C-YuC reviewed the bioinformatics and statistical results. S-UC revised the final manuscript, and approved the submitted version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1066530/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The diagram displays the distribution of mtDNA ratios calculated from 1,673 embryos, and two outliers are excluded due to out of scale. A Kolmogorov-Smirnov test is applied for confirming normality. The skewness and kurtosis are 4.95 and 51.61, respectively ($p<0.05$).

SUPPLEMENTARY FIGURE 2
Since the scales between qPCR and NGS are very different, correlation between mtDNA ratios derived from two methodologies is displayed in a log-log plot. The x scale denotes the log values of mtDNA ratios derived from the NGS platform, and the y scale as that derived from the qPCR platform.

SUPPLEMENTARY FIGURE 3
Three cell lines with different ploidies are tested on the NGS platform for evaluating the repeatability of established mtDNA ratio. Ten replicates of each cell line involve ten different sequencing runs.

SUPPLEMENTARY TABLE 1
Profile of patients for correlation analysis.

SUPPLEMENTARY TABLE 2
Profile of embryos for algorithm establishment.

SUPPLEMENTARY TABLE 3
Profile of embryos for correlation analysis.

SUPPLEMENTARY TABLE 4
Reproductive outcomes of cryotransfer with day 5 single euploid embryo (expansion score 5).

SUPPLEMENTARY TABLE 5
Reproductive outcomes of cryotransfer with day 6 single euploid embryo (expansion score 5).

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Recurrent implantation failure: A comprehensive summary from etiology to treatment

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Implantation is the first step in human reproduction. Successful implantation depends on the crosstalk between embryo and endometrium. Recurrent implantation failure (RIF) is a clinical phenomenon characterized by a lack of implantation after the transfer of several embryos and disturbs approximately 10% couples undergoing *in vitro* fertilization and embryo transfer. Despite increasing literature on RIF, there is still no widely accepted definition or standard protocol for the diagnosis and treatment of RIF. Progress in predicting and preventing RIF has been hampered by a lack of widely accepted definitions. Most couples with RIF can become pregnant after clinical intervention. The prognosis for couples with RIF is related to maternal age. RIF can be caused by immunology, thrombophilias, endometrial receptivity, microbiome, anatomical abnormalities, male factors, and embryo aneuploidy. It is important to determine the most possible etiologies, and individualized treatment aimed at the primary cause seems to be an effective method for increasing the implantation rate. Couples with RIF require psychological support and appropriate clinical intervention. Further studies are required to evaluate diagnostic method and he effectiveness of each therapy, and guide clinical treatment.

KEYWORDS

recurrent implantation failure, immunology, thrombophilias, endometrial receptivity, microbiome, anatomical abnormalities, aneuploidy

1 Introduction

Implantation is the first step of crosstalk between the embryo and endometrium, which is the key point for a successful pregnancy. The implantation process includes apposition, adhesion, and invasion (Figure 1) (1). Successful implantation is identified as an intrauterine gestational sac seen on ultrasonography. Implantation failure may occur

during the attachment and migration process, with a negative urine or blood test for human chorionic gonadotropin (hCG) or failure to form an intrauterine gestational sac with positive hCG. Recurrent implantation failure (RIF) is a clinical phenomenon with no widely accepted definition. The key factors that need to be considered while establishing the definition of RIF are the number of embryos transferred or unsuccessful *in vitro* fertilization-embryo transfer (IVF-ET) cycles, the quality of embryos, fresh or frozen embryos, and maternal age, which are disputed points. The increase in the cumulative live birth rate with more IVF-ET cycles showed a progressive decline (2). Other analyses showed that after three IVF-ET cycles, cumulative pregnancy rates did not increase significantly, and the pregnancy rate per cycle tended to decrease after three cycles of unsuccessful treatment (3–5). When RIF was defined as two or more implantation failures, the live birth rate was significantly lower than when RIF was defined as three or more implantation failures, which was considered an excessively increased denominator (6). Hence, a blind increase in IVF-ET cycles may not lead to a successful pregnancy, and we need to set a cut-off point for treatment cycles to recognize patients with RIF. Owing to the different quality of embryos, the number of transferred embryos varies from 3 to 10 or more (7). A good-quality embryo has the proper developmental status according to the day of its development (8). A poor-quality embryo implies that patients need to go through more embryos that are transferred to acquire a successful pregnancy. Another factor that should be considered when defining RIF is maternal age. It is well known that pregnancy rates decrease with maternal age (9); older patients required more cycles of blastocyst transfer to reach the same implantation rate as young women (10). Defining RIF without considering maternal age is meaningless. Based on the above considerations, the widely accepted definition of RIF, as presented by Coughlan, is the failure to achieve a clinical pregnancy after the transfer of at least four good-quality embryos in a minimum of three fresh or frozen cycles in a woman under 40 years of age (11). The preimplantation genetic diagnosis consortium of the European Society of Human Reproduction and Embryology (ESHRE) defined RIF as when more than three good-quality embryo transfers or ten embryos in multiple transfer cycles are performed without achieving a clinical pregnancy (12). In clinical practice, an international survey of clinicians and embryologists showed that the majority defined RIF as failed embryo transfer with three cycles, both fresh and frozen, with no agreement on the cutoff upper age (7).

IVF-ET success rates have improved over the decades due to technical improvements, which affect RIF definition, mainly in the number of embryos transferred. In clinical practice, different centers often adjust their definitions according to their own status (7). Overall, we defined RIF as failure to become clinically pregnant after the transfer of at least three good-quality embryos in three fresh or frozen cycles in women under 40 years of age. Here, a good-quality embryo means day 3 embryo ≥ 8 cells, symmetric, with <10% fragmentation (8), or blastocyst with a grade ≥ 3BB (13). However, further analysis of multiple center clinical data with large sample size is needed to process a more internationally accepted definition of RIF. This review summarizes the etiology of RIF and the current clinical treatment.

2 Discussion

2.1 Risk factors

Known risk factors for RIF include body mass index (BMI), smoking, alcohol consumption, and stress.

2.1.1 Body mass index

Body mass index is associated with implantation. Obesity affects the female reproductive system. Pre-pregnancy obesity is associated with abnormal menstruation, anovulation, and pregnancy complications (14, 15). In IVF-ET, obese patients tend to have a lower pregnancy rate than normal-weight patients (16). Furthermore, when BMI was ≥ 30 kg/m^2, patients undergoing IVF-ET had significantly decreased odds of implantation (17). In addition, obesity can alter the markers of uterine receptivity and decidualization, which may contribute to a decrease in the implantation rate in obese patients (18).

2.1.2 Smoking

In women undergoing IVF, it is difficult to assess the amount of smoking owing to inaccurate responses to questionnaires, which makes the association between smoking and IVF uncertain. However, for patients who smoked for > 5 years, smoking was associated with fewer oocytes retrieved, a higher cycle cancellation rate, and a lower implantation rate (19). Meanwhile, for male partners, smoking negatively affects sperm motility and counts and increases sperm DNA damage (20).

2.1.3 Alcohol

Alcohol has a negative effect on pregnancy. In developed countries, alcohol use is a risk factor for stillbirth (21) and can also affect the neurocognitive function of the offspring, such as hyperactivity, impulsivity, and lack of awareness of social cues (22). Therefore, couples trying to conceive are advised to quit drinking before pregnancy (23).

2.1.4 Stress

Cortisol production increases in response to stress, which is believed to be a risk factor for pregnancy. Maternal stress, measured by the level of cortisol, increased the risk of

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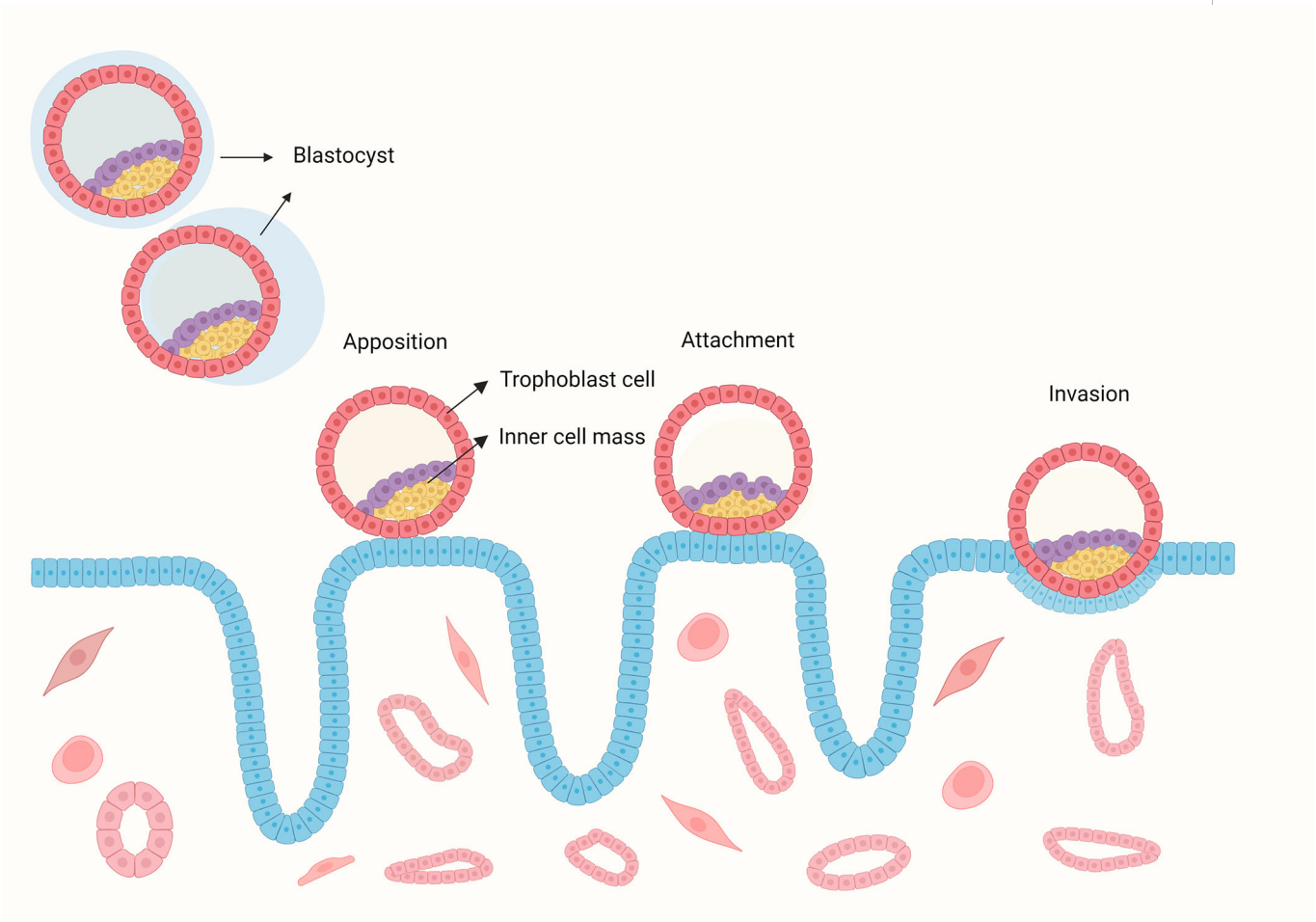

FIGURE 1
The process of human blastocyst implantation. The blastocyst hatches from the zona pellucida and contact with the endometrium, then the embryo bind to the endometrium during the attachment stage, during with the crosstalk between the embryo and endometrium induces up-regulation of surface receptors and the secretion of signalling molecules and hormones. This signalling directs epithelial withdrawal and trophoblast invading the endometrium.

miscarriage by 2.7-fold (24). However, another study showed that stress did not affect the outcomes of patients undergoing the first cycle. Failure of the last IVF cycle leads to a high risk of stress (25).

2.2 Etiology of recurrent implantation failure

RIF is a complex clinical phenomenon with several different etiologies, including maternal factors, paternal factors and embryo factor. There may not be one single cause, but several factors working together lead to RIF. Among the etiologies, maternal factors include different aspects. Though a good quality embryo is foundation for successful implantation, the state of mother is also crucial, which we will focus on,

2.2.1 Immunology

Successful implantation is a process of maternal-fetal immune tolerance involving various molecules. Trophoblast invasion can activate the maternal immune response to fetal antigens. Local immune cells at the implantation site in the endometrium, which are activated by the embryos, mediate maternal-fetal immune tolerance and promote placental development. They involve in regulating the differentiation of decidual cell, remodeling uterine vascular, promoting epithelial attachment and regulating immune activation. In this stage, immune cells, including innate lymphocytes, T cells, decidual

dendritic cells, and macrophages, are activated, and they are also associated with adverse pregnancy outcomes such as RIF (26).

2.2.1.1 Innate lymphocytes

Innate lymphocytes (ILCs) have been proved to exist in human decidua (27). They are divided into two subtypes: natural killer (NK) cells and non-cytotoxic helper ILCs (ILC1s, ILC2s, and ILC3s) (28). NK cells in the uterus (uNk cells) account for over 70% of all endometrial leukocytes in early pregnancy (28, 29) and possess unique functions that differentiate them from peripheral NK cells. They secrete specific chemokines, express unique cell surface markers, and display a large granule morphology. However, they show poor cytotoxicity because they are unable to polarize granules into the immune synapse (30).

NK cells in the decidua stroma secrete cytokines and express receptors mediating maternal-fetal immunity. uNK cells are not directly cytolytic to fetal extravillous trophoblast (EVT) cells (31). They prompt the low cytotoxicity of uNK cells necessary for semi-allogeneic fetus. Specifically, uNK cells express killer cell immunoglobulin-like receptors (KIRs) that can bind to selectively expressed ligands on EVT, such as human leukocyte antigen-C (HLA-C), human leukocyte antigen-G (HLA-G), and human leukocyte antigen-E (HLA-E) (32, 33). The function of uNK cells depends on the balance between inhibitory and activating receptors (34), as KIR genes are highly polymorphic. Each pregnancy involves different maternal/fetal genetic combinations that deliver activating or inhibitory signals to uNK cells. KIR genes can be grouped into two main haplotypes, A and B (35). The maternal KIR genotype could be AA (inhibition of KIR), AB, or BB (activation of KIR). Trophoblast invasion is regulated by interactions between the maternal KIR and fetal HLA-C. Women with the KIR AA genotype have a higher risk of preeclampsia and other pregnancy-related complications (36). About 78% of patients with more than five unsuccessful IVF treatments or embryo transfers lacked three KIR-activating receptors (2DS1, 2DS3, and 3DS5) (37). Moreover, the KIR genotype of Tel AA combined with the HLA-C2C2 genotype was more prevalent in patients with RIF (p/pcorr. = 0.004/0.012, OR = 2.321) (38). This specific combination of polymorphic KIR and HLA-C genotypes can also affect decidual vascular remodeling (39).

Angiogenesis is the foundation for implantation. uNK cells are the main source of angiogenic growth factors such as placental growth factor, vascular endothelial growth factor (VEGF)-A, and angiopoietin, which may direct angiogenesis during embryo implantation (40, 41). In early pregnancy, uNK cells aggregate around spiral arteries, and animal studies have shown that uNK is involved in spiral artery remodeling (42). These findings suggested that uNK cells play a role in mediating vascular changes during implantation. The number of uNK cells, which was no correlation with peripheral NK level, increases in patients with RIF (43, 44).. However, the production of angiogenic factors, such as VEGF, by uNK cells was lower in patients with RIF than in fertile women, which may be attributed to the increased cytotoxicity of $CD16^+$ uNK cells (45, 46). The angiogenic factors produced by uNK cells may be located at the implantation site and move toward the embryo, directing the development of maternal vasculature to the implantation site (47). Hypoxia-inducible factor 1-alpha (HIF-1α) is a transcription factor expressed under hypoxic conditions and can promote angiogenesis by increasing VEGF expression in the tumor tissue. HIF-1α inhibitors can activate the anti-tumor functions of NK cells by elevating interferon-γ (IFN-γ) production (48). In early pregnancy, trophoblasts secrete HIF-1α under hypoxic conditions (49). In the uteri of patients with RIF, both HIF-1α expression and angiogenesis are reduced (50). Therefore, we assume that a decrease in HIF-1α may be involved in RIF *via* the reduction of VEGF secreted by uNK cells or *via* an increase in uNK cell cytotoxicity. This may be due to abnormal interactions between trophoblasts and uNK cells (Figure 2). However, further studies are required to support this hypothesis. Other studies suggested that patients with RIF showed more abnormal vascular parameters as estimated by the Doppler test, with more uNK cells producing more IL-12 and IL-18. Dysfunction of cytokine signaling may impair vascular remodeling, leading to excessive or insufficient recruitment of uNK cells (51–53). However, another study reported different conclusions. Analysis of uNK cell numbers using standard immunohistochemistry protocols showed that there was no difference in uNK cell numbers and distribution relative to endometrial arterioles between patients with RIF and women with successful IVF cycles. Furthermore, uNK cell numbers were significantly decreased in women who had successful pregnancies compared with those who did not (54). Overall, uNK cells might impair vascular remodeling *via* abnormal recruitment of NK cells to endometrium, with dysregulated cytokine signaling.

2.2.1.2 T cells

T cells play an important role in immunity during pregnancy. They are divided into four main types: T helper 1 (Th1) cells, Th2, Th17, and regulatory T (Treg) cells. T cells constitute 5–20% of $CD45^+$decidual lymphocytes, which display different functions compared to peripheral blood T cells (55). Th2 cell dominance is essential for normal pregnancy (56). An imbalance of Th1/Th2 is associated with reproductive dysfunction. In patients with RIF, the Th1/Th2 ratio increases in the peripheral blood with an increasing Th1 immune response (57). Meanwhile, anti-inflammatory factors, such as IFN-γ and tumor necrosis factor-α (TNF-α), mainly secreted by Th1 cells, were increased in the peripheral blood of patients with RIF (58).

Th17 cells can produce an anti-inflammatory factor, interleukin-17 (IL-17), which promotes the expression of

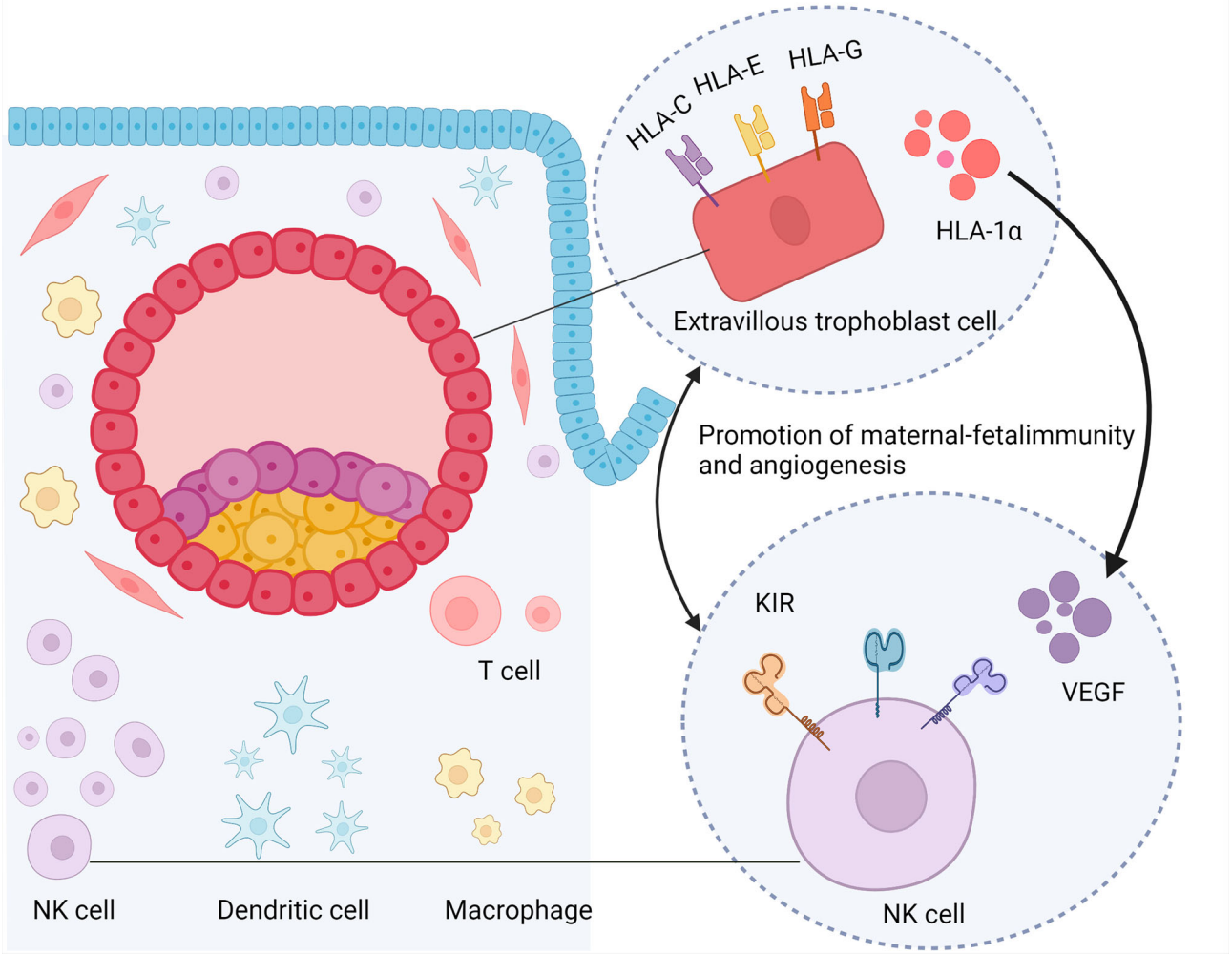

FIGURE 2
Promotion of maternal-fetal immunity and angiogenesis. NK cell,nature kill cell; DC, dendritic cell; KIR, killer cell immunoglobulin-like receptors; HLA, human leukocyte antigen; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible factor 1-alpha.

inflammatory mediators. An abnormal Th17 increase in the peripheral blood and decidua is associated with recurrent miscarriages (59–61). In the peripheral blood of patients with RIF, higher numbers of Th17 cells co-exist with exhausted Treg cells (62). Treg cells are known to mediate pregnancy tolerance, and can potently suppress Th1/Th17-mediated immunity (63). More evidence has shown that exhausted Treg cells may lead to adverse pregnancy outcomes; reduced capability of Treg cells to control over-activated T cells may lead to implantation failure (64). The reduced suppressive capability of Treg cells is associated with CD279/PD-1 expression (65), which may play a role in the RIF mechanism. Moreover, intravenous immunoglobulins (IVIG) can improve the implantation rate by increasing Treg cells in the peripheral blood of patients with RIF, verifying the effect of Treg cells in RIF from another side (66).

2.2.1.3 Decidual dendritic cells

DCs account for 10–20% of decidual leukocytes. As antigen-presenting cells, uterine DCs are involved in the recognition of paternal antigens (26). A decrease in immature DCs and an increase in mature DCs were observed in the decidua of women with recurrent spontaneous abortion (67). Few studies have examined the role of DCs in RIF. Depletion of DC in the uterus led to severe impairment of implantation in mice (68). $ILT4^{+}$ DCs were significantly increased in the peripheral blood

and endometrium of patients with RIF compared to that in the fertile control group, probably due to the induction of Treg cells (69). Further studies are required to confirm the relationship between DCs and RIF.

2.2.1.4 Macrophages

Macrophages regulate implantation, placentation, fetal development, and vascular remodeling at the maternal-fetal surface (70). Macrophages located close to invade trophoblasts and spiral arteriesto promote implantation during early pregnancy (71). The proportion of uterine macrophages was high in patients with RIF with chronic endometritis and adenomyosis, indicating that macrophages are involved in the pathological process of implantation failure in these patients. However, the underlying mechanism remains unknown (72, 73).

2.2.2 Thrombophilias

Pregnancy is a hypercoagulable condition. Thrombophilias are conditions that predispose individuals to inappropriate blood clot formation (74). Thrombophilia is involved in recurrent pregnancy loss (RPL), but the association between thrombophilia and RIF remains to be elucidated. Thrombophilias are believed to affect implantation by impairing vascularization of the embryo and disturbing blood flow to the decidual vessels (75).

2.2.2.1 Inherited thrombophilias

Inherited thrombophilia commonly refers to a condition in which genetic mutations affect the function or quantity of proteins in the coagulation system (76). The common forms of inherited thrombophilias are genetic mutations in factor V Leiden, proteins S and C, prothrombin, and methylenetetrahydrofolate reductase (MTHFR). Mutations in these genes are increased in women with RPL, including RIF (77–79). Moreover, patients with RIF with thrombophilia most commonly harbor the MTHFR C677T variant, which impairs implantation by disturbing vascularization (78, 79). On the other hand, hyperhomocysteinemia caused by the MTHFR C677T variant is also considered a risk factor for RPL (80).

2.2.2.2 Acquired thrombophilias

The most prevalent acquired thrombophilia is the antiphospholipid syndrome (APS). It is an autoimmune hypercoagulable state diagnosed by the presence of antiphospholipid antibodies, such as anticardiolipin antibodies, lupus anticoagulant antibodies, and/or anti–2-glycoprotein I antibodies. It has been proven that APS is associated with RPL and patients with previous arterial or venous thromboembolic events have a higher risk of pregnancy complications (81). However, the role of APS in RIF remains unclear. Antiphospholipid antibodies can be detected in patients with RIF (82). In a previous study, the frequency of antiphospholipid antibodies inpatients with RIF was significantly higher than that in the fertile group (83). Nevertheless, other studies have not reported an association between APS and RIF. When APS was analyzed in women with a mean of seven failed IVF-ET cycles, there was no significant association between thrombophilias and RIF (84). Therefore, a clinical practice guideline by the Canadian Fertility and Andrology Society does not recommend testing for thrombophilia in patients with RIF (85).

2.2.3 Endometrial receptivity

The endometrium is critical in pregnancy, as it provides an environment for the implantation of developing embryos. Impaired endometrial receptivity is estimated to account for two-thirds of implantation failures (86). Suboptimal endometrial receptivity has been confirmed as a cause of RIF (87). An endometrial biopsy obtained from patients with RIF on the seventh day of progesterone administration revealed 313 genes that were differentially expressed between patients with RIF and the control group (88). Another study revealed differences in several fertility-related genes in cultured endometria of RIF versus patients who became pregnant after IVF-ET (89). Bioinformatical analyses demonstrated that PTGS2, FGB, MUC1, SST, VCAM1, MMP7, ERBB4, FOLR1, and C3 were the key differentia expression genes related to RIF (90). Transcriptomic studies have indicated that patients with RIF express a different endometrial profile compared to the fertile control group on special days of the menstrual cycle. This is assumed to be due to the displacement of the window of implantation (WOI), which affects more than 25% of patients with RIF (87, 91). Furthermore, prostaglandin synthesis appears to be disturbed in patients with RIF and may lead to poor endometrial receptivity (92).

2.2.4 Microbiome and chronic endometritis

The human microbiome, called “the other human genome,” has been involved in normal physiology and homeostasis, associated with states of health and disease (93, 94). The female reproductive tract contains distinct bacterial communities that form a continuous microbiota changing from the vagina to the ovaries (95). Alterations in the vaginal microbiome are involved in female reproductive system diseases such as bacterial vaginosis, urinary tract infections, and also in pregnancy complications (96–98). Hence, we can assume that microbiota might be involved in several steps of IVF-ET, including gametogenesis, implantation, and delivery.

The vagina is dominated by the *Lactobacillus* genus, which has a probiotic influence on the vaginal microenvironment (95). It can inhibit the invasion of bacteria by producing high concentrations of lactic acid and short-chain fatty acids, which maintain the acidic environment of the vagina (99, 100). Infertile women display abnormal vaginal microbiota. *Ureaplasma* spp. in the vagina and *Gardnerella* spp. in the cervix appeared to be related to women with a history of infertility (101). Investigating

the vaginal microbiota in patients with unexplained RIF indicated that vaginal *Lactobacillus* (found to be positively correlated with pregnancy rates) was significantly decreased compared to patients who became pregnant in the first frozen embryo transfer (FET) cycle. Patients with RIF presented higher microbial α-diversity than the control group (99). Meanwhile, vaginal *Lactobacillus* in patients with RIF was significantly decreased compared with healthy women, and the vaginal microbiota profiles in patients with RIF had significantly higher levels of five bacterial genera than in healthy women (102). Therefore, the number of vaginal *Lactobacillus* spp. is assumed to be a predictive biomarker of implantation.

The endometrium contains four orders of magnitude fewer bacteriathan the vagina; the vagina harbors approximately 10^{10}-10^{11} bacteria (95, 103). High numbers of *Lactobacillus* spp. in the endometrium during the implantation window were associated with higher successful implantation rates, whereas non-*Lactobacillus*-dominated microbiota, such as *Streptococcus*, during the implantation window resulted in negative pregnancy outcomes (104). Bacterial pathogens alter endometrial microbiota, which can result in chronic endometritis. Chronic endometritis is often asymptomatic, leading to inconsistencies in prevalence. The reported prevalence in patients with RIF ranges from 7.7% to 66% (105–109), with a prevalence of 2.8% in patients with general infertility (110). The uterine immune status in chronic endometritis is altered (72). A study on chronic endometritis has shown abundant immune cells in the endometrium and an increase in $CD83^+$ mature DCs, $CD68^+$ macrophages, $CD8^+$ T cells, and $Foxp3^+$ Treg cells; these results might be reasonable for impaired endometrial receptivity and recurrent pregnancy failures (72). Furthermore, microbial alterations in chronic endometritis may also disturb immune status by increasing the synthesis of lipopolysaccharide, an important immunomodulator (111).

During pregnancy, the gut microbiota can change in composition or abundance (112). It may also be involved in embryo implantation by affecting the immune system, coagulation system, and endometriosis pathology (113–115). Patients with RIF display abnormal gut microbiota (116), but the relationship between gut microbiota and implantation failure needs to be further investigated.

2.2.5 Anatomical abnormalities

Several types of uterine abnormalities can affect implantation rates, including fibroids, polyps, intrauterine adhesions, Mullerian abnormalities, adenomyosis, and hydrosalpinges. The proportion of unidentified intrauterine abnormalities in patients with RIF varied between 14% and 51% (117–120). Most patients are asymptomatic and remain undiagnosed until they undergo transvaginal ultrasound or hysteroscopy.

2.2.5.1 Uterine fibroids

Fibroids can lead to deformation of the uterine cavity and adhesion, which can prevent the attachment of the embryo to the endometrium. The effect of fibroids on pregnancy outcomes is related to their location. Intramural and subserous fibroids may not have an impact on pregnancy outcomes. Submucosal fibroids can decrease implantation and pregnancy rates in patients undergoing IVF. The mechanism hindering implantation includes increased uterine myometrial contractions, abnormal vascularization, and a disordered cytokine profile (121). A systematic review concluded that patients with submucosal fibroids had lower implantation and live birth rates than the control group. Therefore, the removal of submucosal fibroids before IVF-ET seems to confer benefits (122).

2.2.5.2 Polyps

Polyps in the endometrium are the most frequent uterine lesions in patients with RIF that interfere with embryo implantation (121, 123). They not only affect the deformation of the uterine cavity, but also disturb the implantation process by altering cytokines secreted by the endometrium, such as insulin-like growth factor 1 binding protein and TNF-α (124, 125). The removal of endometrial polyps before intrauterine insemination is believed to improve clinical pregnancy rates (126).

2.2.5.3 Intrauterine adhesion

Intrauterine adhesion often occurs after the curettage of the gravid uterus to terminate the pregnancy. It impairs the functional layer of the endometrium and prevents embryo attachment for successful implantation. A study of 210 patients with RIF who underwent hysteroscopic evaluation showed that the frequency of intrauterine adhesions was 8.5% (127).

2.2.5.4 Mullerian abnormalities

Mullerian abnormalities, such as septate and bicornuate uteri, should be considered in patients with RIF. Compared with other congenital uterine anomalies, partial septate and septate uteri appear to have the poorest reproductive outcomes, such as reduced pregnancy rate, increased risk of first-trimester miscarriage, and preterm birth (128). Among 144 patients undergoing IVF-ET who experienced implantation failure, uterine abnormalities (mainly septate) were found in 14 (9.7%), which led to the assumption that uterine septate may be a factor involved in implantation failure (129). However, a bicornuate uterus is more likely to have less influence on pregnancy. The major risk factors for a bicornuate uterus are mid-trimester abortion and preterm birth (130).

2.2.5.5 Endometriosis

Endometriosis is an estrogen-dependent inflammation with an incidence of up to 50% in infertile women (131, 132). It can affect female IVF-ET in several aspects, including the number of

oocytes retrieved, fertilization, and implantation rate (133). The mechanism involves anatomic distortion, oviduct occlusion, abnormal secretion of cytokines involved in endometrial receptivity, and poor oocyte quality (134). In addition, patients with endometriosis-related infertility display different reproductive tract microbiota, which may disturb endometrial receptivity (95). Adenomyosis, defined by the presence of a heterotopic endometrium in the myometrium, is a special form of endometriosis. This can lead to implantation failure in young patients (135). Nevertheless, surgical operation in adenomyosis may not improve clinical outcomes because there is no defined capsule and part of the uterine wall has to be removed (11).

2.2.5.6 Hydrosalpinges

Hydrosalpinges can negatively impact implantation, mainly due to the impairment of embryo development by innutritious fluid (136). Other mechanisms include disturbing endometrial receptivity and physically flushing the embryo out (137). Infertile patients with hydrosalpinges express significantly less αvβ3 integrin, HOXA 10, and leukemia inhibitory factor (LIF) during WOI compared with fertile women (138–140). In IVF-ET, hydrosalpinges are associated with negative outcomes, including lower implantation rates, lower pregnancy rates, and increased spontaneous abortion rates (141, 142). However, the influence of hydrosalpinges on implantation rates appears to be associated with the extent of the hydrosalpinges. One study showed that implantation rates of patients undergoing salpingectomy were not significantly higher than those in the non-intervention group. However, subgroup analysis indicated significantly increased implantation rates when patients with ultrasound-visible hydrosalpinges underwent surgery (143).

2.2.6 Male factors

Although studies have shown that sperm affects early embryogenesis and placental function, the relationship between male factors and RIF remains poorly understood. Sperm DNA damage is related to poor embryo development, and sperm DNA integrity testing is considered to be associated with reproductive failure (144). However, a prospective study with a small number of patients showed that a high DNA fragmentation index was not correlated with RIF (145), which was consistent with another prospective study (146). Therefore, routine testing for DNA fragmentation is not recommended by the American Society for Reproductive Medicine (ASRM) (147). While sperm aneuploidy rates were evaluated by fluorescence *in situ* hybridization techniques, there was a significant increase in the incidence of sex chromosome disomies in patients with a previous history of RIF; however, the implantation rates did not significantly increase in patients who underwent subsequent IVF-ET cycles (148).

In addition, protamines are the largest number of nuclear proteins in human sperm, which are divided into protamine 1 (P1) and protamine 2 (P2). They can package compacted chromatin more efficiently and protect sperm from oxidative damage. Recently, the P1/P2 ratio has been identified as a new parameter of sperm function that can partly predict the fertilization outcome of IVF-ET (149, 150). An abnormal P1/P2 ratio is related to infertility (151). A decreased P1/P2 ratio was associated with poor pregnancy outcomes, including a lower fertilization rate of IVF and a lower implantation rate per embryo in patients undergoing IVF-ET (152). Moreover, the sperm of male partners of women with RPL contained significantly higher P1 and P2, and a lower P1/P2 ratio, indicating that protamines are not only important for fertilization, but also play a role in early embryogenesis (153).

In conclusion, there is insufficient evidence for an association between male factors and RIF. We hypothesized that impaired sperm parameters are more likely to be involved in RIF by affecting the chromosomal constitution of embryos, which will be discussed in the following section.

2.2.7 Embryo factor

Embryos with abnormal chromosomes are recognized as important factors that cause implantation failure or pregnancy loss (154). The probability of chromosomal aneuploidy in embryos also increases with age. In the first trimester, a spontaneous abortion rate as high as 76% has been attributed to chromosomal abnormalities (155).

Chromosomal abnormalities, including translocations, inversions, deletions, and mosaicism, are more common in patients with RIF than in the general population (156). In cleavage embryos, the incidence of complex chromosomal abnormalities, such as three or more abnormal chromosomes, was independent of age but increased in embryos from patients with a history of RIF (157). This complex abnormality is considered mitotically derived because it is more common in embryos than in retrieved oocytes. However, the exact cause of this remains unknown. Furthermore, embryonic mosaicism is the presence of two or more genetically different cell lineages, usually one with an abnormal chromosome and the other with a normal chromosome, and is common in human preimplantation embryos (158, 159). Due to chromosomal abnormalities in this type of embryo, it is reasonable to suspect that mosaicism can influence the implantation rate. Mosaic embryos have lower implantation rates and live births than euploid embryos, and their implantation potential is affected by the extent of mosaicism (160). Typically, embryos with whole-chromosome aneuploidy display negligible implantation potential (161).

2.3 Therapy of recurrent implantation failure

The treatment of patients with RIF presents a challenge to clinicians. Various therapeutic options have been proposed to

manage RIF, including lifestyle intervention, immunotherapy, anticoagulant, improving endometrium receptivity and sperm quality and preimplantation genetic testing for aneuploidies (PGT-A). Experienced clinicians and embryologists should discuss therapeutic options with patients to address their questions and offer an individualized treatment plan. We discuss below the different interventions that can be used in the management of RIF.

2.3.1 Lifestyle intervention

2.3.1.1 BMI

Patients should be informed that obesity (BMI ≥ 30 kg/m^2) or underweight (BMI < 19 kg/m^2) can negatively impact reproduction outcomes. Patients should be advised to return to a normal BMI before IVF-ET treatment. Multidisciplinary approaches include low-energy diets, pharmacotherapy, and bariatric surgery (162). Weight loss before clomiphene treatment in patients with PCOS resulted in improved ovulation and live births (163). Moreover, short-term weight loss before IVF-ET was associated with the retrieval of more metaphase II oocytes (164). For safety, some countries do not allow public funding for IVF-ET treatment in obese infertile patients unless their BMI is within a certain level (165).

2.3.1.2 Smoking

Women planning pregnancy should stop smoking and avoid secondhand smoke for better IVF-ET outcomes (166). Male partners should also abstain from smoking, as smoking increases the production of reactive oxygen species in seminal plasma, alters sperm microRNA content, and increases DNA fragmentation in sperm (167).

2.3.1.3 Alcohol

More than one unit of alcohol per day can reduce the efficiency of IVF-ET, including fertilization and pregnancy rates, and excessive alcohol intake can be harmful to semen quality. Therefore, couples with RIF should reduce alcohol intake to one or two units per week or total abstinence from alcohol before IVF-ET.

2.3.1.4 Stress

Stress is also associated with RIF. Lifestyle interventions such as a healthy diet, regular exercise, and even psychological interventions may reduce psychological distress and improve future IVF-ET outcomes (168).

2.3.2 Optimal IVF-ET procedure

2.3.2.1 Ovarian stimulation protocol

An appropriate controlled ovarian hyperstimulation (COH) protocol should be considered. The stimulation protocol and dose of gonadotrophin require reconsideration if patients have a suboptimal response. Gonadotropin-releasing hormone agonist (GnRHa) combined with human menopausal gonadotropins (HMG) appeared to widen the implantation window compared to a single HMG protocol, resulting in improved IVF-ET success (169). Moreover, the use of long-acting GnRHa for a few months before IVF-ET may increase the pregnancy rate in patients with endometriosis (170). Administration of a single dose of GnRHa in the luteal phase can improve the implantation rate in intracytoplasmic sperm injection (ICSI) cycles (171). This might be partially due to differences in gene expression caused by different luteal support protocols (172). Therefore, it is important to select a specific protocol that includes ovarian stimulation and luteal support in patients with RIF, which may be related to the success rate.

2.3.2.2 Assisted hatching

Assisted hatching (AH) is a technique that includes zona thinning and zona drilling/opening, using chemical, mechanical, or laser energy. The effects of AH remain unclear. Embryos that underwent drilling treatment in frozen/thawed embryo transfer displayed a higher implantation rate but no increase in pregnancy rate (173). A recent meta-analysis showed that it was uncertain of the effect of AH on live birth rates (174). In selected patients such as those with RIF, AH might be beneficial. In patients with RIF older than 38 years, AH caused by partial zona dissection led to a significant increase in implantation and clinical pregnancy rates (175). However, ASRM considered that there is insufficient evidence for the benefit of AH in patients with poor prognosis, including poor-quality embryos, more than two previous IVF-ET failures, and advanced maternal age (176). Generally, considering the influence of AH on the embryo and its controversial effect on RIF, AH should be used cautiously.

2.3.2.3 Embryo transfer

Abnormally elevated estrogen levels in fresh cycles may influence endometrial morphology and receptivity (177). The endometrium in fresh cycles shows a premature secretory phase followed by dyssynchronous stromal and glandular differentiation in the mid-luteal phase (178). Therefore, the implantation rates in fresh embryo transfer were lower than in frozen-thawed cycles (179). Moreover, the embryo transfer stage is important for successful implantation. Implantation rates were higher in the blastocyst transfer group than in the cleavage embryo transfer group in patients with RIF (180). In patients with RIF with a good ovarian response, the implantation rates of fresh cycles were significantly higher in blastocyst transfer; however, the cycle cancellation rates also increased (181). Thus, transfer of blastocysts in frozen-thawed cycles might be a choice for patients with RIF, and sequential cleavage and blastocyst embryo transfer appeared to be beneficial. It can improve clinical pregnancy rates compared to cleavage embryo transfer. Patients with sufficient embryos may attempt this method (182).

2.3.3 Immunotherapy

Maternal-fetal immune tolerance is a necessary condition for successful implantation. Several immunological therapies have been explored to increase implantation rates. Endometrial biopsies and peripheral blood sampling for NK cell type and count or Th cell proportion offer a method to assess the maternal immune status and a rationale for immune-modulating therapies (183).

2.3.3.1 Glucocorticoids

Glucocorticoids are a type of immunomodulator. They can bind to the glucocorticoid receptor on uNK cells and decrease the number of uNK cells (184, 185). A meta-analysis showed that administration of glucocorticoids during routine IVF-ET cycles did not improve live birth rates. However, the use of glucocorticoids in a subgroup of IVF, not ICSI cycles, was related to increasing pregnancy rates with borderline statistical significance, suggesting that specific subgroups of patients might benefit from glucocorticoid therapy (186). Using prednisolone in patients with serum anti-ovarian antibody positivity and at least two previous IVF failures could decrease the serum anti-ovarian antibody level and improve pregnancy outcomes (187). Prednisolone could also improve the implantation rates in patients undergoing ICSI with high-level peripheral $CD69^+$ NK cells (188). However, in a selected group of patients (failure to obtain clinical pregnancy after transfer of at least two embryos in at least two fresh or frozen cycles) with elevated uterine NK cells, prednisolone could decrease uNK cell concentration, but with no significant benefit on pregnancy outcomes (185). Therefore, glucocorticoids should be carefully administered to patients with specific indications, the dosage and time are arbitrary.

2.3.3.2 Intravenous immunoglobulin

Intravenous immunoglobulin (IVIG) is produced by the extraction of IgG fractions from the plasma of healthy donors. It can protect the fetus from the maternal immune system by promoting the expansion of suppressor T cells, inhibiting complement deposition, protecting paternal genes by neutralizing anti-HLA antibodies, and reducing the adhesion of T cells to the human placental extracellular matrix (189, 190). In RPL, IVIG is an efficient therapy for improving pregnancy outcomes by affecting the Th1/Th2 ratio and increasing Treg cells (189, 191). Furthermore, IVIG can improve implantation and pregnancy rates in patients with RIF and immune abnormalities (192). The combined application of IVIG, aspirin, and heparin could increase the pregnancy rates and peripheral blood Treg cell proportion in patients with RIF, compared with patients using only aspirin and heparin (66). In addition, IVIG can decrease NK cell percentage and cytotoxicity, and improve pregnancy and live birth rates in patients with reproductive failure (193, 194). IVIG was administered at 200–500 mg/kg body weight (usually 400 mg/kg) 7 days-24 hours before embryo transfer and lasted until fetal pulse detection or every 3 weeks during pregnancy (192).

2.3.3.3 Tacrolimus

Pregnancy is a type of semi-allograft. The maternal immune system treats the fetus as a foreign agent. Excessive immune activation results in implantation failure. Tacrolimus, an immunosuppressant, has been demonstrated to suppress immunological rejection by inhibiting cytotoxic T cell generation, alloantigen-induced lymphocyte proliferation, and the production of IL-2 and IFN-γ (195). It has been used as a plausible treatment for patients with RIF who have an elevated Th1/Th2 ratio and appears to improve pregnancy outcomes (196, 197). However, further evidence is required to support the use of tacrolimus for RIF. Further, its dose and safety need to be carefully assessed.

2.3.3.4 Cyclosporine

Cyclosporine is a typical immunosuppressant that induces immune tolerance in patients with autoimmune diseases and organ transplantation. It can promote the invasion and migration of villous trophoblasts, thereby improving implantation (198). The production of IL-4, a Th2 cytokine, and chemokine CXCL12 is increased by cyclosporine at the maternal-fetal surface (199, 200). Cyclosporine could improve pregnancy outcomes in patients with RPL with an elevated Th1/Th2 ratio in peripheral blood (201). Patients with unexplained RIF receiving cyclosporine treatment since the transfer day showed an obvious improvement in implantation rates, especially of non-high-quality embryos (202). However, in patients with only one unsuccessful transfer cycle of high-quality embryos, cyclosporine treatment did not display benefits for clinical pregnancy outcomes in the following FET cycles (203). Therefore, it is not recommended administration of cyclosporine in RIF patients routinely.

2.3.3.5 Intralipid

Intralipids are fat emulsions containing glycerin, soybean oil, and egg phospholipids, which are used for parenteral nutrition. It can modulate NK cell cytotoxicity and suppress pro-inflammatory cytokine activity (204, 205). In RIF patients with overactivation of NK cells, intralipids can decrease the biomarkers of immune overactivation in the endometrium and increase live birth rates (206). A recent meta-analysis showed that intra-venous intralipid therapy could improve the clinical pregnancy and live birth rates, but the sample sizes of included studies were small, and the treatment protocols were variable (207). However, not all studies showed an improvement after treatment with intralipid. A randomized controlled trial (RCT)

in patients with RIF that used 20% (100 mg) intralipid in 500 mL NaCl on the day of embryo transfer demonstrated that the increase in the clinical pregnancy and live birth rates was not significant after intralipid infusion therapy (208). Coulam believed that intralipid is not appropriate for all patients with RIF but for those with some kind of immune abnormality; identifying such patients is essential (209). Overall, there is insufficient evidence regarding the routine use of intralipid therapy in patients with RIF, and a standard treatment protocol is lacking. Large-scale studies are required to explore the effects and safety of intralipids in RIF treatment.

2.3.3.6 Lymphocyte immunization therapy

LIT is an active immunotherapy that can modulate maternal fetal interface immune balance by administering lymphocytes obtained from mother's partner. It was initially conceived to improve immune tolerance and better for implantation. This immunotherapy was first used to treat RPL, but its current application is controversial. The 2017 ESHRE guidelines for RPL do not recommend the use of LIT in affected patients. Meanwhile, some studies have found LIT to be beneficial for RIF (210, 211), but RCTs analyzing the efficiency of LIT in treating this condition are still lacking. In general, there is insufficient evidence to recommend LIT in patients with RIF, and we should be aware of the possible complications such as infections, autoimmune disorders and formation of irregular antibodies.

2.3.4 Anticoagulants

2.3.4.1 Aspirin

Aspirin is classified as a non-steroidal anti-inflammatory drug. It can inhibit the activity of cyclooxygenase and is, therefore, used as an antithrombotic agent. In terms of reproduction, aspirin contributed to reduce the inflammation in uterus and improve uterine perfusion, which may improve endometrial receptivity (212, 213). Although aspirin can decrease endometrial and uterine arterial blood flow resistance in patients with unexplained RIF (214), no significant differences were found between the aspirin treatment group and control group with respect to implantation and pregnancy rates (215–218).

2.3.4.2 Low molecular weight heparin

LMWH has an activity similar to that of heparin, with an increased half-life and depolymerization. LMWH possess antithrombin or anticoagulation activities. It is speculated that LMWH might prevent placental thrombosis and infarction and modulate decidualization of the endometrium (219, 220). A prospective randomized trial in patients with previous IVF failure and thrombophilia showed a significant increase in implantation and pregnancy rates (221). In RIF patients, LMWH significantly improved live birth rates and reduced miscarriage rates, even though implantation rates were not significantly improved (222). In patients with two or more unexplained failed fresh embryo transfers, LMWH administration from the day after oocyte retrieval led to a tendency of a higher live birth rate with no significant difference, and the implantation rate was also not different (223). Therefore, LMWH may be a potential intervention for patients with RIF, at a dosage of 40mg/day from the day of oocyte retrieval or embryo transfer to 8-12 weeks of gestation.

2.3.5 Endometrial receptivity improvement

2.3.5.1 Intrauterine infusion

2.3.5.1.1 Human chorionic gonadotropin

Generally, hCG can bind to the LH receptor in the endometrium, induce the secretion of cytokines during implantation window and regulate endometrial receptivity and embryo implantation. It is usually administered 0.25–72 hours before embryo transfer at a dosage ranging from 500 to 1000 IU. Administration of hCG appears to regulate embryo implantation among patients with RIF. It can increase the invasion potential of trophoblast cells by modulating the secretion of matrix metalloprotein-2 and tissue inhibitor of metallopeptidase-1 (224). In a previous study, intrauterine injection of hCG before embryo transfer increased the live birth, clinical, and implantation rates of IVF-ET. The effect of 500 IU hCG was better than that of other dosages. However, the outcomes between the first IVF-ET cycle and RIF subgroups did not significantly differ (225). Another study showed that intrauterine injection of hCG before FET improved pregnancy rates in patients with two more implantation failures. Generally, infertile patients may benefit from intrauterine injection of hCG, but further RCTs are needed to confirm these findings.

2.3.5.1.2 Peripheral blood mononuclear cells

PBMCs, such as monocytes and T and B lymphocytes, can induce the secretion of interleukins and growth factors, which appear to be beneficial to the endometrial thickness and receptivity (226). In addition, intrauterine PBMCs can promote embryo attachment and invasion by creating a pathway while moving towards the endometrial stroma (227). The immune cells recruited to the implantation site may not induce initial inflammation for successful implantation in RIF patients, which can be improved by PBMCs (228–230). A recent meta-analysis showed that implantation and live birth rates of patients with RIF were significantly increased in the PBMCs group (227). Another clinical trial confirmed the effect of PBMCs on patients with RIF. Intrauterine infusion of PBMCs before embryo transfer significantly increased implantation rates in frozen-thawed cycles (230). Thus, PBMCs are considered an effective treatment for patients with RIF that lacks initial

inflammation (Table 1). Blood samples were typically obtained from patients 3 to 5 days before the scheduled embryo transfer, and PBMCs were isolated and cultured with or without hCG, followed by intrauterine infusion. However, larger study populations and more information on the effectiveness and safety of blood products are still needed.

2.3.5.1.3 Platelet-rich plasma

PRP is an autologous blood product containing a high concentration of platelets. The release of platelet-derived growth factors, vascular endothelial growth factors, transforming growth factors, and epidermal growth factors from activated platelets results in angiogenesis, cell

TABLE 1 Researches of treatment options for improving endometrial receptivity of patients with recurrent implantation failure.

Treatment	Year of publication	Number of patients	Age of patients	Number of implantation failure patient experienced	Type of IVF-ET cycle	Approach	Outcomes
Intrauterine infusion of PBMCs	2016 (226)	198	Less than 35 years old	Three or more IVF-ET failure	Fresh ET cycle	PBMCs cultured with hCG for 24 h; intrauterine infusion	IR, CPR and LBR improved
	2006 (229)	35	Unknown	Four or more IVF-ET failure	Fresh ET cycle	PBMCs cultured with hCG for 48 h; intrauterine infusion 3 days before fresh ET	IR, CPR, LBR improved
	2017 (230)	216	Unknown	Three or more IVF-ET failure	Frozen/thawed ET cycle	PBMCs cultured with hCG for 24 h; intrauterine infusion day before frozen/thawed ET	IR, CPR, LBR improved in four or more IVF-ET failure group
Intrauterine infusion of PRP	2022 (231)	85	Age 24 to 52 years old	Unknown	Frozen/thawed ET cycle	Administration of the PRP in day 10-15 of frozen/thawed ET cycles	BPR, CPR and LBR improved, SAR decreased, endometrial thickness increased
	2021 (232)	98	Age 20 to 40 years old	Three or more high-quality frozen-thawed embryo transfers failure	Frozen/thawed ET cycle	Intrauterine infusion of PRP 2 days before frozen/thawed ET	IR, CPR and OPR improved
	2022 (233)	288	Aged 23 to 40 years old	Three or more consecutive implantation failure of at least 6 cleavage-stage embryos or three blastocysts	Frozen/thawed ET cycle	Intrauterine infusion of PRP 2 days before ET	BPR, IR, CPR, LBR improved
Subcutaneous administered G-CSF	2016 (234)	112	Less than 40 years old	Three or more consecutive implantation failure with three high-grade quality embryos per cycle	Fresh ET cycle	300μg of subcutaneous G-CSF administered 1 h before fresh ET	BPR, IR and CPR improved
	2011 (235)	89	Less than 39 years old	Three previous IVF-ET failure with at least 7 good embryos	Fresh ET cycle	1.5 mg/kg/daily of subcutaneous G-CSF from the day of fresh ET to day of pregnancy test; if positive, continued for 40 days	CPR improved
Endometrial scratch	2022 (236)	933	Age 18 to 44 years old	One previous IVF-ET failure	Fresh ET cycle	Endometrial scratch at 5–10 days before the expected menstrual	LBR improved
	2012 (237)	200	Less than 39 years old	Two or more previous ICSI cycle failure	Fresh ET cycle	Endometrial scratch at Day 4-7 in the menstrual cycle before ET cycle	IR, CPR and LBR improved

PBMCs, peripheral blood mononuclear cells; PRP, platelet-rich plasma; G-CSF, granulocyte-colony stimulating factor; IVF-ET, in vitro fertilization-embryo transfer; hCG, human chorionic gonadotropin; IR, implantation rate; CPR, clinical pregnancy rate; LBR, live birth rate; OPR, ongoing pregnancy rate; BPR, biochemical pregnancy rate; SAR, rate of spontaneous abortion; ICSI, intracytoplasmic sperm injection.

proliferation, differentiation, and modification of the local immune response (238–241). It can also promote the expression of tissue remodeling genes and reduce fibrosis in mice with Asherman's syndrome (242). It has been reported that PRP can improve clinical pregnancy rates and endometrium thickness in patients with RIF (231). The benefit of PRP on implantation rates in patients with RIF has been confirmed by other studies (Table 1) (232, 233, 243). Thus, PRP is a promising treatment option. In addition, more high-quality and large-scale studies are needed to further assess the effects and safety of PRP.

2.3.5.1.4 Granulocyte colony-stimulating factor

G-CSF is a cytokine produced by endothelial cells, stromal cells, macrophages, and other immune cells (244). It is also produced by decidual cells, which prompted its use as an adjunct treatment (locally or systemically) for patients with a history of RIF or RPL and thin endometrium (85, 245, 246). A meta-analysis showed an increase in clinical rates of intrauterine infusion or subcutaneous injection of G-CSF during both fresh and frozen embryo transfer (247). However, the method of administration and dosage of G-CSF should be carefully selected. G-CSF is typically administered at a dosage ranging from 60 to 300 mg on the day of hCG trigger or embryo transfer. Implantation and pregnancy rates in patients with RIF were not improved by G-CSF intrauterine infusion in two RCTs (248, 249). Furthermore, subcutaneous administration of G-CSF 1 h before fresh embryo transfer resulted in an improvement in clinical pregnancy and implantation rates compared to the control group, which is consistent with the results of other studies (Table 1) (234, 235).

2.3.5.2 Endometrial scratch (Biopsy)

Endometrial scratch before implantation appears to cause decidualization and prepares the endometrium for implantation by increasing cytokines such as LIF and IL-11, which are involved in endometrial receptivity (250), and delaying endometrium maturation caused by COH, which might cause synchronization between the endometrium and embryo (251). Moderate-quality evidence has been demonstrated in previous studies. Endometrial scratch on day 7 of the previous cycle and day 7 of the ET cycle appeared to improve the live birth and pregnancy rates in patients with two previous ET cycles, with no evidence of increasing miscarriage rates or bleeding (251). In patients with one previous IVF cycle failure, higher live birth rates were obtained in the endometrial scratch group, with slightly higher expenditures (236). Endometrial scratches performed during hysteroscopy in the cycle preceding ICSI also improved implantation rates in patients with two or more ICSI cycle failures (237). Therefore, this widely used treatment is safe for improving the IVF-ET outcomes. However, the number and degree of injury and the procedure timing need further investigation.

2.3.5.3 Endometrial receptivity assay

An ERA is a transcriptomic analysis of gene expression at different stages of the endometrium that detects WOI and can facilitate "personalized" embryo transfer for every patient. Patients with RIF appeared to have a lower receptivity proportion compared to the control group in the ERA test (74.1% vs. 88%). In RIF patients with a "receptive endometrium" diagnosed by ERA, embryo transfer conducted at the receptivity time led to similar clinical pregnancy rates as in general patients undergoing IVF (87). A 5-year multicenter RCT demonstrated that personalized embryo transfer after ERA diagnosis reached higher implantation and live birth rates at the first embryo transfer cycle in infertile patients (252). Thus, ERA is a unique procedure for endometrial evaluation that can improve endometrium-related implantation failure.

2.3.6 Antibiotics

Antibiotics can cure infections in most patients with chronic endometritis (253). Chronic endometritis is common in patients with RIF, which can be diagnosed and evaluated by hysteroscopy, and the most frequent infectious agents are bacteria and mycoplasmas (109). Patients with RIF and chronic endometritis received oral antibiotic treatment, and the effect was assessed by hysteroscopy with biopsy. In the cure group, a significant increase in pregnancy and live birth rates was reported compared to the group with continuous chronic endometritis after antibiotic treatment (109). A recent meta-analysis also showed that the implantation and clinical pregnancy rates of patients with RIF with cured chronic endometritis were significantly higher than those of patients with continuous chronic endometritis (254). However, different administration routes have led to different results. Intrauterine antibiotic infusion combined with oral antibiotic administration could not improve clinical pregnancy rates, which may be due to the disturbance of intrauterine infusion in the intrauterine environment (255). In general, chronic endometritis is curable in most patients with RIF, which results in a significant increase in the pregnancy outcomes of IVF-ET performed after treatment.

2.3.7 Hysteroscopy

A few patients with normal hysterosalpingogram results show abnormal hysteroscopy findings (127). Hysteroscopy is a valuable diagnostic and treatment tool that can remove small uterine lesions and restore the shape of the uterine cavity in patients with uterine lesions. Correction of the T-shaped uterus was related to high live birth rates and low miscarriage rates in patients with both primary infertility and recurrent miscarriage (256, 257). Although outpatient hysteroscopy did not improve IVF outcomes in patients with RIF with normal ultrasound of the uterine cavity, which may be due to the high proportion of normal uterine cavity (258). For uterine lesions that affect

implantation rates, it is necessary to remove them before the next IVF-ET cycle (122, 126, 259, 260).

2.3.8 Male factor

Normal sperm has smooth nuclei with normal chromatin content and head shape. Moreover, severe abnormalities in sperm are related to low fertilization, implantation, and pregnancy rates (261). Intracytoplasmic morphologically selected sperm injection (IMSI) is a non-invasive method that examines sperm under 6000× magnification before injection to obtain optimal sperm. The IMSI procedure before ICSI appears to be beneficial for implantation and clinical rates in patients with repeated IVF-ICSI failure (262). However, other studies did not draw the same conclusions (263). Thus, no specific microscopic criterion exists for evaluating sperm morphology, and more studies are needed to assess the effect of IMSI on IVF-ET outcomes.

2.3.9 Preimplantation genetic testing for aneuploidies

Aneuploidy accounts for implantation failure and early pregnancy loss, as high as 76% in first-trimester spontaneous abortions (155). PGT-A is a technology that can analyze the chromosomes of embryos in IVF-ET and select euploid embryos for subsequent transfer. Single euploid embryos selected by array comparative genomic hybridization were transferred to patients with RIF, which resulted in implantation rates similar to those in the group without RIF (264). Another retrospective cohort study showed the benefit of using PGT-A in patients with RIF and recurrent miscarriage, leading to a significant increase in implantation rates (265). The cumulative implantation rate of patients with RIF who underwent euploid embryo transfer was 95.2%, which means that most RIFs are due to chromosome aneuploidy and can be improved by transferring euploid embryos (266). Therefore, PGT-A appears to be a considerable treatment option for patients with RIF (12). Furthermore, PGT-A should be administered after a careful assessment of the circumstances of each patient. And the influence of mosaicism must be considered.

3 Conclusions and future perspectives

RIF remains a complex, growing problem that affects several patients. There are various etiologies, mechanisms, and treatment options (Table 2). Identifying the causes of RIF and providing individualized treatment can improve the implantation rate. However, the treatment of RIF remains challenging, and further research on treatment options is

TABLE 2 Summary of etiologies and treatment options of recurrent implantation failure.

		Treatment	Reference
Risk factors	Body mass index	Low-energy diets, Pharmacotherapy, and Bariatric surgery	Cheah et al. (2022), Legro et al. (2016)
	Smoking	Stop smoking and avoid secondhand smoke	Fullston et al. (2017), Budani et al. (2017)
	Alcohol	Reduce alcohol intake to one or two units a week or abstinence from alcohol	National Collaborating Centre for Women's and Children's Health (UK) (2013)
	Stress	Healthy diet, Regular exercise, Psychological interventions	Frederiksen et al. (2015)
Maternal factors	Immunology	Glucocorticoids, Intravenous immunoglobulin, Tacrolimus, Cyclosporine, Intralipids	Forges et al. (2006), Alhalabi et al. (2011), Ahmadi et al. (2017), Abdolmohammadi-Vahid et al. (2019), Nakagawa *er al.* (2015), Cheng et al. (2022), Ledee et al. (2018)
	Thrombophilias	Aspirin, Low molecular weight heparin	Zhang et al. (2022), Potdar et al. (2013),
	Endometrial receptivity	Frozen-thawed embryo transfer, Peripheral blood mononuclear cells, Platelet-rich plasma, Granulocyte colony-stimulating factor, Endometrial scratch, Endometrial receptivity assay	Shapiro *er al.* (2011), Pourmoghadam Z *er al.* (2020), Li *er al.* (2017), Russel et al. (2022), Zamaniyan et al. (2021), Aleyasin et al. (2016), Hou et al. (2021), Shohayeb et al. (2012), Simon et al. (2020)
	Microbiome	Antibiotics	Cicinelli et al. (2015), Vitagliano et al. (2017)
	Anatomical abnormalities	Hysteroscopy and surgery	Garzon et al. (2020), Pritts et al. (2009), Bosteels et al. (2010)
Male factors		Intracytoplasmic morphologically selected sperm injection	Shalom-Paz, et al. (2015), Teixeira et al. (2020)
Embryo factor		Optimize ovarian stimulation protocol, Assisted hatching, Preimplantation genetic testing for aneuploidies	Stein et al. (1995), Practice Committee of the American Society forReproductive Medicine (2022), Greco et al. (2014), Pirtea et al. (2021)

needed to assess the potential of each treatment and establish a standard protocol for each patient.

Author contributions

JM, DL, and WG performed the literature search and data extraction and played a major role in writing the manuscript. DL critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Association between transferred embryos and multiple pregnancy/live birth rate in frozen embryo transfer cycles: A retrospective study

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Background: Physicians need an appropriate embryo transfer strategy to address the challenge of reducing multiple birth rates, while maintaining the couples' live birth rate during assisted reproductive technology.

Methods: We included 10,060 frozen embryo transfer cycles from January 2015 to March 2020 in reproductive medical center of Ruijin hospital, Shanghai, China. Patients were grouped according to the number and grade of cleavage-stage embryo or blastocysts transferred. Live birth rate and multiple live birth rate were compared among groups of women of different ages. Multivariable logistic regression models were used to estimate the risk of multiple live birth using different combinations of transferred embryos.

Results: The transfer of double good-quality embryos was an independent predictor for multiple birth in women aged <30 years and those aged 36–39 years [<30 years: aOR =1.54 (95% CI: 1.14–2.06, P < 0.01); 36–39 years: aOR =1.84 (95% CI: 1.0–3.4, P < 0.01)]. Further, for women aged <36 years, the transfer of good-quality + poor-quality blastocysts was an independent predictor for multiple birth rate [<30 years: aOR=2.46 (95% CI: 1.45–4.18, P < 0.01); 31–35 years: aOR =4.45 (95% CI: 1.97–10.06, P < 0.01)].

Conclusions: Single-good-quality blastocyst transfer is recommended for women of all ages. When good-quality cleavage embryos are available, the choice of single or double embryo transfer with good- or average-quality embryo should depend on the age of women. Double embryo transfer with the highest possible grade of embryos is recommended for women aged ≥40 years.

KEYWORDS

assisted reproductive technology, blastocyst, embryo transfer, live birth rate, pregnancy outcomes, retrospective cohort study

Introduction

With improvements in assisted reproductive technology (ART), the strategy of single embryo transfer (SET) has been promoted in many countries, since the goal of ART is to produce a healthy baby. According to a study, SET can greatly decrease multiple pregnancy (MP) rates from 26–29% to 2%, and results in lower risks of miscarriage, preterm delivery, morbidity, and mortality of mothers and children (1). Many studies have shown that the risk of live birth is lower in the SET cycle than in the DET cycle (2–4). According to systematic reviews which included five randomized trials on pregnancy outcomes of SET and DET in fresh IVF cycle, the live birth rate (LBR) per cycle with DET was significantly higher than that with SET (OR: 2.10, 95% CI: 1.65 to 2.66), with MP rate being significantly lower in women who had SET compared with DET (OR: 0.04, 95% CI: 0.01- 0.11) (5). However, there are also data suggesting that a more liberal use of SET does not lead to a fall in LBR per cycle (6, 7). It was suggested that SET strategy has minimum impact on overall outcomes when it is applied in women with a better prognosis (8, 9), such as those younger than 35 years old, or having good quality blastocyst to be transferred. Thus, embryo transfer strategy should be personalized which was associated not only with LBR, but also with cumulative live birth rate (CLBR).

The comparison of LBR in SET and DET in frozen embryo transfer (FET) cycles has been reported by others (7). In this study, we conducted a larger retrospective analysis to investigate both LBR and multiple birth rate (MBR) under the combination of several parameters (DET/SET + embryo quality at different stages + women's age), providing more accurate and valuable information to develop better cost-effective embryo transfer strategies.

Materials and methods

Ethics statement

The study protocol was approved by the Ethics Committee (Institutional Review Board) of Shanghai Ruijin Hospital.

Participants

In this retrospective cohort study, we included 10,600 FET cycles (7631patients) performed between January 2015 and March 2020 at the reproductive medical center of Ruijin Hospital affiliated with Shanghai Jiao Tong University School of Medicine. Details of ovulation induction, embryo culture and evaluation, and embryo freeze-thaw have been described in our previous articles (10, 11).

Endometrial preparation

Artificial hormone replacement therapy (HRT) cycles were used to prepare the endometrium. Briefly, estradiol valerate at a dose of 4–6 mg/day was started from day 2 of the menstrual cycle, and endometrial thickness was examined by ultrasound scan on days 12–14. If the thickness was ≥7 mm, progesterone gel (90 mg/day; Crinone, MERCK) was administered. Cleavage embryos were transferred on day 4, and blastocyst transfer was performed on the sixth day of progesterone supplementation. The administration of estradiol valerate and progesterone was continued until 10 weeks of gestation if pregnant.

Patient groups

The patients were grouped according to the number and grade of embryos transferred, as detailed in Table 1. Embryo grading was performed just before embryo transfer using an Olympus microscope with 200× magnification. The score of cleavage embryos was based on the following three criteria (12): (A) Blastomere number (BL): 4 BL=1, 5 BL=2, 6–7 BL=3, and 8–10 BL=4; (B) Fragmentation (FR): <5%=4, 5–10%=3, 11–25%=2, 26–50%=1, and > 50%=0; and (C) symmetry (SY): perfect symmetry=score 1, and asymmetry=score 0. The score for an embryo was the sum of BL, FR, and SY. The group criteria were as follows: poor-quality embryo (PQE)= score 5; average-quality embryo (AQE)= score 6–7; and good-quality embryo (GQE)= score ≥8.

TABLE 1 Patients groups based on the number and grade of embryos transferred.

Cleavage-stage	DET (double embryo transfer)			SET (single embryo transfer)
	GQE	AQE	PQE	/
GQE	B1	/	/	A1
AQE	B2	C1	/	A2
PQE	B3	C2	C3	A3
Blastocyst-Stage	**DET (double embryo transfer)**			**SET (single embryo transfer)**
	GQB	PQB		/
GQB	E3	/		E1
FQB	E5	E4		E2

GQE, good-quality embryo; AQE, average-quality embryo; PQE, poor-quality embryo; GQB, good-quality blastocyst; FQB, fair-quality blastocyst.

The evaluation of blastocysts was based on the classification of Gardner et al. (13): AA, AB, BA, and BB were defined as good-quality blastocyst (GQB), and BC and CB were defined as fair-quality blastocyst (FQB). Two embryologists double-checked all embryo evaluations.

The number of embryos transferred was determined by the physician's approach, the patient's desire, and other clinical factors such as clinical history and previous embryo transfer cycle outcomes.

Outcomes

Live birth was defined as live birth of at least one baby at ≥28 weeks of gestation. LBR was calculated as the number of cycles with live birth/number of embryo transfer cycles. MBR was defined as the number of cycles with multiple births/number of clinical pregnancy cycles. Clinical pregnancy was defined as the presence of an intrauterine gestational sac and active fetal heartbeat.

Statistical analysis

Baseline characteristics are presented as mean (standard deviation [SD]) for continuous variables and as percentage for categorical variables; these characteristics were compared between the groups using the chi-square test or analysis of variance (ANOVA). Bonferroni multiple comparison test was conducted to compare the groups between which statistically significant differences existed. Multivariate logistic regression analysis was performed to investigate the risk of different embryo combinations in multiple births. The results were reported as adjusted odds ratios (aORs) with 95% confidence intervals (CIs). All statistical analyses were performed using the two-sided 5% level of significance and the statistical package Stata version 12 (StataCorp, College Station, TX, United States).

Results

We retrospectively collected the data on 10,060 FET cycles including 6,408 cycles of cleavage-stage embryo transfer and 3,652 cycles of blastocysts transfer between 2015 and 2020. The baseline characteristics of patients in all cohorts are summarized in Table 2. The most common indication for *in vitro* fertilization (IVF) was tubal factor (44.24%). The percentage of primary infertility was 54.9%, and the ovarian reserve indicator [anti-Müllerian hormone (AMH)] level ranged from 0.32 to 12.75 ng/ml which declined as age increased.

Age distributions of FET cycles

The group-wise distribution based on women's age is presented in Table 3. For women aged ≥40 years, the proportion of single-cleavage embryo transfer cycles (A1, A2, and A3 groups) was 21.2%, while for those aged <30 years, the proportion was only 4.5%. Additionally, for all age groups, double AQE transfer cycles (C1 group) accounted for the highest proportion, followed by single poor-quality blastocyst (PQB) transfer cycles (E2 group).

LBR and MBR based on different embryo transfer groups

Comparison of LBRs and MBRs among the cycles is presented in Table 4. Significant differences were observed among LBRs or MBRs in all cleavage embryos and single blastocyst transfer cycles. However, no significant difference was observed in the double blastocyst transfer cycle.

LBRs and MBRs based on different embryo transfer groups and ages

LBRs and MBRs based on embryo transfer groups and age are shown in Tables 5, 6. Overall, for each embryo combination, LBRs and MBRs decreased with increasing maternal age. The highest LBR and MBR were 64.7% and 54.8%, respectively, both of which were noted in women aged <30 years receiving DET-GQB+PQB transfer. For those aged ≥40 years, the highest LBR was 35.3% in the SET-GQB group and 25% in the DET-GQE group.

Multiple comparisons of LBRs and MBRs based on embryo transfer groups and ages

Multiple comparisons with Bonferroni correction were performed, and groups involving a very small number of cases to be analyzed (B3, C3, and E3) were excluded. As shown in Tables 7, 8, for women <30 years, the double AQE and AQE+PQE groups showed similar MBRs, whereas the double AQE group had a significantly higher LBR. In women aged 31–35 years, the double GQE and GQE+AQE groups had similar LBRs, with a higher MBR noted in the double GQE group. In women aged 36–-39 years, the double GQE group had a similar LBR but a higher MBR compared with the double AQE or AQE+PQE groups. In cycles of blastocyst transfer, GQB+PQB transfer (E5) had a similar LBR as SET-GQB transfer (E1), regardless of age. When we compared the SET-PQB (E2) and double PQB transfer (E4) groups, the DET group had a higher LBR in women aged <35 years but not in those aged >36 years. In women aged >40 years, no significant difference was noted in either MBRs or LBRs among either of the two groups, regardless of cleavage embryo or blastocyst transfer.

Risk of multiple birth based on age and embryo transferred

Figure 1 presents the adjusted ORs for associations between the combinations of transferred embryos and

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TABLE 2 Baseline characteristics of study cycles.

	<30 Years (N=3775)	31-35 Years (N=3744)	36-39 Years (N=1575)	>=40 Years (N=966)	P
BMI (kg/m2)					<.0001
<18.5	239 (6.3)	211 (5.6)	76 (4.8)	31 (3.2)	
18.5-23.9	2988(79.2)	2983 (79.7)	1290 (81.9)	803 (83.2)	
24-27	359 (9.5)	303 (8.1)	145 (9.2)	92 (9.5)	
>=27	189 (5.0)	247 (6.6)	64 (4.1)	40 (4.1)	
Type of infertility (%)					<.0001
Primary	2540 (67.3)	2104 (56.2)	641 (40.7)	237 (24.5)	
Secondary	1235 (32.7)	1640 (43.8)	934 (59.3)	729 (75.5)	
AMH	6.0±4.10	5.1±3.85	3.6±3.05	2.2±2.16	<.0001
Indication n (%)					<.0001
Tubal factor	1666 (44.1)	1670 (44.6)	736 (46.7)	376 (38.9)	
Endometriosis	226 (6.0)	202 (5.4)	108 (6.9)	61 (6.3)	
Ovulation disorders	109(2.9)	92 (2.5)	58 (3.7)	32 (3.3)	
Male factor	597 (15.8)	548 (14.6)	196 (12.4)	118 (12.2)	
Unexplained	223 (5.9)	199 (5.3)	100 (6.3)	66 (6.8)	
combination	954 (25.3)	1033 (27.6)	377 (23.9)	313 (32.4)	

Data are expressed as mean ± SD or as percentage of women (percentage) for categorical variables. †One-way ANOVA for continuous variables and chi-squared test for categorical variables.
BMI, body mass index; AMH, anti-Müllerian hormone.

TABLE 3 Distribution of groups based on the number and quality of embryos transferred in women of different ages.

	<30 Years (N=3,775)	31–35 Years (N=3,744)	36–39 Years (N=1,575)	>=40 Years (N=966)	P	Heat Map
	n (%)	n (%)	n (%)	n (%)		
A1	46 (1.2)	74 (2.0)	53 (3.4)	44 (4.6)	<0.001	
A2	103 (2.7)	164 (4.4)	105 (6.7)	142 (14.7)	<0.001	
A3	22 (0.6)	32 (0.9)	30 (1.9)	18 (1.9)	<0.001	
B1	562 (14.9)	433 (11.6)	148 (9.4)	72 (7.5)	<0.001	
B2	457 (12.1)	430 (11.5)	162 (10.3)	120 (12.4)	0.2407	
B3	12 (0.3)	20 (0.5)	9 (0.6)	7 (0.7)	0.2829	
C1	983 (26.0)	918 (24.5)	388 (24.6)	259 (26.8)	0.2798	
C2	155 (4.1)	171 (4.6)	93 (5.9)	57 (5.9)	0.0102	
C3	36 (1.0)	43 (1.1)	22 (1.4)	18 (1.9)	0.1033	
E1	311 (8.2)	336 (9.0)	119 (7.6)	34 (3.5)	<0.001	
E2	557 (14.8)	687 (18.3)	292 (18.5)	152 (15.7)	<0.001	
E3	42 (1.1)	26 (0.7)	6 (0.4)	1 (0.1)	0.0016	
E4	404 (10.7)	318 (8.5)	112 (7.1)	33 (3.4)	<0.001	
E5	85 (2.3)	92 (2.5)	36 (2.3)	9 (0.9)	0.0376	

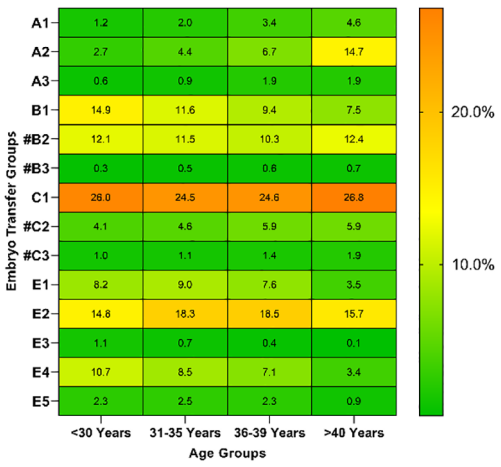

TABLE 4 Live birth rate and multiple birth rate of different embryo transfer groups.

	Live birth rate n/N(%)	P	Multiple birth rate n/N(%)	P
A1	49/217 (22.6)	X^2= 9.9179 P=0.0070	–	–
A2	83/514 (16.1)		–	
A3	9 /102 (8.8)		–	
B1	547/1215 (45.0)	X^2= 105.0364 P<0.001	261 /643 (40.6)	X^2= 35.7663 P <0.001
B2	438/1169 (37.5)		177 /527 (33.6)	
B3	14 /48 (29.2)		6 /19 (31.6)	
C1	860/2548 (33.8)		311/1038 (30.0)	
C2	104 /476 (21.8)		30 /136 (22.1)	
C3	24/119 (20.2)		3/30 (10.0)	
E1	389 /800 (48.6)	X^2= 38.0476 P<0.001	–	
E2	602/1688 (35.7)		–	
E3	39/75 (52.0)	X^2= 3.6147 P< 0.1641	19 /45 (42.2)	X^2= 1.0215 P=0.6001
E4	446 /867 (51.4)		246/543 (45.3)	
E5	130 /222 (58.6)		73 /148 (49.3)	

multiple births in women of different ages. Multivariate logistic analysis revealed that the transfer of double GQE was an independent predictor for multiple birth (MB) in women aged <30 years and those aged 36–39 years [<30 years: aOR=1.54 (95% CI: 1.14-2.06, P < 0.01); 36–39 years: aOR=1.84 (95% CI: 1.0–3.4, P < 0.01)]. DET-PQB was positively associated with MB in women aged <40 years, and this effect remained statistically significant in the multivariate analysis (P < 0.01). In addition, for women aged <36 years, the transfer of GQB+PQB was an independent predictor for MB (<30 years: aOR=2.46 (95% CI: 1.45–4.18, P < 0.01); 31–35 years: aOR=4.45 (95% CI: 1.97–10.06, P < 0.01)].

TABLE 5 Live birth rate of different embryo transfer groups based on women's age.

	<30 Years n/N(%)	31–35 Years n/N(%)	36–39 Years n/N(%)	>=40 Years n/N(%)	Heatmap
A1	15/46 (32.6)	23/74 (31.1)	10/53 (18.9)	1/44 (2.3)	
A2	23/103 (22.3)	35/164 (21.3)	15/105 (14.3)	10/142 (7.0)	
A3	5/22 (22.7)	3/32 (9.4)	1/30 (3.3)	0/18 (0.0)	
B1	282/562 (50.2)	194/433 (44.8)	59/148 (39.9)	12/72 (16.7)	
B2	194/457 (42.5)	182/430 (42.3)	49/162 (30.2)	13/120 (10.8)	
B3	5/12 (41.7)	8/20 (40.0)	1/9 (11.1)	0/7 (0.0)	
C1	400/983 (40.7)	304/918 (33.1)	125/388 (32.2)	31/259 (12.0)	
C2	42/155 (27.1)	37/171 (21.6)	23/93 (24.7)	2/57 (3.5)	
C3	10/36 (27.8)	9/43 (20.9)	3/22 (13.6)	2/18 (11.1)	
E1	157/311 (50.5)	171/336 (50.9)	49/119 (41.2)	12/34 (35.3)	
E2	231/557 (41.5)	260/687 (37.8)	86/292 (29.5)	25/152 (16.4)	
E3	23/42 (54.8)	16/26 (61.5)	0/6 (0.0)	0/1 (0.0)	
E4	213/404 (52.7)	173/318 (54.4)	49/112 (43.8)	11/33 (33.3)	
E5	55/85 (64.7)	52/92 (56.5)	20/36 (55.6)	3/9 (33.3)	

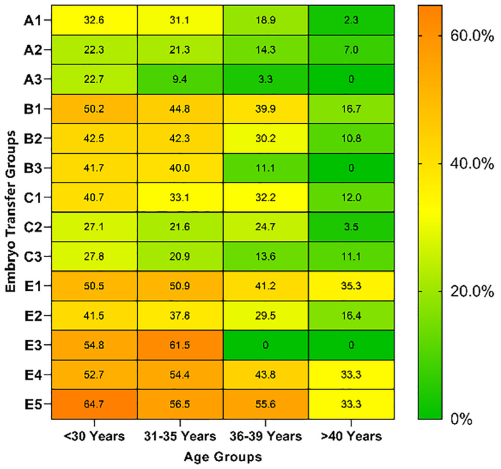

TABLE 6 Multiple birth rates of embryo transfer groups based on maternal age.

	<30 Years n/ N(%)	31–35 Years n/ N(%)	36–39 Years n/ N(%)	>=40 Years n/ N(%)	Heatmap
B1	142/239 (43.2)	89/223 (39.9)	25/71 (35.2)	5/20 (25.0)	
B2	91/230 (39.6)	65/212 (30.7)	17/60 (28.3)	4/25 (16.0)	
B3	2/5 (40.0)	3/11 (27.3)	1 /3 (33.3)	0/0 (0.0)	
C1	151/454 (33.3)	116/372 (31.2)	36/158 (22.8)	8/54 (14.8)	
C2	12/50 (24.0)	13/51 (25.5)	4/30 (13.3)	1/5 (20.0)	
C3	1/10 (10.0)	1/14 (7.1)	1/3 (33.3)	0/3 (0.0)	
E3	12/25 (48.0)	7/19 (36.8)	0/1 (0.0)	0/0 (0.0)	
E4	124/258 (48.1)	93/204 (45.6)	26/63 (41.3)	3 /18 (16.7)	
E5	35/64 (54.7)	29/58 (50.0)	9/23 (39.1)	0/3 (0.0)	

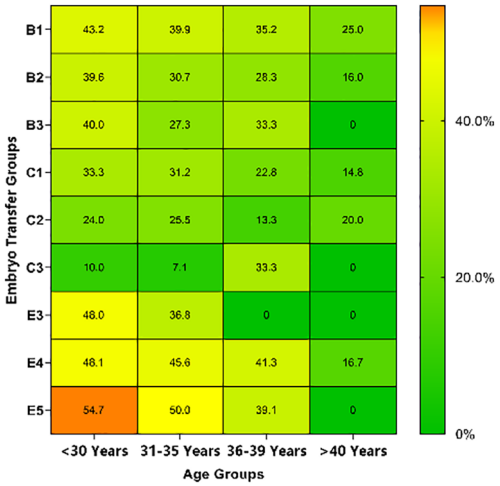

TABLE 7 Multiple comparisons of live birth rate in embryo transfer groups with p-value adjustments by Bonferroni correction.

Maternal age (years)	Cleavage embryo transfer (B1, B2, C1, and C2)				Blastocyst transfer (E1, E2, E4, and E5)			
<30 Years	**B1**	**B2**	**C1**	**C2**	**<30 Years**	**E2**	**E4**	**E5**
A1	0.214	1.000			**E1**	0.298	1.000	0.544
A2			0.001	1.000	**E2**		**0.016**	0.002
B1		0.140	0.003	<0.001	**E4**			1.000
B2			1.000	0.005				
C1				0.009				
31–-35 Years	**B1**	**B2**	**C1**	**C2**	**31–35 Years**	**E2**	**E4**	**E5**
A1	0.262	0.681			**E1**	**0.002**	1.000	1.000
A2			0.020	1.000	**E2**		**<0.001**	0.020
B1		1.000	0.000	<0.001	**E4**			1.000
B2			0.011	<0.001				
C1				0.022				
36–39 Years	**B1**	**B2**	**C1**	**C2**	**36–39 Years**	**E2**	**E4**	**E5**
A1	0.043	1.000			**E1**	0.656	1.000	1.000
A2			0.001	0.646	**E2**		0.204	0.071
B1		0.770	0.974	0.151	**E4**			1.000
B2			1.000	1.000				
C1				1.000				
>=40 Years	**B1**	**B2**	**C1**	**C2**	**>=40 Years**	**E2**	**E4**	**E5**
A1	0.099	0.690			**E1**	0.550	1.000	1.000
A2			1.000	1.000	**E2**		1.000	1.000

(Continued)

TABLE 7 Continued

Maternal age (years)	Cleavage embryo transfer (B1, B2, C1, and C2)				Blastocyst transfer (E1, E2, E4, and E5)			
B1		1.000	1.000	0.134	**E4**			1.000
B2			1.000	0.985				
C1				0.431				

Discussion

Principal findings

Our results analyzed the LBRs and MBRs in a variety of transferred embryo combinations in women of different ages. In women ≤ 35 years old, transfer of single good quality embryo, either cleavage or blastocyst, could result in an LBR comparable to double embryo transfer. In those older than 40 years, double embryo transfer could shorten the time interval to achieve live birth with relatively low MBR, except for good quality blastocyst available. However, for women between 36-40 years, the selection of transferred embryo should be dependent on the quality of all available embryos.

Results in the context of what is known

In recent years, the goal of ART has been to achieve a healthy singleton gestation. The American Society for Reproductive Medicine guidelines recommends that patients aged ≤37 years should be encouraged to undergo SET regardless of the embryo stage (14). In 2018, an expert consensus formulated by the Committee of Chinese Society of Reproductive Medicine suggested that SET in the first embryo transfer cycle and ≤2 embryos should be transferred in each cycle, regardless of age (15). Despite this, the SET strategy is not widely accepted in China, and clinicians face the challenge of reducing MBR without impairing LBR in couples receiving ART treatments.

TABLE 8 Multiple comparison of multiple birth rate for embryo transfer groups based on maternal age.

Maternal age (years)	Embryo transfer groups			
<30 Years	**B2**	**C1**	**C2**	**E5**
B1	0.396	0.005	0.010	
B2		0.103	0.039	
C1			0.184	
E4				0.343
31–35 Years	**B2**	**C1**	**C2**	
B1	0.044	0.030	0.055	
B2		0.896	0.468	
C1			0.408	
E4				0.552
36–39 Years	**B2**	**C1**	**C2**	
B1	0.401	0.049	0.026	
B2		0.394	0.113	
C1			0.246	
E4				0.858
>=40 Years	**B2**	**C1**	**C2**	
B1	0.453	0.307	0.815	
B2		0.891	0.827	
C1			0.758	
E4				0.552

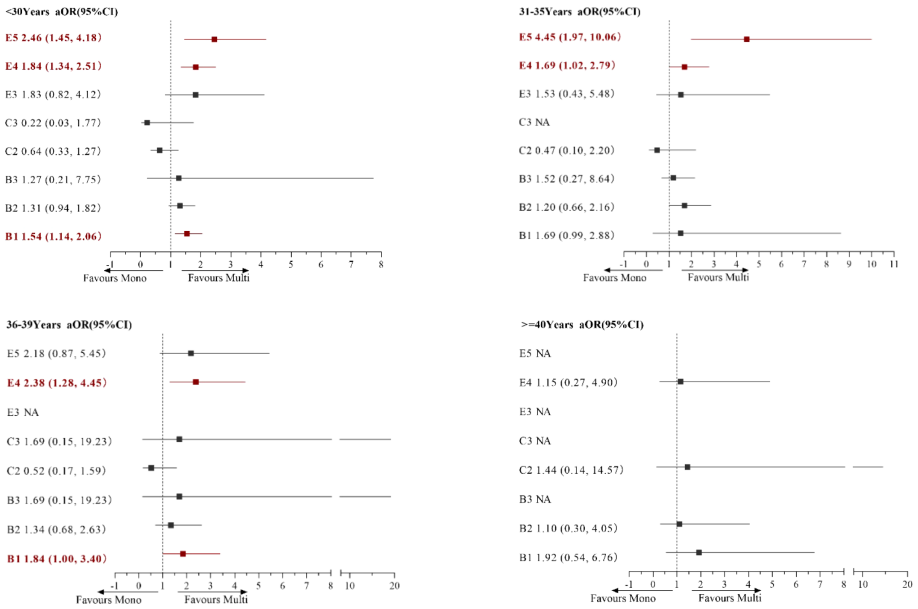

FIGURE 1
Logistic regression analysis for multiple birth rate (MBR) among patients based on age and embryo transfer group. *AOR: adjusted odds ratio for type of infertility, indications for IVF, body mass index (BMI), and anti-Müllerian hormone (AMH) in logistic regression analysis.

In advanced age of women, the proportion of single good cleavage embryo transfer cycles increased (from 1.2% in 30y to 4.6% in ≥40y), while the proportion of single GQB and two GQE decreased (from 8.2% and 14.9% in 30y to 3.5% and 9.4% in ≥40y, respectively). This was due to the low number of available embryos or intentional SET to avoid multiple pregnancies due to previous cesarean section or family planning. The distribution of embryo grades in the cleavage phase was comparable among women of different ages. However, the proportion of high-quality transferred blastocysts decreased with increasing maternal age. The reasons for the lower blastocyst formation rate in older women include increased aneuploidy rate, mitochondrial genome D-loop loci mutations, and low levels of stored maternally transcribed mRNA, which is involved in trophectoderm function and maintenance of the blastocoel (16). Blastocyst formation from good-morphology embryos has been reported to decrease significantly from 66.9% in women aged <35 years to 53% in those aged >39 years (17). As an embryo selection technique, blastocyst culture does not improve embryo viability, but can elevate the efficiency of embryo transfer, especially in advanced-age women.

A meta-analysis published in 2022, including 14 randomized controlled studies, focused on the association of LBR and MBR with the age or number of embryos transferred (18). Overall, the probability of live birth and multiple pregnancies decreased in women of advanced age or those receiving SET. However, only few studies (18, 19) have provided suggestions on how to make the best use of available embryos, based on both age and embryo grade. Here, we performed multiple comparisons and logistic regression analyses to explore individualized embryo transfer strategies. Give that the cases of GQE+PQE (B3), double PQE (C3), and double GQB (E3) were very few, these situations were considered as B2, C2, and E5, respectively, for convenience.

SET-GQB achieved an LBR similar to double blastocyst transfer (E3, E4, and E5) groups in women of all ages. Thus, SET is recommended when at least one GQB is available, which is consistent with the American Society for Reproductive Medicine guidelines (20). Some studies have suggested that compared with a single GQB transfer, DET-GQB+PQB results in almost the same LBR at the expense of a marked increase in the likelihood of multiple pregnancies (21, 22). However, Zhu et al. reported inconsistent findings, which showed a higher LBR after double GQB than after single GQB, with an adjusted OR of 1.76 (CI: 1.20, 2.57) (23). A policy of selective blastocyst culture or patient age may result in diversity. The more valuable meaning of SET is the sharply decreased MBR and improved CLBR in consecutive transfer cycles. It has been reported that two cycles of single GQB transfer could reach an LBR of 48.5% and a CLBR of 64.7%, whereas the LBR of double GQB transfer was only 48.9% (24).

Somewhat unexpectedly, the transfer of double PQB (E4) did not result in compromised LBR or MBR compared with the transfer of GQB + PQB (E5), indicating that the replacement of a good-quality blastocyst with a poor one in the double blastocyst transfer cycle did not compromise the chances of live birth. Based on a report enrolling 2,582 blastocyst transfer cycles from China, the transfer of double PQB and GQB+PQB had similar LBR and MBR, regardless of the age of ≤37 years or older (25). Theodorou et al. also reached the same conclusion (26). It seems

	Cleavage embryo	Blastocyst
≤35 years	SET-GQE>DET-AQE>DET-AQE +PQE>DET-PQE	SET-QGB>SET-PQB >DET-PQB
36–40 years	DET-GQE+AQE>DET-AQE>DET-AQE+PQE>SET-GQE	
>40 years	DET with embryos as high grade as possible	SET-QGB>DET-PQB> SET-PQB

that the predictive value of the morphology score for LBR and MBR was diminished when double blastocysts were transferred. Thus, the DET of blastocysts should be avoided, except for women older than 40 years, and when no GQBs are available.

In the cycles of cleavage embryo transfer, the relationship between embryo grade and LBR and MBR depended on the age of the women. For women aged <35 years, the addition of another embryo is not helpful for LBR when good-quality embryos are available. However, in women 36-40 years of age, double GQE transfer could increase the LBR compared with SET. Thus, DET-GQE should be avoided in patients aged ≤35 years and should be performed in those aged 36–40 years after full consultation. In addition, considering the CLBR and MBR risk, the combination of high- and low-quality embryos is preferred to DET-GQE in women aged ≤40 years, as the reserved high-grade embryo could bring more chances of LBR in the following embryo transfer cycle. In fact, for women with poor prognosis and decreased ovarian reserve, cumulative LBR was more important than LBR of a single cycle (27).

Clinical implications

The proposed embryo transfer practices based on different ages are listed as follows.

The principle is that, in the case of similar LBRs, embryo combinations with lower MBRs are preferred, and in the case of similar MBRs, embryo combinations with higher LBRs are preferred.

For women aged >40 years, the relationship between transferred embryos and LBR and MBR becomes indistinct, which is mainly related to the high rate of embryo chromosomal abnormalities (28–30). Preimplantation genetic testing for aneuploidy (PGT-A) is the best choice for women of advanced age, but it requires specific qualifications in China. Blastocyst culture is another good strategy because the aneuploidy rate is much lower in high-quality blastocysts than in high-quality D3 embryos (31). The elimination of cleavage embryos with poor developmental potential through blastocyst culture may increase the efficacy of embryo transfer. Chen et al. (32) reported that blastocyst culture and transfer did not increase the CLBR in women aged ≥38 years, but significantly increased the pregnancy rate per embryo transfer cycle. In 2012, data from 32,732 cycles with double embryos transferred were analyzed, which showed that the ORs and absolute risk differences for multiple births, preterm births, and low birth weight were all smaller in women ≥40 years than in younger women (33). Thus, if only cleavage embryos are available, DET should be suggested in women older than 40 years, as it tends to achieve higher LBR per cycle and shorten the time to achieve a live birth.

Research implications

The currently used criteria for embryo morphological score are of very limited value for women with advanced age. Thus, novel non-invasive methods for embryo development should be investigated to improve the efficiency of embryo transfer.

Strengths and limitations

To the best of our knowledge, this is one of the largest retrospective studies evaluating LBR and MBR based on maternal age and embryo grade in both cleavage-stage embryo and blastocyst transfer cycles. However, our study was limited by its retrospective design, and the fact that it was performed at a single center. Another major limitation was the decision of the number of the transferred embryos which was influenced by complicated factors from both physicians and infertile couples. Moreover, we did not exclude patients with repeated implantation failure (RIF) associated with adverse pregnancy outcomes. In addition, other potential confounders such as the pre-thrombotic state and polycystic ovary syndrome (PCOS) were not analyzed.

Conclusions

Based on the current evidence, SET should be selected for high-quality blastocysts in women of all ages. When good-quality cleavage embryos are available, the choice of SET or DET with GQE or AQE should depend on the age of the woman. For elderly women aged ≥40 years, if no GQB is available, DET with embryos as high a grade as possible is recommended. It should be noticed that since our data were from FET cycles, its validation in fresh cycles needs more evidence. Overall, the choice of embryo transferred should be jointly made by patients and physicians based on individualized transfer strategies, which need to be verified by further high-quality randomized controlled trials or national registry-based cohort studies.

Data availability statement

The relevant data involves patient privacy, so it is not disclosed or uploaded to the database. Requests to access the datasets should be directed to Zhi-hong Niu.

Ethics statement

The studies involving human participants were reviewed and approved by Ruijin Hospital Ethics Committee Shanghai JiaoTong University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

Author contributions

XW and W-JZ contributed equally to this work. Z-HN and A-JZ designed the study. B-FX, QC, LX,H-HX and Z-HN collected data and participated in parts of analysis of the data. XW and W-JZ analyzed data and wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Sperm telomere length as a novel biomarker of male infertility and embryonic development: A systematic review and meta-analysis

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Background: Telomeres have an essential role in maintaining the integrity and stability of the human chromosomal genome and preserving essential DNA biological functions. Several articles have been published on the association of STL with male semen parameters and clinical pregnancy. The results, however, are either inconclusive or inconsistent. Therefore, this meta-analysis aimed to systematically assess the accuracy and clinical value of sperm telomere length (STL) as a new marker for diagnosing male infertility and predicting the quality of embryonic development.

Methods: We performed a comprehensive systematic search for relevant publications in PubMed, the Cochrane Library, Web of Science, Embase, Scopus, and Ovid, from database build to August 2022. All experimental studies exploring the association of STL with male semen quality, male infertility, or embryonic development were included.

Results: Overall, Twelve prospective observational cohort studies (1700 patients) were eligible for inclusion in the meta-analysis. The meta-analysis showed a positive linear correlation between STL and semen parameters. The optimal cut-off value for STL diagnosing male infertility was 1.0, with a sensitivity and specificity of 80%. Regarding STL and embryonic development, the clinical pregnancy rate was associated with longer STL, and there was no significant difference between the two groups regarding fertilization rate.

Conclusion: Our study showed that STL has good diagnostic and predictive value for male fertility and clinical pregnancy and could be used as a new biomarker for diagnosing male infertility and predicting embryonic development.

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KEYWORDS

infertility, male, spermatozoa, telomere, embryos, diagnosis

Introduction

The World Health Organisation defines sterility as "the inability to conceive successfully after more than 12 months of unprotected sexual intercourse". It has been reported that over 50 million (approximately 15%) couples worldwide are affected (1). Male factors are involved in 51% of infertility problems (2, 3), of which up to 40% are diagnosed as idiopathic (4, 5). Currently, male fertility is mainly based on the initial assessment of semen analysis. The probability of conception depends on the quality of semen, which is reduced as one of the leading causes of male infertility (6), including reduced sperm concentration (oligospermia), decreased percentage of forward-moving sperm (weak sperm), a lower percentage of morphologically normal sperm (teratozoospermia), and complete absence of sperm in the semen (azoospermia) (7). However, basic diagnostic procedures using semen parameters are often inadequate to differentiate between fertile and infertile men (8). Among the approximately 30-40% of men with idiopathic infertility, standard semen parameters are often assessed in the 'normal' range (1, 9), and even when the semen analysis is below typical average values, it is not a direct indicator or predictor of fertility outcome (10). Hence, finding a new, non-invasive, reliable method to differentiate infertility from normal fertility is urgently needed.

Reactive oxygen species (ROS) are highly reactive oxidative radical, including superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\bullet}$), and hydroxyl radical ($^{\bullet}OH$) radicals, which in spermatozoa are mainly derived from activated leukocytes in the seminal plasma and the mitochondria (11). Due to the limited level of antioxidant defense of sperm, high levels of oxidative stress are highly susceptible to damage to sperm DNA and RNA transcripts, and extensive evidence suggests that reactive oxygen species-mediated sperm damage is a major cause of sperm damage in 30-80% of infertility patients (12). Telomeres are DNA-protein complexes located at the ends of chromosomes and consist of a non-coding hexamer formed by the tandem formation of a highly conserved repetitive DNA sequence (TTAGGG) forming a T-loop structure that interacts with the Shelterin protein complex to form a fully functional hooded structure (13). The specific structure allows cells to distinguish telomeres from sites of DNA damage, protects them from inappropriate DNA repair mechanisms, prevents gene degradation due to incomplete DNA replication, protects chromosome ends from erosion, and plays a crucial part in the integrity of the structure and stability of the chromosomal genome itself and preserving essential biological functions of DNA (14). Oxidative damage can disrupt telomere integrity and interfere with telomerase activity, leading to telomere shortening (15). The results of an *in vitro* test conducted by Lafuente et al (16)showed that the addition of hydrogen peroxide to sperm resulted in a reduction in measured sperm telomere length and a negative correlation between sperm telomere length and sperm reactive oxygen content (17). More recent studies have suggested that STL may be a promising marker of male reproductive biology (18). Most studies have concluded that STL is shorter in men with idiopathic infertility compared to fertile men. Telomere length is positively correlated with sperm anterograde motility and sperm count and negatively associated with sperm DNA fragmentation, and can be used as a marker of sperm quality (19–22). However, some scholars have suggested that telomere length shortening may be a sign of sperm damage rather than a cause of sperm alteration (23). In contrast, some studies have concluded that STL is unrelated to sperm parameters (24).

The telomere length has been attracting more and more attention in the reproductive field. Several studies have shown a greater preponderance of telomere length and telomerase activity in the cumulus cells (25), granulosa cells (26), and peripheral lymphocytes (27) of fertile women compared to infertile women. Nevertheless, limitations in the collection of material, particularly cumulus cells, make it challenging to apply these findings to clinical predictions of embryonic developmental quality and pregnancy outcome. Compared to cumulus cells and other cells, semen samples are easier to collect and assay. The telomere length of sperm cells typically correlates positively with the high-quality and transferable embryo ratio (28). In addition, the incidence of sustained pregnancy after *in vitro* fertilization (IVF) treatment among patients with relatively abnormal STL was zero, compared to 35.7% in samples with STL in the normal range, which may indicate that STL plays an essential role in reproduction (29). Whether STL is recommended as a diagnostic and predictive clinical outcome for male infertility remains controversial. To date, there have been no meta-analyses to assess the value of STL in the field of reproduction. In this study, we comprehensively analyzed and

evaluated the current studies on STL concerning male infertility and embryonic development to clarify STL's accuracy and clinical value in diagnosing male infertility and predicting embryonic developmental quality.

Materials and methods

The study is transparent and original, adhering to the Cochrane Manual version 6.2 and Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines on systematic reviews and meta-analyses (30, 31). Systematic reviews and meta-analyses should be registered to avoid publication bias (32). Therefore, we have completed registration with the International Prospective Register of Systematic Reviews (PROSPERO) under the registration ID CRD42022303333. As this study involved only the collection and collation of clinical study data, institutional review board (IRB) approval was not required.

Search strategy

We performed a comprehensive systematic search for relevant publications in PubMed, the Cochrane Library, Web of Science, Embase, Scopus, and Ovid, from database build to August 2022. The search formula was the following terms: (((sperm telomere length) OR (telomere length)) OR (STL)) AND (((((Male Infertility) OR (Sub-Fertility Male)) OR (Embryo)) OR (embryonic development)) OR (Pregnancy)) and further consulted their references to expand the search without language and year restrictions.

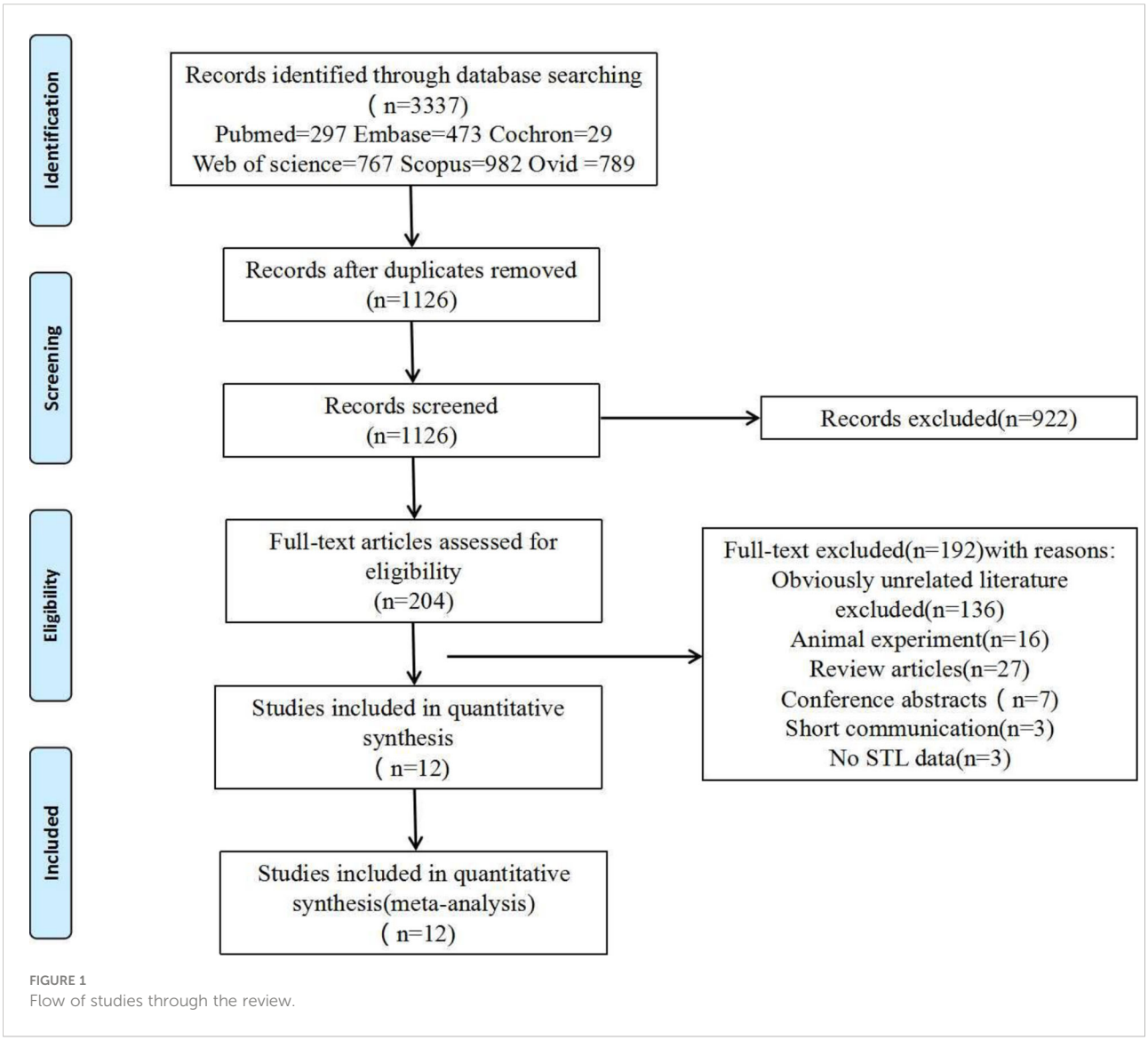

FIGURE 1
Flow of studies through the review.

Study selection and data extraction

Inclusion criteria (1): all experimental studies exploring the association of STL with male semen quality or male infertility or embryonic development (2); the diagnosis of infertility or subfertility included all degrees of altered semen parameters (except azoospermia) (3); the study reported at least one extractable outcome such as sperm count, percentage of forward-moving sperm (a+b%) and sperm concentration.

Exclusion criteria (1): Reviews, conference abstracts, or duplicate publications (2). Insufficient data or inability to download the full text (3). The subject of the literature is not human. The specific reasons excluded are illustrated in Figure 1. two independent reviewers (X.Q and H.L) screened the searched literature to remove duplicates and find the full text of the articles. When disagreements arise, a consensus is reached through thorough discussion and analysis with a third reviewer (X.Y).

Extract data of interest from each included study. Include authors, publication year, study region, type of study design, experimental method, inclusion or exclusion criteria, and the number of subjects. The assessment of STL was used as the primary endpoint for inclusion in the literature search. In addition, (X.Q., H.L., and X.Y.) separate quality checks were performed on the extracted data.

Assessment of methodological quality

The investigators (Qiu and Luo) applied the Cochrane risk of bias tools to assess the risk of bias for all included studies independently. Review manager 5.3 was used to represent the risk of bias assessment results visually. The assessment was based on the following quality assessment criteria: I Randomisation methods, although not an inclusion criterion; II Concealed allocation; III Double-blinding process for subjects and staff; IV Participants recruited, number of analyzed or dropped from the track; V Selective reporting VI Other risks of bias.

Statistical analysis

Data analysis using Review Manager 5.3 software. All of the papers included in this study are observational and prospective cohort studies, with weighted mean differences (WMD) applied to continuous variables and relative risk ratios (RR) applied to dichotomous variables, and all with their 95% confidence intervals (CI) as effect indicators. The I^2 test to analyze heterogeneity across studies, Choice of effect model based on the size of heterogeneity (fixed-effects model when $P>0.1$ or $I^2<50\%$; random-effects model when $P \leq 0.1$ or $I^2>50\%$). An IBM SPSS Statistics 25.0 (SPSS Inc., Chicago, IL, U.S.A.) software was used to plot to scatter plots to test the correlation between STL and semen parameters, thus further validating the statistical results. The Receiver operating characteristic curve (ROC) was applied to test the diagnostic accuracy of STL for male infertility. The Uden index was used to calculate the ROC threshold to determine the best pairing of sensitivity and specificity to identify better the best cut-off value for diagnosing infertile men. Meanwhile, the Hosmer and Lemeshow tests were applied for goodness-of-fit to test the working of the scoring model. A sensitivity analysis was finally performed to identify sources of heterogeneity, using Review Manager 5.3 funnel plots tools to assess publication bias. If $P<0.05$ is considered to be statistically significant.

Results

Literature search

In total, literature searches identified 3,337 papers, and 12 articles (10, 18, 29, 33–41) were finally included after excluding duplicate studies, irrelevant literature, review articles, and short communications adhering strictly to the inclusion and exclusion criteria. Details of the screening are illustrated in Figure 1.

Characteristics and qualitative results of included studies

Overall, a total of 887 fertile men (control group) and 813 infertile men (experimental group) were compared in the 12 studies finally included in the meta-analysis, including patients from Italy, Spain, Portugal, the UK, Iran, China, and India. The publication dates of the literature varied from 2012 to 2021, and the sample sizes changed from 10 to 345 patients. The primary characteristics and study features of the included studies are listed in Table 1. The quality of each study was calculated using the Cochrane Risk of Bias Assessment Tool, with studies categorized as 'low risk, 'high risk,' or 'unclear risk. Six of the 12 studies had a high risk for at least one department, and Low bias risks accounted for 83.3% of all departments. Ultimately, a low overall risk of bias was revealed in (Supplementary Figure 1).

Semen parameters

Sperm count data were available from seven studies (10, 29, 34, 36–38, 40). The mean sperm count was $(160.64 \pm 149.32) \times 10^6$/per ejaculation in the fertile group and $(94.82 \pm 103.55) \times 10^6$/per ejaculation in the infertile group. Meta-analysis indicated that the sperm count of the fertile group was superior as compared to the infertile group (WMD 2.73, 95%

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TABLE 1 Characteristics of studies included in the analysis.

					STL	Fertile men(CONTROL GROUP)			Infertile men(STUDY GROUP)		
Author	N.R	Country	Year	Study design	Method	N	Age (years)	Inclusion criteria	N	Age (years)	Inclusion criteria
Lopes, AC. et al. (10),	68	Portugal	2020	O.C.S. (P)	qRT-PCR	33	39.3 ± 4.1	normozoospermic	45	39.3 ± 4.1	non-normozoospermic
Cariati, F. et al. (29),	45	England	2016	O.C.S. (P)	qRT-PCR	54	39.4 ± 5.5	normozoospermic	19	39.3 ± 5.3	oligozoospermic
Ferlin, A. et al. (33),	35	Italy	2013	O.C.S. (P)	qRT-PCR	61	Range 18-19	normozoospermic	20	Range 18-19	oligozoospermic
Thilagavathi, J. et al. (18),	19	India	2012	O.C.S. (P)	qRT-PCR	25	N.A.	Proven fertility	32	N.A.	Unexplained infertility
Liu, SY. et al. (34),	18	China	2015	O.C.S. (P)	qRT-PCR	138	Range 22-52	Proven fertility	126	Range 23-57	Unexplained infertility
Torra-Massana, M. et al. (35),	27	Spain	2018	O.C.S. (P)	qRT-PCR	60	24.3 ± 5	Positive	60	24.3 ± 5	Negative
Rocca, MS. et al. (36),	44	Italy	2021	O.C.S. (P)	qRT-PCR	30	36.1 ± 6.8	Proven fertility	35	39.0 ± 6 5.4	Oligozoospermic normozoospermia
Amirzadegan, M. et al. (37),	30	Iran	2021	O.C.S.(P)	qRT-PCR	10	40.3 ± 3.75	Proven fertility	10	35.46 ± 5.59	oligozoospermic
Mishra, S. et al. (38),	31	India	2016	O.C.S.(P)	qRT-PCR	102	32.2 ± 4.0	Proven fertility	112	31.71 ± 4.45	Unexplained infertility
Yang, Q. et al. (39),	54	China	2016	O.C.S. (P)	qRT-PCR	345	30.4 ± 4.0	Positive	306	30.5 ± 3.9	Negative
Darmishonnejad, Z. et al. (40),	59	Iran	2019	O.C.S.(P)	qRT-PCR	10	40.11 ± 3.14	Proven fertility	10	38.10 ± 4.17	Unexplained infertility
Darmishonnejad, Z. et al. (41),	44	Iran	2020	O.C.S. (P)	qRT-PCR	19	40.47 ± 3.82	Proven fertility	38	32.65 ± 6.56	Unexplained infertility

N.R, number of references; O.C.S., observational clinical study; P, prospectively collected data; qRT-PCR, Quantitative Real-Time Polymerase Chain Reaction; N.A., not available; Positive the longer STL, Negative the shorter STL.

CI:1.70-3.76, I^2 = 96%, p < 0.00001), The difference was statistically significant (Supplemental Figure 2).

Seven studies provided data on the percentage of forwarding motile sperm (a+b) % (10, 29, 34, 36, 37, 40, 41). The Meta-analysis results suggested that the rate of forward-moving sperm (a+b) % was significantly better in the fertile individuals (WMD 3.17, 95% CI: 1.84-4.51, I^2 = 96%, p < 0.00001), The difference was statistically significant (Supplemental Figure 3).

Six studies provided data on sperm concentration (10, 34, 36, 37, 40, 41). The mean sperm concentration was (88.67 ± 54.89) $\times 10^6$/ml in the fertile group and (48.50 ± 39.67) $\times 10^6$/ml in the infertile group. As Meta-analysis revealed, Sperm concentrations were likewise visibly higher in the fertile group (WMD 2.77, 95% CI:1.52-4.02, I^2 = 96%, p < 0.00001), The difference was statistically significant (Supplemental Figure 4).

Four out of twelve studies of this analysis report sperm DNA fragmentation value obtained using TUNEL analysis (29, 37, 40, 41). The mean Sperm DNA Fragmentation Index was significantly higher in infertile men (25.96 ± 10.42)% than in fertile individuals (20.98 ± 10.45)%. As Meta-analysis revealed, sperm DNA fragmentation value was likewise visibly more increased in the infertile group (WMD 6.89, 95% CI:3.52-10.26, I^2 = 94%, p < 0.0001), The difference was statistically significant (Supplemental Figure 5).

Sperm telomere length (STL)

STL data were available from the 10 included studies (10, 18, 29, 33, 34, 36–38, 40, 41). The mean STL was (2.24 ± 2.21) in the fertile group of men and (1.67 ± 1.43) in the infertile group. In the Meta-analysis, the fertile group had a higher STL than the infertile group (WMD 1.81, 95% CI:1.18-2.45, I^2 = 93%, p < 0.00001), and the difference was statistically significant (Figure 2). Furthermore, when comparing the five studies that included men of proven fertility with Unexplained infertility (18, 34, 38, 40, 41), the results

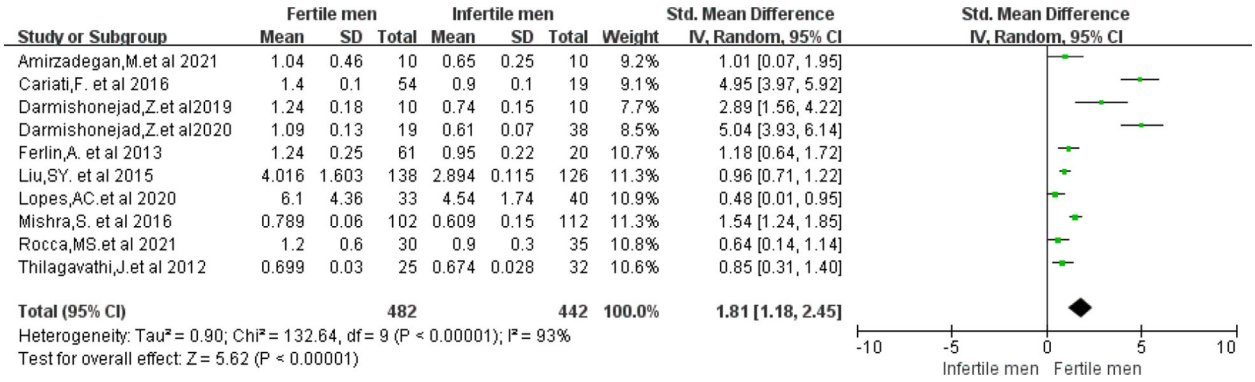

FIGURE 2
Forest plot showing the results of Meta-analysis, comparing fertile and infertile men for differences in STL.

remained significantly different (WMD 2.05, 95% CI:1.21-2.90, I^2 = 93%, P < 0.00001) (Supplemental Figure 6).

Correlation between STL and semen parameters

There might be a correlation between STL and semen analysis parameters, as indicated by the results of the Meta-analysis. Therefore, separate scatter plots were drawn to demonstrate a significant positive linear correlation between STL and semen parameters: a higher sperm count (R^2 = 0.162, P=0.154) (Supplemental Figure 7), percentage of forward-moving sperm (a+b)% (R^2 = 0.033, P=0.501) (Supplemental Figure 8), sperm concentration (R^2 = 0.037, P=0.549) (Supplemental Figure 9) resulted in a longer STL.

Male infertility diagnosis

ROC curves ([AUC]=0.76, p<0.05) for all data from the 12 included studies for STL. The optimum cut-off value for the STL was ascertained by calculating the Jorden index for the best pairing of sensitivity and specificity, which was 1.0. At this threshold, the diagnostic ability of STL showed a sensitivity and specificity of 80% (Figure 3). The Hosmer and Lemeshow tests were also applied for the goodness of fit (P=0.40, P>0.05) (Supplemental Figure 10). Our outcomes demonstrate that the combined sensitivities in this study were generally good and that the scoring model worked well.

Embryonic development

A total of 3 studies provided data on fertilization rates (10, 39, 40). Meta-analysis was performed in 3 studies with Considerable heterogeneity (I^2 = 83%), and a random effects model was applied. Our results revealed that higher STL groups did not show a clear advantage in fertilization rates with no statistically meaningful difference(RR=0.99; 95% CI:0.86-1.14; P=0.88) (Supplemental Figure 11).

Four studies provided data on clinical pregnancy rates (10, 29, 35, 39). Meta-analysis was performed in the four studies with low heterogeneity between studies (I^2 = 0%), and a fixed effects model was applied. Our results revealed that higher STL groups possessed superior clinical pregnancy rates and the two groups showed statistically significant differences (RR=0.87; 95% CI:0.78-0.97; P=0.02) (Figure 4).

Sensitivity analysis and publication bias

A sensitivity analysis was undertaken using the exclusion of individual studies from the outcome analysis to assess whether individual studies would affect the overall results. Meta-analysis results were similar after excluding each study, validating the stability of the Meta-analysis. When the three studies by Cariati F and Darmishonnejad Z (29, 40, 41) were excluded, the recalculated results showed a marked reduction in heterogeneity (WMD 0.97, 95% CI:0.67-1.28, I^2 = 69%, p<0.00001). Using STL as an indicator by funnel plots, we found no significant publication bias (Supplemental Figure 12).

Discussion

Research on STL has recently increased yearly, with more scholars focusing on its role in human reproduction. Although several studies have been previously published on the relationship between STL and male semen and embryos, this is the first study to systematically review and meta-analyze the value of STL as a new biomarker for diagnosing male infertility

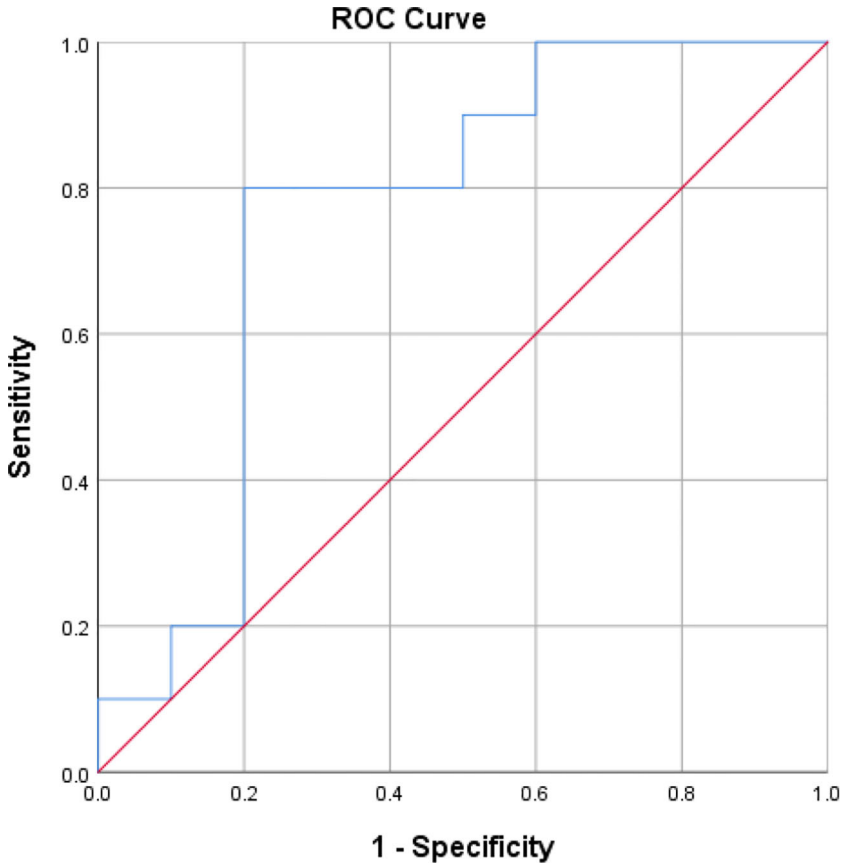

FIGURE 3
The Receiver operating characteristic(R.O.C.) curve for determining the optimal STL cut-off value for the diagnosis of male infertility.

and predicting embryonic development, filling a gap in the field. Our study confirms a clear positive correlation between STL and semen quality parameters and influences embryonic development and clinical pregnancy outcome, which could be considered a new diagnostic tool for diagnosing male infertility and predicting the quality of embryonic development, adding more definitive information to conventional semen analysis.

Male infertility is a global disease that threatens human development with genetic characteristics, and the incidence of male infertility is rising worldwide (42, 43). A meta-analysis showed a 50% to 60% reduction in male sperm count compared to 40 years ago (44). In most cases, clinical patients are found to be infertile only after marriage, but by then, the best treatment and male fertility time may have been missed. In severe cases, this can lead to a decline in marital quality and affect family well-being (45). However, to date, there are still no laboratory indicators for specific early diagnosis, standard semen analysis does not accurately distinguish between fertile and infertile

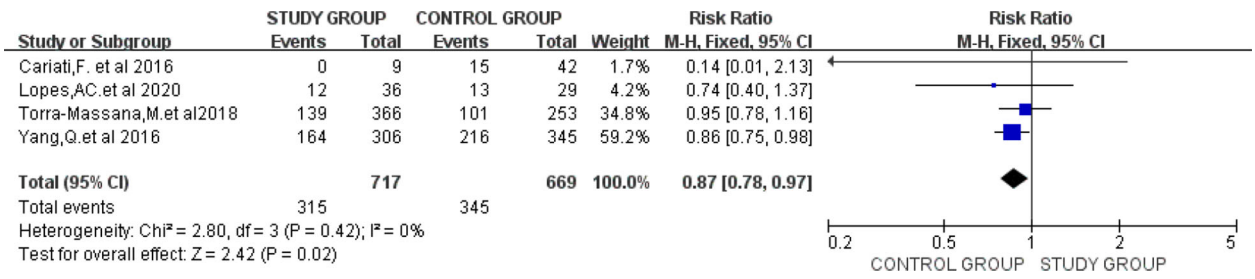

FIGURE 4
Forest plot showing the results of Meta-analysis, comparing study and control men for differences in clinical pregnancy rates.

populations, and diagnostic confusion often exists, as confirmed by the studies we included (18, 34, 36, 38, 40, 41). Therefore, searching for the ideal biomarker is essential in the early diagnosis, treatment, and prognosis of male infertility.

Telomeres protect chromosome ends from erosion and maintain human gametogenesis and fertility (46). The telomere function is largely limited by its length. Once telomere shortening exceeds a critical level, the proteins forming the Shelterin complex are unable to bind to telomeric sequences and cannot perform capping at chromosome ends (47). Therefore, it is essential to maintain telomere length, which is generally achieved by telomerase, a ribonucleoprotein complex consisting of a unique telomerase reverse transcriptase (TERT) and telomerase RNA (TERC) that synthesizes new telomeric repeats by copying its telomerase RNA component copy extension 3' end (48). In humans, telomerase is present in germ cells, stem cells, and about 85% of cancer cells. It is particularly active in germ cells for extended periods, thereby delaying telomere erosion and avoiding chromosomal segregation defects such as aneuploidy or gamete imbalance (49). Due to the delayed closure of telomerase, sperm telomeres are generally longer than somatic cells, with a length of about 10-20 Kb (14). Inactivation of telomerase leads to progressive telomeres shortening, which shorten by about 40-200 bp base pairs with each cell division until they reach a specific limit when the cell stops dividing and dies of senescence (50). Three essays are commonly used in studies to determine STL. The southern blot is mainly used as a reference method to validate newly imported technologies (51); q-fish can only be used for mitotically active cells (52). In contrast, only Q-PCR can be performed in isolated DNA (53). The Q-PCR method was used to measure STL in the literature included in this study. We speculate that this may have increased the risk of bias and limited the predictive strength of the STL cut-off values obtained from the survey to some extent. However, the results were conclusive in the studies that used the Q-PCR method. The Q-PCR method is more suitable for epidemiological studies in large populations due to its relative simplicity, affordability, and ability to use smaller amounts of DNA. It is also used in clinical practice to measure STL in most cases. Therefore, we are still recommending this cut-off value and look forward to additional studies in the future to determine a more comprehensive outcome.

Telomere attrition is an inevitable and normal biological event during cellular aging. Apart from progressive telomere shortening due to cell division problems, telomere length is still influenced by many factors such as age, genetics, environment, and psychosocial stress levels (49, 54, 55). In Ferlin's study (33), by including 61 patients with normospermia and 20 patients with idiopathic oligospermia, it was found that older fathers and mothers had longer STL in their offspring and that STL was directly related to the age of the parents at the time of pregnancy. Still, the relative contribution of paternal and maternal age could not be determined. In certain genetic disorders, abnormal telomere shortening is caused by gene dysregulation that disrupts telomeres' integrity and stability, known as telomeropathies (56). Although no specific mechanisms linking telomeres to the pathogenesis of these diseases have been identified, it has been shown that most of them exacerbate pathological conditions associated with aging, such as cardiovascular disease and diabetes (57). The adverse effects of environmental pollution on telomere length have been confirmed by a systematic review of 12,058 subjects (55), which showed a direct link between air pollution exposure and shortened telomere length. Increased levels of oxidants and poor lifestyles also contribute to telomere erosion (58). On the other hand, humans can reduce telomere erosion through physical activity (59). One meta-analysis of the association between diet and telomere length maintenance showed that the Mediterranean diet protects telomere integrity through its anti-inflammatory and antioxidant properties (60). It has been found that the physiological process of telomere attrition may be accelerated under certain pathological conditions. A recent study of 38 infertile men and 19 fertile men reported that a comparative analysis of STL and semen quality in male patients found that infertile men generally had shorter STL than fertile men (41). Several meta-analyses have previously reported that sperm DNA fragmentation indices can influence fertilization, embryonic development, and pregnancy outcome (61, 62), and a high proportion of DNA fragmentation was found in sperm from men with shorter telomeres (63). This finding further supports the correlation between STL and male fertility. Our results showed that infertile men had a significantly higher sperm DNA fragmentation index than fertile men, which is consistent with the findings of previous studies. Animal studies have also found that long telomeres are only inherited in male mice whose parents have longer telomeres (64) and that longer telomeric sperm can lead to higher rates of morulae and blastocysts (65). In some studies (20, 22, 33, 40), a comparative analysis of the relationship between STL and semen quality in infertile male patients suggested that telomeres could be considered as a biomarker of abnormal spermatogenesis quality and quantity. Our analysis also showed that fertile men had a higher STL in comparison to infertile men (2.24 ± 2.21 vs. 1.67 ± 1.43, $p < 0.001$), and STL was significantly associated with sperm count ($R^2 = 0.162$), percentage of forward-moving sperm (a+b) % ($R^2 = 0.033$) and sperm concentration ($R^2 = 0.037$). A positive linear correlation was calculated, and statistical analysis showed that the Cut-off value = 1.0 could be used to predict male fertility, the same as the results of several previous studies.

A controversial viewpoint: STL may be able to predict successful implantation and embryo quality after assisted reproductive treatments (ART), such as *in vitro* fertilization (IVF), among infertile couples (21). Selecting sperm with longer

telomeres facilitates the production of better-quality embryos and may influence pregnancy outcomes and the success of ART (66). However, some scholars have argued that STL is not helpful in predicting the outcome of intracytoplasmic sperm injection therapy (ICSI) (35), with no significant correlation between STL and clinical outcome. Two different conclusions may be explained by the fact that part of the study did not relate male STL to the physical characteristics of the female partner. Telomeres have been reported to be shorter in the oocytes of women who are not pregnant after IVF (67), and telomere length may limit the ability of fertilized eggs to develop into healthy embryos. Indeed, many complications of advanced age are associated with shorter embryonic telomere lengths, including Down's syndrome (68) and recurrent miscarriage (69). A recent meta-analysis (70) that included 105 studies involving 271,632 pregnant women suggested that higher body mass index (BMI) values may indicate poor pregnancy outcomes and that higher BMI is linearly associated with higher miscarriage rates, lower clinical pregnancy rates and lower live birth rates. Therefore, certain variables (e.g., female age, BMI, embryo morphology, etc.) should be included in future ART studies. The results of this study showed clinical pregnancy rates associated with longer STL (RR=0.87; 95% CI:0.78-0.97), and two groups did not show a large difference in fertilization rates (RR=0.99; 95% CI:0.86-1.14). This may be due to a smaller sample size and more significant heterogeneity between studies. Notably, our findings clarify the relevance of STL to clinical pregnancy outcomes and herald a potentially crucial mechanistic role in embryonic development. A recent study suggests that the increased risk of IVF failure and recurrent miscarriage may be associated with embryonic aneuploidy. Short telomeres significantly cause increased aneuploidy abnormalities and delayed embryo development (25). Thus, we hypothesize that STL may be a promising predictor of embryonic developmental quality, both for natural conception and IVF, as it may reflect embryonic quality to some extent and predict pregnancy success.

Our meta-analysis has several advantages over existing published meta-analyses. First, our meta-analysis is the first systematic assessment of STL in diagnosing male infertility and predicting embryonic developmental quality. Secondly, we applied the area under the ROC curve and the Hosmer and Lemeshow test to a comprehensive test of the accuracy and feasibility of STL in diagnosing male infertility, providing a comprehensive assessment of its clinical diagnostic ability. However, the study still showed several limitations: firstly, the inclusion criteria were not homogeneous, and there were significant individual differences between the patients enrolled in the study, resulting in heterogeneity between studies, which needs to be further eliminated in future studies by rationalizing the design and increasing the sample size. In addition, subgroup analysis of the assay was not performed, leading to a higher likelihood of false positive or negative results.

Conclusions

In conclusion, STL has diagnostic and predictive value for males in fertility and clinical pregnancy. In conjunction with the specific clinical situation, it may be possible in the future to combine tests with other biomarkers in the clinic, such as the combined testing of semen parameters and sperm DNA fragmentation index, thus further improving the diagnostic sensitivity and specificity of male infertility and the ability to assess and predict pregnancy outcomes, which will play a vital role in the future diagnosis and treatment of human reproductive disorders.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

YY was a significant contributor to the writing of the manuscript, contributed to the design and data acquisition of the study, drafted and critically revised the article, and organized the final approval of the version to be published. YT contributed to the study concept and design, data acquisition, and data analysis and interpretation. XQ, HL, YL, and RL assessed the quality of the included studies and critically revised the manuscript for important intellectual content. XY had full access to all of the data in the study and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1079966/full#supplementary-material

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Controlled ovarian hyperstimulation parameters are not associated with *de novo* chromosomal abnormality rates and clinical pregnancy outcomes in preimplantation genetic testing

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Objective: This study aimed to determine whether controlled ovarian hyperstimulation (COH) parameters influence the incidence of *de novo* chromosomal abnormalities (> 4 Mb) in blastocysts and, thus, clinical pregnancy outcomes in preimplantation genetic testing (PGT).

Methods: Couples who underwent preimplantation genetic testing for structural chromosome rearrangements (PGT-SR) and monogenic disorders (PGT-M) were included in this study. The relationships of maternal age, paternal age, stimulation protocol, exogenous gonadotropin dosage, duration of stimulation, number of oocytes retrieved and estradiol (E_2) levels on human chorionic gonadotropin (hCG) trigger day with the incidence of *de novo* chromosomal abnormalities were assessed. Blastocysts were biopsied, and nuclear DNA was sequenced using next-generation sequencing (NGS). Clinical pregnancy outcomes after single euploid blastocyst transfers under different COH parameters were assessed.

Results: A total of 1,710 and 190 blastocysts were biopsied for PGT-SR and PGT-M, respectively. The rate of *de novo* chromosomal abnormalities was found to increase with maternal age ($p < 0.001$) and paternal age ($p = 0.019$) in the PGT-SR group. No significant differences in the incidence of *de novo* chromosomal abnormalities were seen for different maternal or paternal age groups between the PGT-SR and PGT-M groups ($p > 0.05$). Stratification analysis by gonadotropin dosage, stimulation protocol, duration of stimulation, number of retrieved oocytes and E_2 levels on hCG trigger day

revealed that *de novo* chromosomal abnormalities and clinical pregnancy outcomes were not correlated with COH parameters after adjusting for various confounding factors.

Conclusion: The rate of *de novo* chromosomal abnormalities was found to increase with maternal or paternal age. COH parameters were found to not influence the incidence of *de novo* chromosomal abnormalities or clinical pregnancy outcomes.

KEYWORDS

controlled ovarian hyperstimulation (COH), *in vitro* fertilization, preimplantation genetic testing (PGT), *de novo* chromosomal abnormality, clinical pregnancy outcomes

Introduction

In assisted reproductive technology (ART), aneuploidy is one of the most significant causes of pregnancy failure and miscarriage. Aneuploidy occurs due to meiotic errors during gametogenesis, the fertilization of unbalanced gametes or mitotic errors during embryonic development (1, 2). Therefore, a major goal of controlled ovulation hyperstimulation (COH) is to achieve maximal follicular development during a single menstrual cycle. It is thought that aneuploidy can be avoided through the collection of many oocytes, thereby increasing the likelihood of obtaining euploid embryos. However, embryonic chromosomal abnormalities are thought to also occur due to iatrogenic factors (3), with the *in vitro* fertilization (IVF) process itself and associated COH increasing the risk of meiotic or mitotic errors. It has been speculated that the use of exogenous gonadotropins (Gn) to stimulate multifollicular development can interfere with the natural selection of dominant follicles, thereby increasing the retention of aneuploid oocytes (4, 5). Moreover, COH has been hypothesized to increase oocyte division errors and affect genomic imprinting (6). However, the specific nature of such adverse effects of COH remains unclear.

As preimplantation genetic testing (PGT) is an important prenatal diagnostic method used to detect aneuploid embryos (7), it can prevent the transmission of pathogenic genetic mutations or an unbalanced chromosome to the offspring, increasing the chances of a successful, healthy pregnancy. Therefore, PGT is widely used in clinical practice (7, 8). Many studies have explored the relationship between COH and aneuploid, but have yielded inconclusive results. It has been suggested that higher Gn dosage increases the incidence of aneuploid (9, 10). In contrast, other studies have reported that the incidence of embryonic aneuploidy was not affected by exogenous Gn, with equivalent aneuploidy rates across unstimulated and stimulated IVF cycles, and different Gn dosages (11, 12). However, most studies have focused on the relationship between ovulation induction and aneuploidy (6, 13), although many other intrinsic factors can influence ploidy. Aneuploidy can be inherited from a parental carrier of genetic abnormalities (14), or it can occur *de novo* (15, 16). PGT for aneuploidy (PGT-A) is typically carried out following recurrent miscarriages and repeated implantation failures or in cases of advanced maternal age. In contrast, PGT for structural chromosome rearrangements (PGT-SR) is carried out for individuals known to have high rates of unbalanced gametes after meiotic segregation, resulting in embryos with abnormal chromosomal composition. Therefore, we sought to study the effect of COH on the rate of *de novo* chromosomal abnormalities identified by PGT-SR. As patients undergoing PGT for monogenic disorders (PGT-M) have known monogenetic mutations but normal karyotypes, they typically do not have fertility issues and are not at risk of elevated embryonic aneuploidy rates. We therefore used patients undergoing PGT-M as our control group.

In this study, we aimed to determine whether COH parameters are associated with *de novo* chromosomal abnormality rates in the Chinese population served by our reproductive center at The Third Affiliated Hospital of Zhengzhou University. We compared patients undergoing unstimulated and stimulated PGT-SR and PGT-M and explored various COH parameters for their associations with aneuploidy rates and clinical pregnancy outcomes. To our knowledge, this is the first systematic evaluation of the effects of specific COH parameters on *de novo* chromosomal abnormalities rates and clinical pregnancy outcomes involving PGT using next-generation sequencing (NGS) techniques. This research will inform the development of improved stimulation protocols that may reduce the occurrence of *de novo* aneuploidy following COH.

Materials and methods

Study population

This retrospective study involved 430 patients who underwent PGT-SR and 47 patients who underwent PGT-M from January 2017 to December 2021 (Table 1). Anonymous data were obtained from the Center for Reproductive Medicine at The Third Affiliated Hospital of Zhengzhou University. Chromosomal karyotyping of all participants was performed by standard Giemsa banding techniques prior to ovarian stimulation. Couples where at least one partner was a known carrier of a reciprocal translocation (REC), Robertsonian translocation (ROB) or inversion (INV) were assigned to the PGT-SR group. Patients with monogenetic mutations but normal karyotypes were assigned to the PGT-M group. Basic patient information was gathered, and other parameters, including the number of oocytes retrieved, fertilization rate, embryo formation rate and blastocyst formation rate, were recorded.

Ethics approval

The study was reviewed and approved by the Ethics Committee of the Third Affiliated Hospital of Zhengzhou University, and all patients underwent genetic counseling. Written informed consent for participation was not required for this study, in accordance with national legislation and institutional requirements.

Basic clinical characteristics

Baseline demographic information was collected, including maternal and paternal age (years, y) and maternal and paternal body mass index (BMI; kg/m^2). Basal plasma follicle stimulating

TABLE 1 Patient characteristics and corresponding PGT data.

	PGT-SR	PGT-M	t/χ^2	*P*
No. of cycles	430	47		
Maternal age (years, $\bar{x}$± SD)	30.94 ± 4.06	30.83 ± 3.78	0.373	0.709
Maternal BMI (years, $\bar{x}$± SD)	23.56 ± 3.11	23.53 ± 3.15	0.142	0.887
Paternal age (years, $\bar{x}$± SD)	31.56 ± 4.01	31.86 ± 3.44	1.109	0.269
Paternal BMI ($\bar{x}$± SD)	25.45 ± 4.08	25.77 ± 4.09	0.970	0.332
FSH (mIU/mL, $\bar{x}$± SD)	6.55 ± 1.71	6.72 ± 1.51	1.292	0.196
LH (mIU/mL, $\bar{x}$± SD)	6.28 ± 4.06	6.66 ± 2.85	1.671	0.096
E_2 (pmol/L, $\bar{x}$± SD)	147.44 ± 86.69	139.35 ± 57.12	1.256	0.209
AMH (pmol/L, $\bar{x}$± SD)	28.57 ± 14.24	27.20 ± 13.51	1.312	0.191
No. of retrieved oocytes (n)	6,899	733		
No. of blastocysts for PGT analysis (n)	1,734	191		
Oocytes in MII stage (%, n)	79.58% (5,490/6,899)	79.26% (581/733)	0.040	0.841
2PN fertilized oocytes (%, n)	81.77% (4,489/5,490)	81.58% (474/581)	0.012	0.913
Day 3 available embryos (%, n)	80.98% (3,635/4,489)	79.54% (377/474)	0.574	0.449
Available blastocysts (%, n)	49.63% (1,804/3,635)	61.01% (230/377)	17.696	<0.001*
Blastocysts with genetic results (%, n)	98.62% (1,710/1,734)	99.48% (190/191)	0.994	0.319
Euploid blastocysts (%, n)	33.63% (575/1,710)	60.53% (115/190)	53.505	<0.001*
Aneuploid blastocysts (%, n)	55.03% (941/1,710)	23.68% (45/190)	67.300	<0.001*
Mosaic blastocysts (%, n)	11.34% (194/1,710)	15.79% (30/190)	3.248	0.072
No. of blastocysts with *de novo* chromosomal abnormalities (%, n)	22.98% (393/1,710)	22.63% (43/190)	0.012	0.913

Value are presented as means ± standard deviations or number, n (%).
*p< 0.05 was considered statistically significant with respect to the PGT-SR group.
PGT-SR, Preimplantation Genetic Testing for Structural Rearrangements; PGT-M, Preimplantation Genetic Testing for Monogenic; BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E_2, estradiol; AMH, Anti-Mullerian hormone; PGT, Preimplantation Genetic Testing; MII, second metaphase; PN, pronucleus.

hormone (FSH; mIU/mL), luteinizing hormone (LH; mIU/mL) and estradiol (E_2; pmol/L) concentrations were measured on day two or three of the menstrual cycle, and anti-Mullerian hormone (AMH; pmol/L) concentrations were measured on any day of the menstrual cycle. COH parameters, including the ovulation stimulation protocol used, Gn dosage (IU), duration of ovarian stimulation (days, d), number of retrieved oocytes and peak E_2 concentrations (pmol/L), were documented for all patients.

Controlled ovarian hyperstimulation

Ovarian stimulation and gonadotrophin (Gn) administration was performed by experienced clinicians taking into consideration maternal age, antral follicle count (AFC), basal FSH concentration, the cause of infertility and ovarian reserve function. COH cycles for PGT require the administration of more Gn to ensure that more oocytes are collected and enough transferable embryos remain after testing. Three stimulation protocols were used: a gonadotropin-releasing hormone (GnRH) antagonist, a GnRH agonist and progestin-primed ovarian stimulation (PPOS). Gn dosing and induction duration were adjusted with ovarian response, as monitored using transvaginal ultrasound and circulating E_2. Patients were categorized into different groups according to the total amount of Gn administered (< 2,000, 2,000–3,000 and > 3,000 IU), stimulation duration (< 10, 10–12 and > 12 d), number of oocytes retrieved (< 10, 10–15 and > 15 oocytes) and peak E_2 concentrations (< 10,000, 10,000–15,000 and > 15,000 pmol/L). hCG was administered by injection to promote oocyte maturation when the diameter of at least two follicles exceeded 18 mm.

Oocyte collection, intracytoplasmic sperm injection (ICSI), embryo culture, and blastocyst biopsy

Transvaginal ultrasonography-assisted oocyte aspiration was performed approximately 36 hours after the hCG injection. After oocyte retrieval, cumulus-oocyte complexes were cultured for 4 h and then inseminated by intracytoplasmic sperm injection (ICSI). If the oocytes were at the metaphase-I (MI) or germinal vesicle (GV) stage, they were cultured *in vitro* until mature for an additional 24 h and then fertilized. Fertilization was confirmed by the presence of two pronuclei (2PN) 17–18 h post-insemination. Embryo cleavage was evaluated 41–44 h (Day 2) and 65–68 h (Day 3) after ICSI. For the first 3 days post-ICSI, the embryos were cultured in G1™ plus (Vitrolife, Sweden) in a humidified incubator with 5% O_2 and 6% CO_2. All cleavage embryos were transferred into G2™ plus (Vitrolife, Sweden) sequential media and cultured until they reached the blastocyst stage. Blastocysts were scored using the Gardner grading system. Blastocysts graded above 3BC were used for subsequent biopsies. Trophectoderm biopsies were performed from day 5 to 7 of development, based on the time of gastrulation, using the laser method. For genetic analysis, 5–8 cells were biopsied and analyzed using NGS (17).

Sample preparation and NGS analysis

The biopsied samples were washed with G-MOPS™ plus medium and placed in 0.2-mL polymerase chain reaction (PCR) tubes with 2 μL PBS. NGS allows direct quantification of the sequenced DNA fragments based on read numbers. In accordance with the Illumina NGS protocol, raw data were further processed using computational bioinformatic algorithms to map and align the short sequence reads to a linear human reference genome sequence. A small minority of cases, for which DNA amplification failed, were excluded from the study. The minimum detection range was 4 Mb. An embryo was considered "abnormal" when the result deviated from the reference baseline. Embryos with< 20%, 20%–80% and > 80% aneuploid cells were classified as euploid, mosaic and aneuploid, respectively. In the case of aneuploid embryos, if the chromosome involved was the same as that of an affected parent, it was classified as a genetic abnormality. In contrast, if the imbalanced chromosome was normal in the parents, it was classified as a *de novo* abnormality.

Endometrium preparation and frozen embryo transfer

Natural cycle tracking, hormone replacement therapy and ovarian stimulation were used for endometrium preparation. Luteal-phase support was initiated when endometrial thickness reached at least 7 mm and was continued until 3 months of gestation. A single euploid embryo was chosen for transfer. Biochemical pregnancy was defined as a serum concentration of ß-hCG > 30 mIU/ml measured 2 weeks post-embryo transfer. Clinical pregnancy was confirmed by the ultrasonographic observation of a gestational sac 35 days post-embryo transfer. Spontaneous abortion was defined as a pregnancy with a gestational sac that did not result in a live birth. Live birth was defined as the delivery of at least one live birth at ≥ 28 weeks of gestation.

Statistical analysis

Statistical analyses were performed using SPSS 24.0. Continuous variables are presented as the mean ± standard deviation (SD). Categorical variables are presented as absolute values and percentage frequencies.

The chi-square test was used to compare differences in categorical variables, and one-way analysis of variance (ANOVA) to compare differences in continuous variables, between the groups. $P< 0.05$ was considered statistically significant. The relationships between various COH parameters and the incidence of *de novo* chromosomal abnormalities and clinical pregnancy outcomes were analyzed using logistic regression. [a]*p*-values were calculated using a mixed logistic model adjusted for maternal age, maternal BMI and blastocyst quality. [b]*p*-values were calculated using a mixed logistic model adjusted for maternal age, maternal BMI, method of endometrial preparation, endometrial thickness transfer day of blastocyst and blastocyst quality.

Results

General characteristics of study subjects

From January 2017 to December 2021, 477 PGT cycles were initiated at the study center for which blastocysts were subsequently biopsied. No significant differences were seen between the PGT-M and PGT-SR groups in terms of maternal age or BMI, paternal age or BMI, basal FSH, E_2 and LH or AMH. A total of 7,632 oocytes were collected, and 6,071 MII oocytes were subsequently used for ICSI. In the PGT-SR and PGT-M groups, 81.77% and 81.58% of MII oocytes were successfully fertilized and developed into normally fertilized oocytes with two pronuclei (2PN), of which 49.63% and 61.01% fertilized oocytes developed into blastocysts suitable for biopsy, respectively. In the PGT-SR group, 1,710 (98.62%) of blastocysts had genetic results, of which 575 (33.63%) were euploid, 941 (55.03%) were aneuploid, 194 (11.34%) were mosaic and 393 (22.98%) had *de novo* chromosomal abnormalities. In the PGT-M group, 190 blastocysts had genetic results, of which 115 (60.53%) were euploid, 45 (23.68%) were aneuploid, 30 (15.79%) were mosaic and 43 (23.63%) had *de novo* chromosomal abnormalities. The total number of blastocysts, number of euploid blastocysts and number of aneuploid blastocysts were significantly different in the PGT-SR and PGT-M groups (all $p< 0.05$), as shown in Table 1.

Parent's age and the occurrence of *de novo* chromosomal abnormalities

As shown in Table 2, no significant differences were seen between the PGT-SR and PGT-M groups in the incidence of *de novo* chromosomal abnormalities in each maternal age group and paternal age group. However, within the PGT-SR group, there was a statistically significant increase in such abnormalities with increasing maternal age ($p< 0.001$). Similarly, the rate of *de novo* chromosomal abnormalities

TABLE 2 Incidence of *de novo* chromosomal abnormality in different maternal and paternal age groups.

	The incidence of *de novo* chromosomal abnormalities			
	PGT-SR cycles	PGT-M cycles	χ^2	*p*
Maternal age				
<30	17.55% (109/621)	16.39% (10/61)	0.052	0.820
30–35	24.29% (221/910)	24.35% (28/115)	0.000	0.988
>35	35.20% (63/179)	35.71% (5/14)	0.002	0.969
χ2	26.302	2.878		
p	<0.001*	0.237		
Paternal age				
<30	20.19% (105/520)	19.61% (10/51)	0.010	0.921
30–35	22.86% (216/945)	23.01% (26/113)	0.001	0.971
>35	29.39% (72/245)	26.92% (7/26)	0.069	0.793
χ2	7.974	0.549		
p	0.019*	0.760		

Values are presented as number, n (%).
*p< 0.05 was considered statistically significant.
PGT-SR, Preimplantation Genetic Testing for Structural Rearrangements; PGT-M, Preimplantation Genetic Testing for Monogenic.

was found to increase with paternal age in the PGT-SR group (p = 0.019). In contrast, in the PGT-M group, the rate of blastocysts with *de novo* chromosomal abnormalities increased with maternal or paternal age, but these differences were not statistically significant ($p > 0.05$).

COH parameters and the incidence of *de novo* chromosomal abnormalities

Logistic regression analysis was used to analyze the association between ovarian stimulation factors and the incidence of *de novo* chromosomal abnormalities by adjusting for maternal age and BMI and blastocyst quality (Table 3). No significant differences were identified in different stimulation protocols, Gn doses, stimulation durations, number of retrieved oocytes or maximal E_2 concentrations on hCG trigger day between the PGT-SR and PGT-M groups after adjusting for confounding factors.

COH parameters and clinical pregnancy outcomes

After adjusting for confounding factors, we found that COH parameters were not associated with clinical pregnancy outcomes (Figures 1–5). Clinical pregnancy rates ranged from 60.54% to 73.53% ($^bp = 0.293$) for the different stimulation methods, from 63.79% to 69.66% ($^bp = 0.677$) for different Gn dosages, from 63.53% to 67.86% ($^bp = 0.827$) for different stimulation durations, from 65.04% to 68.09% ($^bp = 0.816$) for different numbers of retrieved oocytes and from 63.01% to 73.40% ($^bp = 0.432$) for different maximal E_2 concentrations. Corresponding live birth rates (LBRs) were 52.38%–64.71% ($^bp = 0.320$), 56.52%–58.43% ($^bp = 0.969$), 54.12%–59.69% ($^bp = 0.605$), 56.87%–61.70% ($^bp = 0.600$) and 55.25%–60.64% ($^bp = 0.959$), respectively. Similarly, no significant linear trends of relationship were seen between any of these factors and the rates of miscarriage (12.00%–13.48%, $^bp = 0.780$; 9.01%–16.13%, $^bp = 0.358$; 11.69%–14.82%, $^bp = 0.393$; 9.38%–13.67%, $^bp = 0.498$;

TABLE 3 Incidence of *de novo* chromosomal abnormalities for different ovarian hyperstimulation parameters.

	De novo chromosomal abnormalities in the PGT-SR group					*De novo* chromosomal abnormalities in the PGT-M group				
	Proportion	*p*	OR (95% CI)	^ap	^{a}OR (95% CI)	Proportion	*p*	OR (95% CI)	^ap	^{a}OR (95% CI)
Stimulation protocol										
GnRH agonist	22.70% (183/806)	Ref		Ref		18.64% (11/59)	Ref		Ref	
GnRH antagonist	22.94% (167/728)	0.913	1.013 (0.798–1.287)	0.287	1.143 (0.893–1.463)	27.72% (28/101)	0.199	1.674 (0.762–3.676)	0.076	2.127 (0.923–4.900)
PPOS	24.43% (43/176)	0.622	1.101 (0.752–1.612)	0.962	1.010 (0.680–1.498)	13.33% (4/30)	0.529	0.671 (0.194–2.320)	0.924	0.99 (0.257–3.434)
Gn dose (IU)										
<2,000	21.52% (88/409)	Ref		Ref		13.79% (4/29)	Ref		Ref	
2,000-3,000	22.96% (180/784)	0.571	1.087 (0.815–1.451)	0.936	0.988 (0.734–1.329)	23.00% (23/100)	0.289	1.867 (0.589–5.918)	0.478	1.593 (0.440–5.759)
>3,000	24.18% (125/517)	0.339	1.163 (0.853–1.586)	0.397	0.866 (0.620–1.208)	26.23% (16/61)	0.192	2.222 (0.669–7.376)	0.409	1.826 (0.438–7.624)
Stimulation duration (d)										
<10	22.53% (105/466)	Ref		Ref		22.81% (13/57)	Ref		Ref	
10–12	23.82% (187/785)	0.602	1.075 (0.819–1.412)	0.934	0.988 (0.748–1.306)	23.85% (26/109)	0.880	1.060 (0.496–2.266)	0.698	0.851 (0.377–1.923)
>12	22.00% (101/459)	0.847	0.970 (0.712–1.322)	0.203	0.813 (0.592–1.118)	16.67% (4/24)	0.537	0.677 (0.196–2.337)	0.286	0.484 (0.128–1.836)

(Continued)

TABLE 3 Continued

	De novo chromosomal abnormalities in the PGT-SR group					*De novo* chromosomal abnormalities in the PGT-M group				
	Proportion	*p*	OR (95% CI)	[a]*p*	[a]OR (95% CI)	Proportion	*p*	OR (95% CI)	[a]*p*	[a]OR (95% CI)
No. of retrieved oocytes										
<10	29.57% (55/186)	Ref		Ref		21.62% (8/37)	Ref		Ref	
10–15	24.24% (135/557)	0.150	0.762 (0.526–1.103)	0.230	0.792 (0.541–1.160)	22.86% (8/35)	0.900	1.074 (0.353–3.264)	0.834	1.134 (0.350–3.669)
>15	20.99% (203/967)	0.011	0.633 (0.446–0.899)	0.188	0.780 (0.539–1.129)	22.88% (27/118)	0.873	1.076 (0.440–2.627)	0.305	1.765 (0.596–5.228)
Peak E_2 levels(pmol/L)										
<10,000	24.28% (201/828)	Ref		Ref		25.71% (27/105)	Ref		Ref	
10,000–15,000	20.86% (87/417)	0.178	0.822 (0.619–0.193)	0.483	0.901 (0.674–1.205)	18.18% (6/33)	0.379	0.642 (0.239–1.722)	0.789	0.860 (0.284–2.605)
>15,000	22.58% (105/465)	0.492	0.910 (0.695–1.191)	0.931	1.012 (0.768–1.334)	19.23% (10/52)	0.369	0.688 (0.304–1.557)	0.690	0.837 (0.350–2.006)

Values are presented as number, n (%). Unless otherwise stated, p-values were calculated using a univariable mixed logistic model. [a]p-values were calculated using a mixed logistic model adjusted for maternal age and BMI and blastocyst quality. [a]Odds ratios (OR) were adjusted by maternal age and BMI and blastocyst quality. 95% confidence intervals (CI) are provided. PGT-SR, Preimplantation Genetic Testing for Structural Rearrangements; PGT-M, Preimplantation Genetic Testing for Monogenic; Ref, Reference group; OR, odds ratios; GnRH, gonadotropin-releasing hormone; PPOS, progestin-primed ovarian stimulation; Gn, gonadotropins; E2, estradiol.

and 7.02%–17.39%, [b]$p = 0.089$, respectively). The original data can be found in Supplementary Tables 1, 2.

Discussion

The study comprehensively evaluated the incidence of *de novo* chromosomal abnormalities in blastocysts and clinical pregnancy outcomes for different COH parameters across 477 PGT cycles. The incidence of *de novo* chromosomal abnormalities was 22.98% in PGT-SR cycles and 22.63% in PGT-M cycles. Such abnormalities were found to increase with maternal or paternal age. It was found that the stimulation protocol, Gn dosage, stimulation duration, number of oocytes retrieved and peak E_2 concentrations did not affect *de novo* aneuploidy rates or clinical pregnancy outcomes. We identified a significant age-dependent increase in *de novo* chromosomal abnormalities in the PGT-SR group. To our knowledge, this is the first study examining the relationships between COH parameters and *de novo* chromosomal abnormalities and clinical pregnancy outcomes following PGT.

In terms of ART, the high incidence of chromosomal abnormalities in the resultant embryos and the impact of such

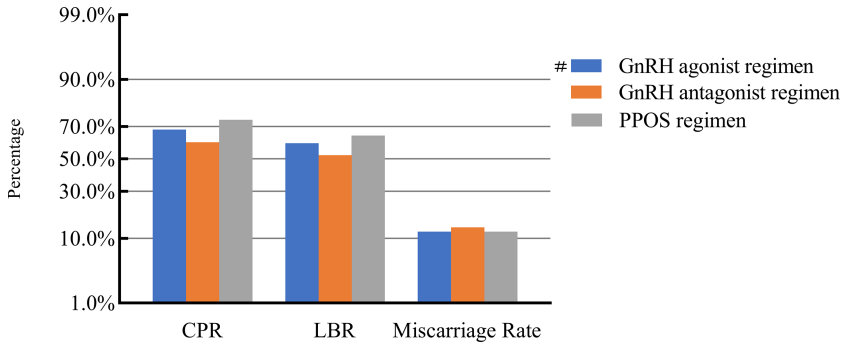

FIGURE 1
Association between stimulation protocols and pregnancy outcomes. #, Reference group [b]*p*-values were calculated using a mixed logistic model adjusted for maternal age and BMI, method of endometrial preparation, endometrial thickness, transfer day of blastocyst and blastocyst quality. CPR, clinical pregnancy rate; LBR, live birth rate; GnRH, gonadotropin-releasing hormone; PPOS, progestin-primed ovarian stimulation.

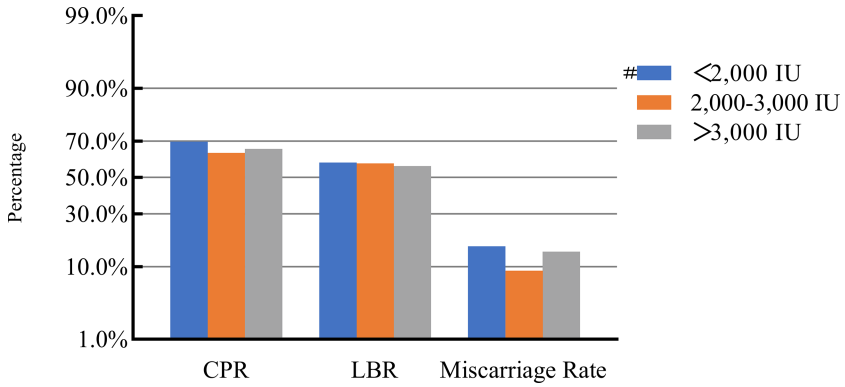

FIGURE 2
Association between gonadotropin dosage and pregnancy outcomes. #, Reference group [b]*p*-values were calculated using a mixed logistic model adjusted for maternal age and BMI, method of endometrial preparation, endometrial thickness, transfer day of blastocyst and blastocyst quality. CPR, clinical pregnancy rate; LBR, live birth rate.

abnormalities on implantation have been widely discussed (18–20). Though PGT-A candidates have been studied frequently in previous studies, such individuals often harbor unexplained confounding factors that lead to high aneuploidy rates, thereby resulting in recurrent miscarriages and repeated implantation failures that necessitate PGT-A (21). Therefore, PGT-A samples were not suitable for accurately examining the relationship between ovarian stimulation and aneuploidy. Additionally, gametes produced by meiotic segregation in patients with unbalanced translocations potentially have an increased risk of aneuploidy. In addition to the direct effects of such translocations on the unbalanced chromosome, they may also affect the meiosis of other structurally normal chromosomes, leading to an increased risk of additional aneuploid gametes. This phenomenon is known as the inter chromosomal effect (ICE) (22). Many studies have attempted to confirm the ICE hypothesis, but it remains disputed (22–24). Therefore, we focused our study on *de novo* chromosomal abnormalities rather than pre-existing translocations or inversions. Additionally, patients undergoing PGT-M were chosen as a control group to verify whether ICE occurs.

It is well known that increased maternal age is an independent factor that negatively impacts the probability of obtaining a euploid embryo (2, 25). In line with this, we found that the incidence of *de novo* chromosomal abnormalities increased with maternal age. This might be because the risk of chromosomal abnormalities in oocytes and embryos increases with maternal age (26) largely due to recombination errors in early meiosis, defective spindle assembly checkpoints at MI and centromere cohesion loss (27). Similarly, our study found that older paternal age also has been associated with an increased incidence of *de novo* chromosomal abnormalities, which is consistent with previous studies. The association between aneuploidy rate and paternal age

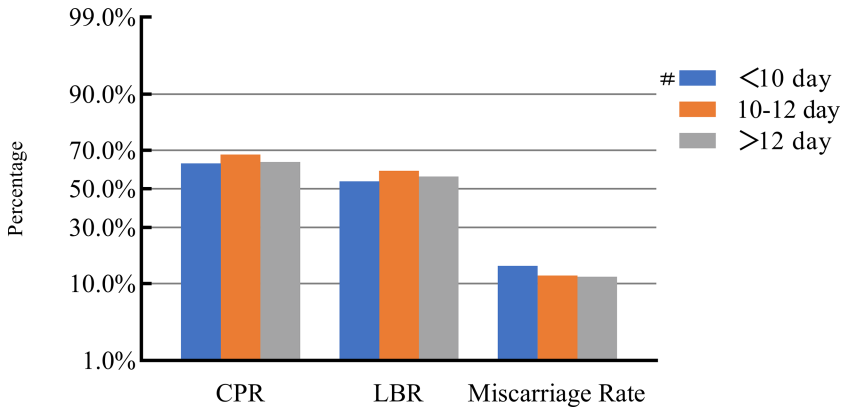

FIGURE 3
Association between stimulation duration and pregnancy outcomes. #, Reference group [b]*p*-values were calculated using a mixed logistic model adjusted for maternal age and BMI, method of endometrial preparation, endometrial thickness, transfer day of blastocyst and blastocyst quality. CPR, clinical pregnancy rate; LBR, live birth rate.

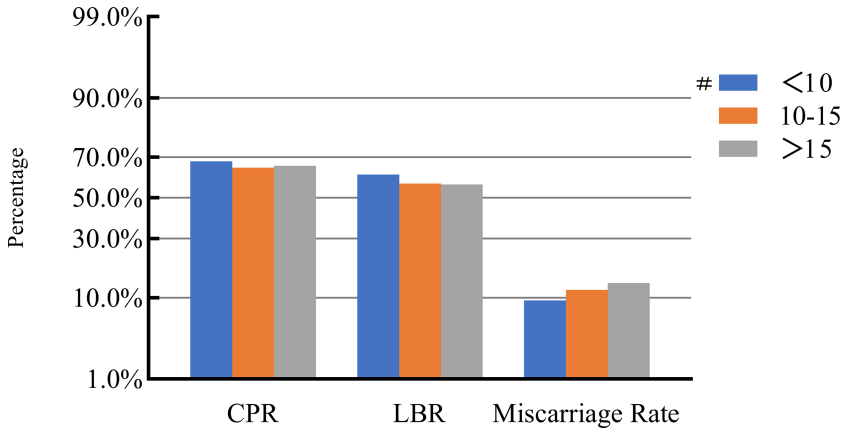

FIGURE 4
Association between the number of retrieved oocytes and pregnancy outcomes. #, Reference group [b]*p*-values were calculated using a mixed logistic model adjusted for maternal age and BMI, method of endometrial preparation, endometrial thickness, transfer day of blastocyst and blastocyst quality. CPR, clinical pregnancy rate; LBR, live birth rate.

may be related to the level of sperm DNA fragmentation, which is theoretically believed that post-fertilization sperm DNA strand breaks can be repaired within the oocyte. However, the level of sperm DNA fragmentation deteriorates with age and the repair capacity of the embryo may not be able to compensate for the decrease in DNA quality, possibly leading to poorer embryo quality and aneuploidy rate increased (28, 29). In contrast, this study found no statistical differences between the PGT-SR and PGT-M groups in terms of the incidence of *de novo* chromosomal abnormalities across different age groups, suggesting that the ICE phenomenon does not occur. Therefore, despite finding evidence that increased *de novo* chromosomal abnormalities can occur in these conditions, they cannot be attributed to ICE.

Other than maternal or paternal age, chromosomal abnormalities have been thought to occur due to various aspects of the ART process, such as the ovarian stimulation protocols used for IVF. Undoubtedly, the goal of ovarian stimulation is to induce the ongoing development of multiple dominant follicles to obtain multiple mature oocytes (30, 31). Such ovarian stimulation is a critical aspect of IVF, particularly PGT cycles. In general, approximately 10–15 days of ovarian stimulation is required, during which E_2 levels increase 10–20-fold compared with natural cycles. Some studies have proposed that oocytes obtained *via* natural or modified natural cycles are superior to those obtained *via* induced ovulation cycles (5). It is also believed that altered regulation of meiotic spindle alignment may occur due to the use of exogenous gonadotropins to stimulate the development of multiple follicles (32). Therefore, we examined whether varied COH parameters are correlated with an increase in *de novo* chromosomal abnormalities and worse pregnancy outcomes.

Our study demonstrated that the incidence of *de novo* chromosomal abnormalities and clinical pregnancy outcomes

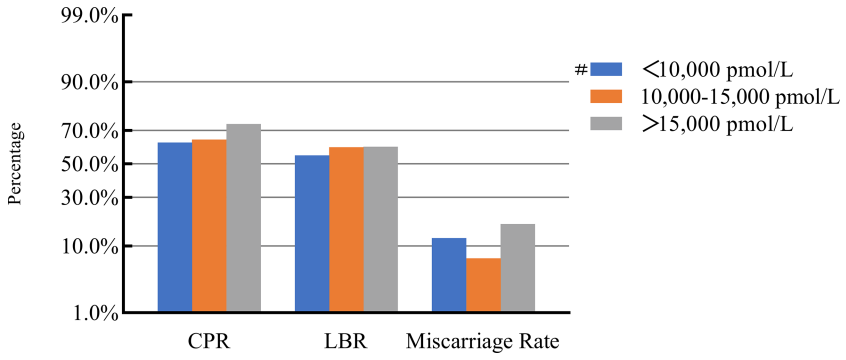

FIGURE 5
Association between maximal E_2 levels and pregnancy outcomes. #, Reference group [b]*p*-values were calculated using a mixed logistic model adjusted for maternal age and BMI, method of endometrial preparation, endometrial thickness, transfer day of blastocyst and blastocyst quality. CPR, clinical pregnancy rate; LBR, live birth rate; E2, estradiol.

are not affected by the COH stimulation protocol used; a result consistent with previous findings (33). We found that the rate of *de novo* chromosomal abnormalities and miscarriages in those treated with a GnRH antagonist was slightly higher than in those treated with a GnRH agonist. However, this difference was not statistically different. Similarly, a Chinese birth cohort study of ART and birth defects reported that GnRH antagonist-based stimulatory regimens were associated with an increased risk of birth defects and that aneuploidy was a major factor resulting in these birth defects (34). Another study revealed that GnRH antagonist treatment was associated with higher aneuploidy rates in early aborted tissues and blastocysts than GnRH agonist treatment (35). These conflicting results may result from heterogenous study populations or variation in the specific methods used by different physicians in different IVF centers. Therefore, a strength of this study is that we focused on *de novo* chromosomal abnormalities rather than aneuploidy.

Previous theoretical studies have speculated that the administration of supraphysiological exogenous Gn interferes with natural follicle selection, potentially leading to chromosomal dysfunction and poor quality oocytes (36). It has also been proposed that low-dose Gn is associated with reduced aneuploidy rates in human preimplantation embryos (9, 37). In contrast, our findings indicate that the Gn dose, duration of ovarian stimulation treatment and peak of estrogen on hCG trigger day were not associated with the incidence of *de novo* chromosomal abnormality and clinical pregnancy outcomes. Other studies have demonstrated that different Gn dosages and the number of oocytes retrieved were not relevant to embryonic aneuploidy rates and pregnancy rates (38, 39). Moreover, another study reported that high-dose Gn leads to an increased risk of embryonic aneuploidy, but only in those with reduced ovarian reserve (6). Such reports of high-dose exogenous Gn leading to increased meiotic segregation errors in oocytes and increasing aneuploidy rates have not been confirmed. Other studies have reported that the duration of ovarian stimulation significantly affects LBRs of fresh cycles, potentially due to the adverse effects of high levels of Gn on endometrium receptivity - effects that are potentially reduced or eliminated in the case of frozen cycles (40). Therefore, in the absence of an alternative, milder method by which to stimulate ovarian maturation, conventional ovarian stimulation remains the recommended approach for most patients undergoing PGT. Our study reinforces previous findings that pregnancy rates are independent of the Gn dosage in frozen embryo transfer cycles.

In the study, we found that the duration of ovarian stimulation was not correlated with the incidence of *de novo* chromosomal abnormalities, while the > 12d group is lowest. It might because longer stimulation times are more conducive to the physiological process of cellular self-repair (41). This hypothesis has been corroborated in this study, as the incidence of *de novo* chromosomal abnormalities was reduced for the longer GnRH agonist protocol. Response to ovarian stimulation was assessed by the number of oocytes retrieved and maximal E_2 concentrations on hCG trigger day. Supraphysiological levels of E_2 are detected due to the maturation of multiple follicles, but more oocytes, and subsequently higher quality blastocysts, are required to obtain at least one euploid embryo for implantation. Clinicians have expressed concern that COH might affect the development and quality of oocytes, as multiple small, nondominant follicles will mature following exogenous Gn treatment (11). Labarta et al. reported (42) that a greater ovarian response, and thus number of oocytes retrieved, results in a greater number of resultant euploid embryos. Moreover, we found that the number of oocytes retrieved and maximal E_2 concentrations on hCG trigger day were not correlated with *de novo* chromosomal abnormality rates and clinical pregnancy outcomes. These findings reinforce the lack of association between E_2 concentrations and embryo quality or pregnancy outcomes seen in previous studies (43, 44). It had been suggested that a reduced oocyte yield represents an appropriate response to some forms of ovarian stimulation, as only the most competent follicles and oocytes can develop. However, older patients and those with a poor ovarian reserve should also be considered.

Conclusion

In conclusion, this study revealed that total Gn dosage, ovarian stimulation duration, peak E_2 concentrations and number of oocytes retrieved did not influence *de novo* chromosomal abnormality rates or clinical pregnancy outcomes. This study will provide clinicians with valuable information to consider when choosing an ovarian stimulation protocol. In addition to maternal age, AFC, baseline FSH levels and known causes of infertility, it is important to consider the potential to retrieve as many oocytes as possible to provide more embryos for biopsy. However, we do not advocate the use of high-dosage stimulation protocols, especially for patients at an increased risk of complications such as ovarian torsion and hyperstimulation syndrome. This is the first clinical study to examine the role of COH parameters in determining the incidence of *de novo* chromosomal abnormalities in Chinese IVF patients. Our findings may inform the development of novel strategies for ovarian stimulation during ART that promote both safety and efficacy. However, the mechanisms underlying *de novo* chromosomal abnormalities that occur during PGT cycles remain unclear.

Limitations

This study was retrospective in nature, had a modest sample size and had a heterogenous study population. As such, larger prospective, multicenter and randomized controlled trials

should be carried out to confirm our findings and further explore the mechanisms underlying the differential effects of different ovarian stimulation protocols.

Data availability statement

The original contributions presented in the study are included in the article/supplementary files, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by The review board of the Third Affiliated Hospital of Zhengzhou University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

YL, RY and YG designed the study. PZ, JS and YZ were involved in the data extraction and analyses. YL and JS were involved in drafting this article. RP, JZ reviewed the data and article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1080843/full#supplementary-material

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Correlation study of male semen parameters and embryo aneuploidy in preimplantation genetic testing for aneuploidy

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Objective: The purpose of this study was to evaluate the influence of abnormal semen parameters on embryo aneuploidy based on single nucleotide polymorphism microarray (SNP array).

Methods: A total of 464 blastocysts from 103 PGT-A cycles were analyzed. The embryo quality and embryo aneuploidy rates were compared between different groups which divided by male semen parameters (sperm concentration, motility, morphology, and DFI) according the WHO criteria (2021).

Results: The total blastocysts chromosome aneuploidy rate was 42.3% (191/452). In the teratozoospermia group, the good-quality embryo and blastocyst formation rate were lower than the normal group(44.4% vs 60.7%, P <0.01; 33.3% vs 43.5%, P <0.05), The good-quality embryo rate in normal DFI group was significantly higher than high-DFI group (59.0% vs 48.4%, P < 0.05). The blastocyst aneuploidy rate in low sperm concentration group, and high DFI group was no differences between with that in normal sperm concentration and DFI group (47.7% vs 37.8% and 44.7% vs 37.8%, P>0.05). The aneuploid rate of blastocyst in teratozoospermic and asthenozoospermia group was significantly higher than that of normal morphology and motility group (50.0% vs 34.0% and 46.7% vs 33.7%, P<0.05).

Conclusion: Our study revealed that sperm DFI were positively correlated with blastocyst aneuploidy rate, while sperm motility and sperm morphology rate were negatively correlated with blastocyst aneuploidy rate. Abnormal semen parameters may affect embryo quality and increase the aneuploidy rate of blastocyst chromosomes, suggesting that in clinical practice of assisted reproduction patients with abnormal semen parameters can be treated in advance to improve sperm quality, so as to reduce the impact on embryo quality and achieve a better pregnancy outcome.

KEYWORDS

semen parameters, embryo aneuploidy, PGT-A, sperm morphology, sperm DNA fragmentation index

Abbreviations: AMH, anti-Mullerian hormone; ART, assisted reproductive technology; BMI, body mass index; DFI, DNA fragmentation index; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; PGT-A, preimplantation genetic testing for aneuploidy; SCSA, sperm chromatin structure assay; SNP, single nucleotide polymorphism.

Introduction

In Assisted Reproductive Technology(ART)treatment, the embryo quality is a key factor for pregnancy outcomes. Studies have shown that the aneuploidy rate in human early embryos is 56-84% during *in vitro* fertilization (IVF) (1). Aneuploidy embryos not only affects embryo implantation and causes pregnancy failure but can also lead to adverse pregnancy outcomes, such as embryonic abortion and miscarriage during early pregnancy (2). The mechanism of embryonic aneuploidy is complex. Most studies have focused on the influence of female factors (oocyte mitosis errors, female advanced age etc.) on embryo aneuploidy (3, 4). Maternal aneuploidy increases exponentially with female age, reaching up to 80% by age 45 (5). While, there was no association between paternal age and aneuploidy rates (6, 7). The male gamete makes up half of the genetic makeup of the embryo; therefore, male factors also play an essential role in embryo chromosomes. Besides age, male sperms are vulnerable to many factors, such as oxidative stress, that result in damage to sperm DNA or increase the number of aneuploid sperms (8, 9), thereby affecting the embryo quality and pregnancy outcomes. Semen parameter is one of the important indicators for male fertility evaluation, which is closely related to embryo development and pregnancy outcome in ART (10). Burrello et al. (11) found that patients with oligo-astheno-teratozoospermia have an increased aneuploidy rate. However, Rossella et al. (12) showed male factor just impairs early embryonic competence in terms of fertilization rate. The euploidy rate and implantation potential of the obtained blastocysts are independent from sperm quality. Studies have shown that a high sperm DNA fragmentation index (DFI) of sperm increases the early miscarriage rate (13, 14), and embryonic chromosomal abnormality is one of the main causes of early pregnancy loss. A recent meta-analysis also showed that couples with unexplained recurrent miscarriage had significantly increased levels of sperm DFI and significantly decreased levels of total motility compared with couples without recurrent miscarriage (15). Therefore, we hypothesized that male factors are also closely related to embryo aneuploidy.

Preimplantation Genetic Testing for Aneuploidy (PGT-A) is an effective method for detecting embryonic chromosomal abnormalities and screening euploid embryos for transfer. In this study, we retrospectively analyzed embryo quality and blastocyst aneuploidy rates with different male semen parameters (sperm concentration, motility, morphology, and DFI) during PGT-A cycles performed at our hospital to investigate the relationship between male semen parameters with the embryo quality and blastocyst aneuploidy rate in assisted reproductive technology.

Materials and methods

Participants and study design

We collected data from 103 PGT-A cycles performed between April 2018 and February 2019 at the Reproductive Center, First Affiliated Hospital of Zhengzhou University. A total of 464 blastocysts were sent for a single nucleotide polymorphism (SNP) array chromosome aneuploidy assay. The inclusion criteria of the cases in this study were: 1) maternal ageless than 35year old; 2) a history of repeated miscarriage or repeated implantation failure; 3) no hereditary disease or chromosomal abnormality detected in either partner. This study was approved by the Ethics Committee of Zhengzhou University.

The male sperm concentration, motility, morphology, and DFI were rated as normal or abnormal according to the World Health Organization (WHO) criteria, 6th Edition (16), as follows: sperm concentration: oligospermia (sperm concentration < 16 x 10^6/mL) and normal density (sperm concentration ≥ 16 × 10^6/mL); sperm motility: asthenospermia (sperm motility < 42%) and normal motility (sperm motility ≥ 42%); sperm morphology: teratozoospermia (sperm cells with normal morphology < 4%) and normal morphology (sperm cells with normal morphology ≥ 4%); and sperm DFI: high DFI (DFI ≥ 15%) and normal DFI (DFI < 15%). In order to avoid research bias, in the grouping comparison of semen parameters, only the grouping parameters were different, and other parameters were normal. The rates of day 3 good-quality embryos, blastocyst formation, implantation and blastocyst aneuploidy were compared between groups.

Semen analysis, sperm morphology assessment and sperm DNA fragmentation assay

All cases in this study were subjected to at least two semen assessments. The men were instructed to abstain from sex for 2-7 days and to collect sperm samples into a sterile container by masturbation. After the semen was completely liquefied, 10 μL of liquefied semen was placed in the sperm Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) for motility and concentration analysis. Hematoxylin and eosin (HE) staining was performed to analyze the sperm morphology according to the Kruger criteria, and the lower limit for normal sperm morphology is 4%. All semen analysis was performed in accordance with the 6th WHO Laboratory Manual for Examination and Processing (2021). Sperm DNA fragmentation was evaluated by DNA fragmentation index (DFI) with the sperm chromatin structure assay (SCSA^{TM}). An appropriate amount of semen was diluted in TNE buffer (0.01M Tris-HCL, 0.15M NaCl, 1 mM EDTA) to adjust the semen density to 0.5-1.0 x 10^6/mL Acridine orange (AO; PH6.0) solution was added for staining, and then the sperm DFI was calculated with fluorescence signals detected with a flow cytometer (BD FACS Canto II). The detailed operation steps refer to the previous description (17).

Ovarian stimulation, fertilization, and blastocyst biopsy

A long luteal phase protocol was used for controlled ovarian hyperovulation. Oocytes were harvested36-38 hours after the doses of 10000 IU human chorionic gonadotropin (HCG) trigger and fertilized with intracytoplasmic sperm injection (ICSI). The embryos were cultured and observed for development. A blastocyst biopsy was performed on day 5 or 6 after the blastocysts were formed, and 3-5 trophectoderm (TE) cells were collected for genetic testing. After biopsy, all blastocysts were vitrified (Cryotop device and solution, Kitazato) and stored in liquid nitrogen. The euploid

blastocysts were transfered during the frozen embryo transfer(FET) cycle. The good-quality embryos rate defined as the number of embryos with Peter grade I or II at the 8-cell stage (day 3 after fertilization)/two pronuclei (2PN) cleavage embryos. According Peter (18), embryos on the third day of development and with six to eight blastomeres, with < 10% of fragmentation, and without multi-nucleation were considered good quality embryos. Blastocyst formation and scoring was followed by Gardner DK, on day 5 or day 6 of development. The embryo develops into a blastocyst with inner cell mass (ICM) and trophoblast cells (TE), and then it can performe a blastocyst scoring according to the Gardner scoring system (19). Good quality blastocyst was defined as having an ICM and TE type A or B.

Whole genome amplification and blastocysts chromosome aneuploidy definition

After biopsy, the ectodermal trophoblasts cells were collected and placed in 5μl of 0.2 N KOH for cell lysis. Then whole-genome amplification (WGA) was performed with the REPL-g Single Cell Kit (QIAGEN, 150345) according to the package insert. The SNP array data were detected using a Human CytoSNP-12DNA microarray array (Illumina, San Diego, CA, USA) and an Illumina HiScanSQ BeadArray Reader. Chromosome aneuploidy in the blastocysts was analyzed with GenomeStudio Software v2011 (Illumina). The specific steps of this method were described previously (20). The aneuploidy rate was defined as the ratio of the number of blastocysts with abnormal chromosome and the total number of blastocysts subjected to SNP array detection.

Statistical analysis

SPSS v19.0 was used for the statistical analysis. The data were expressed as the mean ± standard deviation (x ± s). T-tests were applied in the comparison of basic parameters (female age, BMI, AMH level, male age et al.). Before the t-test, statistical software was used to conduct the normal distribution test and homogeneity analysis of variances (Levence-test) for all the data of each group. And nonparametric test was applied when t test requirements were not met. The good-quality embryos rate, blastocyst formation rate, implantation rate and aneuploidy rate were analyzed with the $\chi 2$ test. It was considered significant difference when $P < 0.05$. Binary logistic regression analysis was performed with the relevant factors that may affect good-quality embryos, blastocyst formation and blastocyst aneuploidy rate as independent variables and $P < 0.05$ was considered statistically significant.

Results

A total of 464 blastocysts obtained from103 PGT-A cycles received diagnostic by SNP-array karyomap chip. The mean age of men and women were 30.3 ± 5.5 and 32.1 ± 5.7 years. The woman's average BMI and AMH level were 23.7 ± 4.0 (kg/m^2) and 3.5 ± 2.7(ng/ml). DNA amplification failed for 12 blastocysts and the failure rate was2.6% (12/464). Of the rest452 blastocysts, 191 were chromosome aneuploid. The total blastocysts aneuploidy rate is 42.3% (191/452).

The basic information in the different sperm morphology and DFI groups

After the normal distribution and F-test, the basic parameter of female age, BMI, AMH level and male age meet the T-test conditions. Non-parametric test and t- test were used to statistically control other parameters of semen (concentration, motility and DFI) in comparison groups in the tables. And there was no significant difference between groups ($P > 0.05$). In Table 1, the good-quality embryo rate and blastocyst formation rate were 44.4% and 33.3% in the teratozoospermia group, which were significantly lower than the rates of60.7% and 43.5%, respectively, obtained in the normal morphology group ($P < 0.05$). The blastocyst aneuploidy rate was 50.0% in the teratozoospermia group, which was significantly higher than the rate of 34.0% obtained in the normal morphology group ($P < 0.05$). Moreover, the good-quality embryo rate was significantly higher in the normal DFI group than in the high-DFI group (59.0%vs48.4%, $P = 0.01$). But there were no significant differences between two groups in the blastocyst formation rate, implantation rate and blastocyst aneuploidy rate ($P = 0.27$, 0.93 and 0.28, respectively) (see Table 2). Figures 1, 2 were clearly shown the comparison of good-quality embryo rates, blastocyst formation rates, implantation rates and chromosome aneuploidy rates in the different sperm morphology and DFI group.

Logistic regression analysis of variables associated with good-quality embryos, blastocyst formation and blastocyst aneuploidy rate

After excluding confounding factors male and female age, sperm concentration and motility, the effects of sperm morphology and DFI on blastocyst euploidy rate, blastocyst formation rate and blastocyst aneuploidy rate were investigated. It can be seen from the Table 3 that compared with normal morphologic sperm, the good-quality embryo and blastocyst formation rate were decreased in the teratozoospermia group (P=0.008 and 0.038), and the risk of blastocyst aneuploidy rate was significantly increased (P=0.005).The good-quality embryo rate of high DFI group was lower than that of normal DFI group (P=0.031), and the risk of blastocyst aneuploidy rate in high DFI group was 3.213 times higher than that in normal DFI group (OR=3.213, P=0.027).

The blastocyst aneuploidy rates with different semen parameters groups

Figure 3 shows the blastocyst aneuploidy rates with different semen parameters groups (sperm concentration, motility, morphology, and DFI). The aneuploidy rate of blastocyst in abnormal semen parameters group were higher than that in normal group, especially in teratozoospermia and asthenospermia group, the blastocyst aneuploidy rate was significantly higher in the normal group ($P < 0.05$).

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TABLE 1 Comparison of parameters between different sperm morphology groups.

	Normal sperm group	Teratozoospermic group	*P*-value
Cycle (n)	54	14	
Female age (y)	31.1 ± 4.7	29.3 ± 5.5	0.29
Female BMI (kg/m^2)	23.6 ± 3.9	24.0 ± 4.1	0.45
Female AMH (ng/ml)	3.3 ± 1.8	3.9 ± 2.8	0.51
Male age (y)	32.4 ± 5.2	31.1 ± 5.9	0.39
Male BMI (kg/m^2)	25.4 ± 3.3	25.8 ± 3.7	0.74
Sperm morphology rate (%)	5.4 ± 0.5	1.8 ± 0.4	<0.01
Sperm DFI (%)	14.1 ± 7.1	22.7 ± 19.7	0.17
Sperm concentration (x10^6/ml)	53.5 ± 22.3	25.9 ± 21.6	0.22
Sperm motility (%)	42.1 ± 16.7	29.6 ± 17.3	0.34
Good-quality embryos rate	60.7%(357/588)	44.4%(84/189)	<0.01*
Blastocyst formation rate	43.5%(256/588)	33.3%(63/189)	0.01*
Implantation rate	60.3%(38/63)	57.1%(8/14)	0.83
Aneuploidy rate	34.0%(87/256)	50.0%(28/56)	0.02*
Amplification failure rate	3.1%(8/256)	5.4%(3/56)	0.67

*Statistical significant.

Discussion

At present, assisted reproductive technology (ART) has become an important way to address infertility. The quality of the embryo is closely related to clinical pregnancy rate in IVF. The high aneuploidy rate of early embryos greatly affects the embryo quality is a major factor for adverse pregnancy outcomes (21). In this study, the blastocyst aneuploidy rate averaged 42.3%, this ratio is similar to the results of Huang's research (22). This high rate may relate to the population in this study, because the aneuploidy rate of embryonic chromosomal is higher in older women and patients with repeated pregnancy failure than in the general population (23). Garcia et al.

TABLE 2 Comparison of parameters between different sperm DFI groups.

	Normal DFI group	High DFI group	*P*-value
Cycle (n)	40	24	
Female age (y)	29.5 ± 5.6	30.7 ± 3.9	0.17
Female BMI (kg/m$^{2)}$	23.4 ± 3.3	23.7 ± 3.5	0.66
Female AMH(ng/ml)	2.9 ± 1.7	3.8 ± 2.6	0.24
Male age (y)	30.7 ± 5.6	33.5 ± 4.4	0.16
Male BMI (kg/m2)	25.4 ± 3.3	25.9 ± 4.0	0.44
Sperm DFI (%)	8.5 ± 4.4	28.5 ± 10.7	<0.01
Sperm morphology rate (%)	5.0 ± 0.6	4.3 ± 0.8	0.29
Sperm concentration (x10^6/ml)	95.5 ± 32.3	62.5 ± 30.6	0.19
Sperm motility (%)	43.6 ± 10.1	34.8 ± 10.3	0.53
Good-quality embryos rate	59.0%(236/400)	48.4%(124/256)	0.01*
Blastocyst formation rate	41.0%(164/400)	36.7%(94/256)	0.27
Implantation rate	51.2%(22/43)	50.0%(10/20)	0.93
Aneuploidy rate	37.8%(62/164)	44.7%(42/94)	0.28
Amplification failure rate	1.2%(2/164)	4.3%(4/94)	0.26

Except for DFI, other semen parameters between normal DFI and high DFI group were normal, and there was no statistical difference (P>0.05). Good-quality embryos rate in normal DFI group was significantly higher than that of the high-DFI group (P<0.05). * Statistical significant.

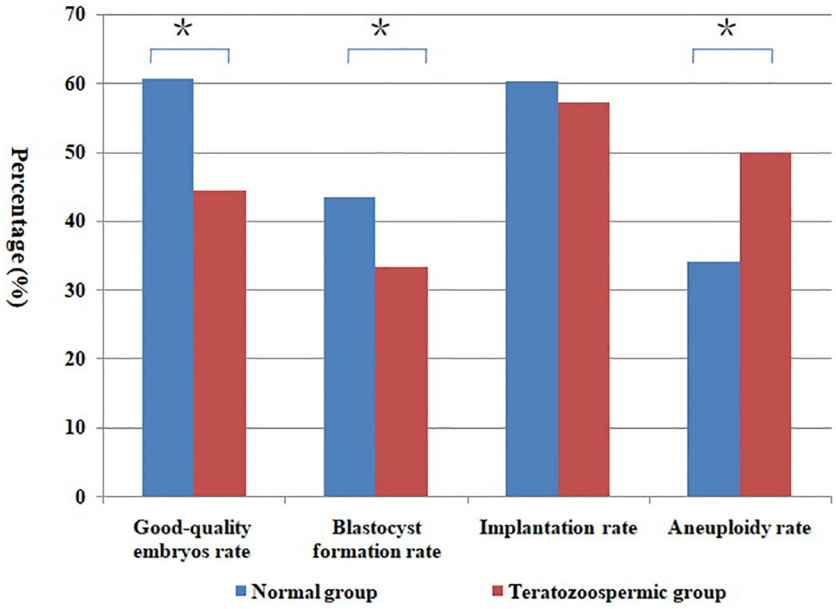

FIGURE 1
Comparison of different rates between normal and teratozoospermic group. *Statistically significant (P < 0.05).

conducted a study in a PGT-A population and also observed a high blastocyst aneuploidy rate (24). Generally, human embryo aneuploidy is believed to derive from the oocyte rather than sperm, which may be related to male versus female differences in gamete meiosis (25). However, studies have shown that as part of the embryo's genetic make-up, the effect of the male sperm nuclear genome can be detected as early as the single cell zygote stage and then throughout 8-cell stage (26). Sperm genomic defects affect centrosome function in the early stage and interfere with the formation of the prokaryotic nucleolus at the zygotic stage, which affects the number and distribution of nucleoli, causes delayed cleavage or increased fragmentation at the cleavage stage, and affects formation of the blastocyst and pregnancy outcomes in the late stage (27). Male factors are known to affect embryonic development, but the extent of this effect is unclear. In this study, we investigated the effect of the male semen quality on the embryo quality (good-quality embryo and blastocyst formation rates) and the blastocyst chromosomal aneuploidy rate during embryonic development and evaluated how male factors affected embryonic development.

Sperm morphology is an important indicator for evaluation of male fertility and is also a key parameter for predicting pregnancy outcomes of assisted reproductive technology (28).

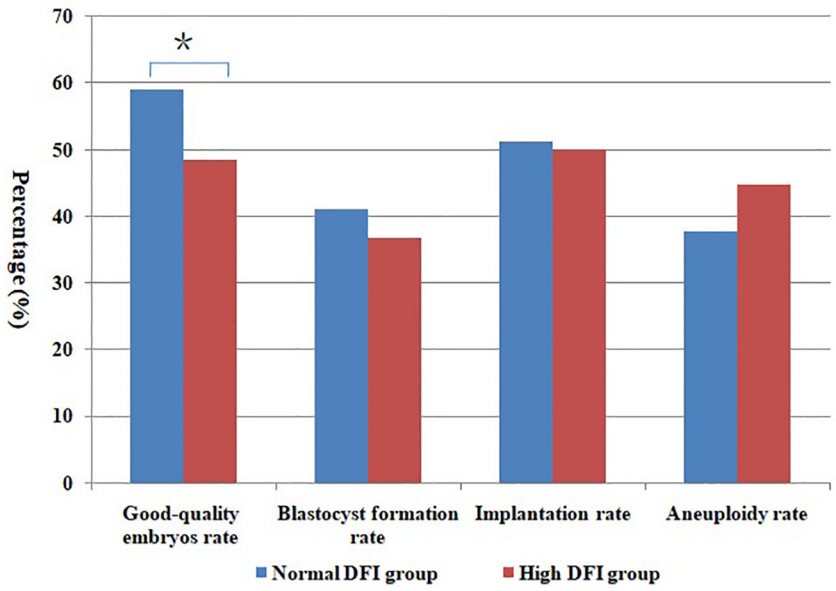

FIGURE 2
Comparison of different rates between normal DFI and high DFI group. *Statistically significant (P < 0.05).

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TABLE 3 Logistic regression analysis of variables associated with good-quality embryos, blastocyst formation and blastocyst aneuploidy rate.

Variable	Good-quality embryos rate		Blastocyst formation rate		Blastocyst aneuploidy rae	
	OR (95%IC)	*P*-value	OR (95%IC)	*P*-value	OR (95%IC)	*P*-value
Sperm morphology						
Normal sperm	Referent		Referent		Referent	
Teratozoospermic	0.335(0.271-0.564)	0.008*	0.851(0.692-0.923)	0.038*	6.747(3.743-10.512)	0.005*
Sperm DFI						
Normal DFI	Referent		Referent		Referent	
High DFI	0.696(0.524-0.812)	0.031*	0.913(0.787-1.406)	0.067	3.213(1.772-5.353)	0.027*

*Statistical significant. Logistic regression analysis excluded confounding factors: male and female age, sperm concentration and motility.

Ditzel et al. (29) showed that globozoospermia was associated with a significantly increased sperm chromosomal abnormality rate. Sperm chromosomes are directly involved in the chromosome composition of fertilized oocytes. Sperm with abnormal chromosome can lead to abnormal chromosome in the embryo after fertilization, with severe impacts on embryonic development, implantation, and the health of the offspring. In this study, the embryo aneuploidy rate was significantly higher in the teratozoospermia group (percentage of normal morphology sperms < 4%) than in the normal morphology group, and was also the cause of adverse pregnancy outcomes and high miscarriage rates in the teratozoospermia group. Dubey et al. (30) conducted a study of 52 IVF- PGD cycles and showed that the percentage of sperms with normal morphology was significantly correlated with the clinical pregnancy and implantation rates, which were lower in the teratozoospermia group than in the normal group. Moreover, the rate of embryonic chromosomal abnormalities on day 3 was higher in the teratozoospermia group than in the normal group. Kahraman et al. (31) showed that PGT-A significantly improved the implantation rate in patients with macrocephalic spermatozoa. Thus, performing PGT-A is may be an advantageous option for patients with a relatively low percentage of normal morphology sperms to screen embryos with a normal karyotype for implantation and improve the pregnancy and implantation rates.

The IVF technique bypasses most of the natural selection mechanisms during sperm-oocyte fertilization, which may be one cause of the increased embryonic aneuploidy rate in assisted reproductive technology. In particular, during ICSI, sperms bypass the biological screening that occurs during natural fertilization. The technique to a large extent relies on the subjective evaluation of sperm morphology by embryologists. However, sperm morphology may not reflect chromosomal abnormalities. Studies have shown that the embryo aneuploidy rate is higher in ICSI than in conventional IVF (32). A low percentage of sperms with normal morphology increase the chance of injecting sperms with chromosomal abnormalities or genetic mutations into the cytoplasm of an oocyte during ICSI, resulting in embryonic chromosomal abnormalities, impaired embryonic development, or embryo aneuploidy. In patients with a high percentage of sperms with normal morphology, the chance of selecting sperms with normal morphology is higher during ICSI, which improves the quality of embryos after fertilization and reduces the blastocyst aneuploidy rate. Recently, new assisted reproductive technologies, such as motile sperm organelle morphology examination (MSOME) and intracytoplasmic morphologically

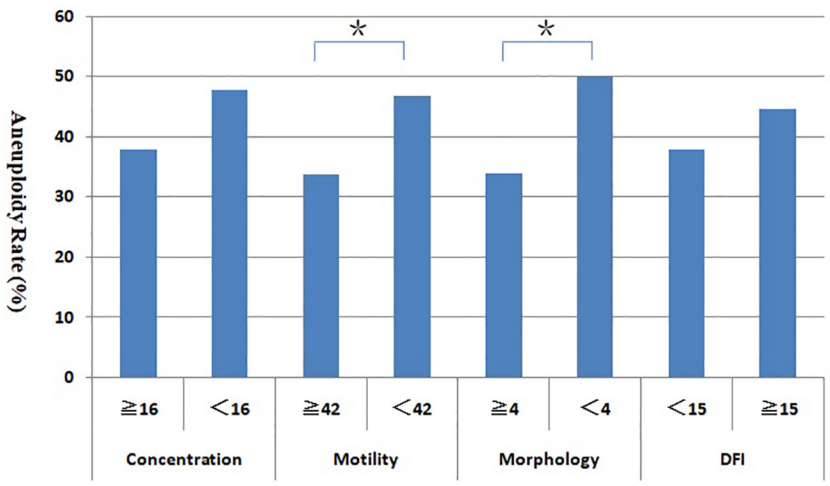

FIGURE 3
Relationship between sperm parameters and blastocyst aneuploidy rate. *Statistically significant (P < 0.05).

selected sperm injection (IMSI), have been introduced in clinical practice with an aim of selecting sperms with normal morphology to improve the quality of embryos and pregnancy outcomes (33). These technologies highlight the important role of sperm morphology in pregnancy outcomes.

Semen parameters are closely related to sperm aneuploidy (34). Studies have shown that the ratio of aneuploid sperms is less than 10% for normal semen but is significantly higher for abnormal semen (35). Aneuploid sperms may result in aneuploid embryos after fertilization. Aneuploidy embryos were significantly higher in couples with abnormal semen parameters (36). Our study showed that the rates of blastocyst aneuploidy was no difference between low sperm concentration group and normal concentration group (47.7% vs 37.8%, P = 0.07). The rate of blastocyst aneuploidy in the low sperm motility group was significantly higher than that in the normal sperm motility group (46.7% vs 33.7%, P<0.05). Dai's research showed that couples with unexplained RM had significantly decreased levels of total motility compared with couples without recurrent miscarriage, but no differences were observed in the semen volume, sperm concentration, and total sperm count between couples with and without recurrent miscarriage (15). This is exactly consistent with the conclusions of our study. The ICSI procedure reduces the need for sperm count, thus reducing the effect of sperm concentration on embryo development and aneuploidy. However, high sperm motility means that sperm have normal function and must be related to embryonic development and euploidy.

Sperm DFI is an important measure of sperm damage and reflects the integrity of sperm genetic materials. During sperm maturation, the histones that bind to DNA are gradually replaced by protamine. Protamine is connected to the DNA strand *via* disulfide bonds to form a unique dense structure of sperm nuclei (37). The instability of disulfide bonds makes sperms prone to damage during maturation and transport. Oxidative stress, endogenous nuclease abnormalities, protamine deficiency, and apoptotic abnormalities associated with various microenvironmental disorders interfere with the highly ordered series of reactions that occur during sperm formation and maturation, which ultimately lead to DNA damage and infertility. Many studies have shown that a high sperm DFI is associated with a high rate of early miscarriage, whereas embryonic chromosomal abnormalities are an important cause of early pregnancy loss. Therefore, we hypothesized that a high sperm DFI was closely related to embryo aneuploidy. This study showed that the blastocyst aneuploidy rate in the high-DFI group is not significant higher than that in normal DFI group, (44.7%vs37.8%, P = 0.28). This is the same result as Bibi's study (36). Increase in DFI did not correlate with embryonic aneuploidy, could be to oocytes' potential to activate mechanism to arrest development of aneuploidy embryos. Researchers are still debating the effect of DFI on pregnancy outcomes in assisted reproductive technology (12, 38). We believe that the method of semen pretreatment may be one of the important factors causing the deviation of results. Density gradient centrifugation and upstream treatment remove most sperms with abnormal morphology and poor motility, thus ensuring the selection of high-quality sperms for ICSI, which reduces the effect of male factors on the embryo quality. Bungum et al. (39) used SCSA to analyze the DNA integrity of sperms selected with density gradient centrifugation and found no significant difference in the sperm DFI between the clinical pregnancy group and the nonpregnancy group undergoing assisted reproductive technology. These results suggest that semen treatment may affect pregnancy outcomes of assisted reproductive technology probably a suitable method of preparation is more effective in removing sperms with potential abnormal morphologies, thereby reducing the embryonic aneuploidy rate.

As a retrospective study, this study has certain limitations. Sample selection bias and the small sample size may affect the accuracy of the results. The sample size is too small. The inclusion criteria of the cases may weaken the reliability and the reason and mechanism are complex or highly variable among different couples. We will try our best to expand the sample size in future studies to exclude the confounding influence of non-research indicators and improve the accuracy of research results. In addition, embryo chromosomal mosaicism may affect the accuracy of the results. In human early embryos, the rate of chromosomal mosaicism is as high as 40% at the cleavage stage and is approximately 10% at the blastocyst stage (40). Embryo mosaicism is very important factor in preimplantation stage. However, in this study, no mosaicism embryos were detected. Firstly, the sample is limited and we performed blastocyst biopsy, which greatly reduced mosaicism rate. On the other hand, a large number of cells were collected during biopsy, which reduced amplification failure, improved the accuracy of the results, and minimized the diagnostic error associated with mosaicism embryos. And most importantly, the detection technique we used (Human CytoSNP-12DNA array from Illumina) is indeed limited in its ability to detect mosaicism embryos, which can only be detected a very high percentage of mosaicism. Our previous strategy was to attribute this high proportion of mosaicism directly to chromosomal abnormalities, so this is the main reason for the absence of mosaicism data in this study and this is also an important limitation of our study. In future studies, we will use NGS, which is more sensitive to embryo mosaicism detection, in order to obtain more accurate results.

Conclusions

This study revealed that high sperm DFI was negatively correlated with good-quality embryo rate and positively correlated with blastocyst aneuploidy rate, while sperm motility and sperm morphology rate were negatively correlated with blastocyst aneuploidy rate. Abnormal semen parameters may affect embryo quality and increase the aneuploidy rate of blastocyst chromosomes, suggesting that in clinical practice of assisted reproduction patients with abnormal semen parameters can be treated in advance to improve sperm quality, so as to reduce the impact on embryo quality and achieve a better pregnancy outcome.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://doi.org/10.6084/m9.figshare.21922695.v1

Ethics statement

This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceived and designed the experiments: GL, HY. Performed the experiments: HY, YL. Collected the data: WN, YW, HJ. Analyzed the data: ZY. Wrote the manuscript: HY. Proofread the manuscript: GL. All authors reviewed and approved this manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Assessment of progesterone levels on the day of pregnancy test determination: A novel concept toward individualized luteal phase support

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Research question: The main objective of the study is to define the optimal trade-off progesterone (P4) values on the day of embryo transfer (ET), to identify low P4-human chorionic gonadotropin (hCG), and to establish whether P4 supplementation started on the hCG day can increase the success rate of the frozen embryo transfer (FET) cycle.

Design: A single-center, cohort, retrospective study with 664 hormone replacement therapy (HRT)-FET cycles analyzed female patients who received vaginal 600 mg/day of P4 starting from 6 days before the FET, had normal P4 values on the day before ET, and whose P4 on the day of the pregnancy test was assessed.

Results: Of the 664 cycles, 69.6% of cycles showed P4 ≥ 10.6 ng/ml, while 30.4% showed P4 < 10.6 ng/ml on the day of the hCG. Of the 411 chemical pregnancies detected, 71.8% had P4-hCG ≥ 10.6 ng/ml (group A), while 28.2% had P4-hCG < 10.6 ng/ml. Of the cycles with P4-hCG < 10.6 ng/ml, 64.7% (group B) were supplemented with a higher dose of vaginal P4 (1,000 mg/day), while 35.3% (group C) were maintained on the same dose of vaginal micronized P4. The live birth rate was 71.9%, 96%, and 7.3% for groups A, B, and C, respectively.

Conclusion: The likelihood to detect P4-hCG < 10.6 ng/ml decreased as the level of serum P4 the day before ET increased. The live birth rate (LBR) was shown to be significantly lower when P4 was low and not supplemented.

KEYWORDS

luteal phase support, artificially prepared endometrium, hCG and progesterone assessment, individualization of luteal phase, progesterone supplementation, live birth rate

Introduction

Approximately 2.4 million assisted reproductive technology (ART) procedures per year are reported worldwide with 500, 000 babies born per year (1). Owing to the improvement in embryo culture, the introduction of vitrification, and the evidence supporting the advantage of freezing in some (elective or non-elective) situations (2), the ratio of frozen embryo transfer (FET) to fresh embryo transfer procedures has increased dramatically over the past few years from 28% to 44.1% (2010–2016) and from 22.9% to 69.4% (2010 –2017), respectively, in Europe and the USA (3) "ART Success Rates | CDC", 2020).

Although FET can be planned with either an artificial cycle (hormone replacement therapy (HRT) FET) or a natural cycle (NC FET) (4), no approach has demonstrated inferiority regarding clinical outcomes (5, 6). HRT FET cycles are mostly used due to their practicality (minimal cycle monitoring and easy scheduling). The two main protagonists of the HRT cycle are estrogens (E_2) and p rogesterone (P4). For the E_2 priming of the endometrium, it has not been demonstrated that serum oestradiol levels are associated with the cycle outcomes (7, 8); this is not the case for serum P4, where multiple studies have confirmed its crucial role in the establishment and maintenance of the pregnancy (9, 10).

Progesterone can be administered in different ways: vaginally, intramuscularly, rectally, orally, or subcutaneously. The most used is the vaginal route; however, the subcutaneous way is becoming more and more popular (11, 12). Intramuscular P4 (IM P4) is still widely used in some parts of the world; however, in Europe, it is quite unpopular. Progesterone has two key features: one is that pharmacokinetics depends on the route of administration and consequently the serum values (13); the other one is the daily variability (14, 15). Despite the high variability of P4, all the recent studies published on luteal serum P4 showed that low serum levels of P4 (below 9–10 ng/ml) are associated with worse cycle outcomes (10, 16–20), independently from the day of the assessment.

Encouraging data collected in a previous prospective study from our group showed the possibility to rescue the FET cycle when the P4 value was found to be <10.6 ng/ml on the day before the FET by supplementing progesterone on the same day (18).

In light of the above, P4 assessment in the luteal phase is of greatest importance as well as the possibility to supplement the deficient cycle with more P4 [progesterone supplementation (PS)], the so-called "rescue protocol" (10, 16, 18, 21, 22).

Nonetheless, until now, all the studies have concentrated on a single measurement of P4, the day before or the day of FET itself. However, considering the important daily variability of the P4 values, and the possibility to individualize the luteal phase (iLP), a single measurement may not be sufficient to establish the appropriateness of the entire luteal phase. Therefore, the aims of the present study were as follows: first, to define optimal trade-off P4 values on the day of embryo transfer (ET) and to identify low P4 values on the day of pregnancy test; second, to investigate the clinical pregnancy rate (CPR) and live birth rate (LBR) in patients with three different conditions, namely, normal P4 not supplemented, low P4 supplemented, and low P4 not supplemented; and third, to determine whether P4 supplementation on the day of pregnancy test improves clinical pregnancy and live birth rate.

Methods

Study setting

This is an exploratory, cohort, retrospective, single-center study performed at a university-affiliated fertility center between January 2018 and June 2020. Patients undergoing FET with artificial preparation of the endometrium (ET-HRT) who had adequate values of P4 (≥10.6 ng/ml) the day before ET were included. The entire study group underwent a second assessment of P4 on the day of the pregnancy test, and in case of positive human chorionic gonadotropin (hCG), whenever P4 was found to be below 10.6 ng/ml, additional vaginal P4 was supplemented on the same day. The study was approved by the institutional review board of Dexeus Mujer (approval number: 072020102604).

Selection of study population

Frozen heterologous ET-HRT (het-ET), homologous ET-HRT (hom-FET), and euploid ET-HRT (eu-FET) after preimplantation genetic testing for aneuploidies (PGT-A) *in vitro* fertilization (IVF) cycles were considered for the analysis.

Patients with known uterine abnormalities or mosaic embryos and with serum P4 extraction taken after 11 a.m. were excluded.

Study protocol

Embryos were kept in culture in a time-lapse incubator, single-step culture media (LifeGlobal®, Paramus, NJ, USA), with 5% oxygen concentration, as described previously (17). Embryos were transferred at the stage of the blastocyst. When performed, the PGT-A procedure was carried out as previously described (23). Embryos were biopsied and vitrified on days 5 and 6 of development. Genetic testing on the embryo was performed by array comparative genomic hybridization (a-CGH) using accessible kits and software (SurePlex® DNA Amplification System, 24Sure® Microarray Pack, BlueFuseMulti®, Illumina®) following the manufacturer's instructions.

Endometrial preparation

Patients received 2 mg/8 h of estrogen valerate (E2) (Progynova®, Schering, Berlin, Germany) for 10–12 days and subsequently underwent a vaginal ultrasound to determine the thickness and the morphology of the endometrium. In case of adequateness of the endometrium (>7 mm and trilaminar), vaginal micronized P (Utrogestan®) treatment (200 mg/8 h) was started at night (D0). Embryo transfer was planned on the sixth day of P4 supplementation (D6). The first assessment of the serum P4 level was performed for all patients included in the analysis on the day prior to the FET (D4) between 8 and 11 a.m., using an electrochemiluminescence immunoassay (Cobas® e-41 analyzer; Roche Diagnostics, Mannheim, Germany). For P4, the lower limit of detection was 0.05 ng/ml (intra- and inter- assay variations of 1.2%–11.8% and 3.6%–23.6%, respectively).

Only cycles with P4 levels ≥10.6 ng/ml on the day prior to FET were included in the study. Then, P4 was analyzed on the β -hCG day, 10 days after the FET (P4-β- hCG). Patients with P4-β- hCG ≥ 10.6 ng/ml continued the luteal phase support with vaginal P4 200 mg/8 h (group A); patients with P4-β- hCG < 10.6 ng/ml were either supplemented with P4 by means of increased dose (1,000 mg/day, divided into 400 mg in the morning, 200 mg in the afternoon, and 400 mg in the evening) of daily vaginal P4 (group B) or maintained on the same dose of vaginal P4 (not supplemented) (group C), as described in Figure 1. The P4-β- hCG cutoff value was stated at 10.6 ng/ml according to Gaggiotti-Marre et al. (22) where the miscarriage rate was higher for patients with low P4 on the day before ET and the live birth rate was lower in the same group.

Treatment discontinuation was usually individualized at around the 10th week of pregnancy, but not rigorously defined.

Outcomes

The primary outcome was to define the optimal trade-off P4 values on the day of ET to identify low p-values on the day of the pregnancy test.

The secondary outcomes of the present study were to investigate CPR and LBR in patients with three different conditions: normal P4 not supplemented, low P4 supplemented, and low P4 not supplemented. Furthermore, this study aimed to determine whether P4 supplementation on the day of pregnancy test improves clinical pregnancy and live birth rate.

CPR was defined as the presence of a vital embryo at 7 weeks of amenorrhea, LBR was described as the delivery of at least one live-born neonate per transfer, and miscarriage rate (MR) was stated as a clinical pregnancy loss before week 23 of gestation (24).

Statistical analysis

Mean and standard deviation were used as continuous variables, while frequencies and percentages were used as categorical variables. Continuous variables were compared through Student's t- test or ANOVA, while the chi-squared test was used to compare categorical variables.

A receiver operating characteristic (ROC) curve and Youden's index were used to find a pre-transfer P4 cutoff point that maximizes sensitivity and specificity for diagnosing low progesterone on the day of pregnancy test assessment. To investigate CPR and LBR in three different conditions, an exploratory comparison between groups A, B, and C was performed. To investigate the effect of P4 supplementation on clinical pregnancy and live birth rate, a between- group comparison (patients with low P4 on the day of pregnancy test receiving versus not receiving P4 supplementation) was performed. For all group comparisons, the analysis was performed in two steps: first, with the entire cohort of pregnant patients (hCG ≥ 10 IU) and second, only with the good prognosis pregnancy (hCG ≥ 100 IU) to control for the hCG levels that were significantly higher in group B compared to groups A and C. All the analyses were exploratory. No formal sample size calculation was performed.

All tests were two- tailed, and a p-value of <0.05 was considered statistically significant. Statistical analyses were performed with IBM© SPSS© Statistics v22.

Results

Patients characteristics

A total of 664 frozen embryo cycles, corresponding to 554 patients, performed under HRT where 200 mg micronized P4 was administered twice a day, and with P4 value ≥10.6 ng/ml on the day before ET, were analyzed, as described in Figure 1 (196 het-ET, 283 hom-FET, and 185 eu-FET).

In Table 1, all the 664 eligible cycles are divided into two groups given P4 levels on the day of pregnancy test assessment, above or under the threshold of 10.6 ng/ml. Specifically, 462 (69.6%) cycles showed appropriate (≥10.6 ng/ml) P4 values, while 202 (30.4%) showed inadequate (<10.6 ng/ml) levels of P4 on the day of the hCG measurement. None of the variables investigated differed in the two groups aside from the serum levels of P4 before the ET, being lower in the group with inadequate levels of P4 on the day of pregnancy test measurement (13.3 *vs.* 15.6, p < 0.001).

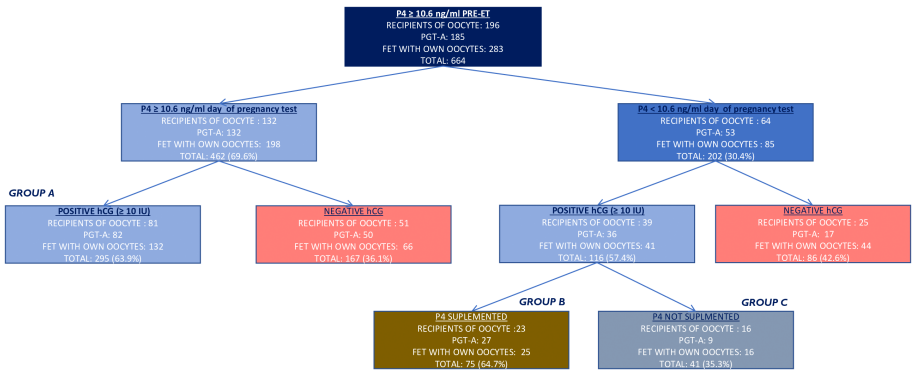

FIGURE 1
Flow chart of the study population and results. In red are the cycles excluded from the primary outcome. In gold is the supplementation on the day of hCG assessment. Positive hCG was considered when ≥ 10 IU. hCG, human chorionic gonadotropin.

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TABLE 1 Baseline characteristics between normal P4 and low P4 on the day of hCG.

	Progesterone ≥ 10.6 ng/ml N: 462 (69.6%)	Progesterone < 10.6 ng/ml N: 202 (30.4%)	p
Age oocyte (years)	33.3 ± 6.2	33.1 ± 6.2	0.685
BMI	24.4 ± 4	25.1 ± 4.8	0.122
P4 day before transfer (ng/ml)	**15.6 ± 5.3**	**13.3 ± 3.9**	**<0.001**
Endometrial thickness (mm)	10.5 ± 1.9	10.6 ± 1.9	0.911
Number of embryos transferred	1.08 ± 0.3	1.04 ± 0.2	0.066
Good-quality embryo transferred	0.6 ± 0.5	0.6 ± 0.5	0.943
P4 day β-hCG (ng/ml)	16.3 ± 6.6	8.9 ± 1.5	
hCG	156.8 ± 227.3	159.8 ± 260	0.881
hCG ≥ 10	63.9% (295)	57.4% (116)	0.117

In bold are statistically significant values. All values are mean ± SD.
BMI, body mass index; P4, progesterone.

Progesterone levels on the day of pregnancy test in the pregnant group

Out of 664 cycles, there were 411 (61.9%) positive hCG (hCG ≥ 10 IU). The second part of the analysis focused on this group. Table 2 shows a division into two subgroups according to the positive (411/664, 61.9%) or negative hCG (253/664, 38.1%). The main difference between the two groups was body mass index (BMI) (24.9 *vs.* 23.9, p = 0.014), mean number of good quality embryos transferred (0.7 *vs.* 0.5, p < 0.001), and P4 levels on the day of the pregnancy test, being all significantly higher in favor of the pregnant group.

Definition of an optimal pre-ET P4 cutoff point to identify low P4 values on the day of the pregnancy test

Results showed that the likelihood to detect P4-hCG < 10.6 ng/ml decreases as the level of serum P4 the day before ET increases.

As the primary outcome, an optimal trade-off of P4 values on the day of ET to identify low P4 values on the day of the pregnancy test was defined. A ROC analysis (Figure 2) showed an area under the curve (AUC) of 0.69 (sensitivity 70.3 and specificity 61.7), indicating that if P4 is measured once (on the day before ET), a value of 13.6 ng/ml is required to assume that P4 will most likely stay above the threshold of 10.6 ng/ml within the first 10 days of the luteal phase.

Exploratory comparison between patients with normal P4 values and low P4 values, receiving or not receiving P4 supplementation on the day of pregnancy test

In Table 3, the population of pregnant women was divided according to P4-hCG and P4 supplementation or not in case the P4 was lower than 10.6 ng/ml. The three groups differed on BMI (24.6 *vs.* 25.9 *vs.* 26.7, p = 0.021), mean P4 on the day before ET (15.7 *vs.* 13.9 *vs.* 12.6, p < 0.001), mean P4 on the day of the pregnancy test (16.9 *vs.* 9.3 *vs.* 8.5, p > 0.001), and mean hCG value (245.3 *vs.* 327.8 *vs.* 185.8, p = 0.009), where group B showed significantly higher hCG values than groups A and C.

P4 levels on the day of pregnancy test with possible P4 supplementation and clinical outcomes (for hCG positive)

Of the 411 chemical pregnancies assessed (hCG ≥ 10 IU), 295 (71.8%) had P4-hCG ≥ 10.6 ng/ml again, while 116 (28.2%) had P4-hCG < 10.6 ng/ml on the same day. Of 116 (64.7%) cycles with an

TABLE 2 Baseline characteristics between hCG positive (hCG ≥ 10 IU) and. hCG negative (hCG < 10 IU).

	hCG ≥ 10 IU N: 411 (61.9%)	hCG< 10 IU N: 253 (38.1%)	p
Age oocyte (years)	32.9 ± 6.1	33.7 ± 6.4	0.141
BMI	**24.9 ± 4.4**	**23.9 ± 3.9**	**0.011**
PGT-A	118/411 (28.7%)	67/253 (26.5%)	0.824
P4 day before transfer (ng/ml)	15 ± 5.2	14.7 ± 4.9	0.395
Endometrial thickness (mm)	10.6 ± 1.9	10.3 ± 1.9	0.094
Number of embryos transferred	1.1 ± 0.3	1.1 ± 0.3	0.644
Good-quality embryo transferred	**0.7 ± 0.5**	**0.5 ± 0.5**	**<0.001**
Score transfer	9.3 ± 1.1	9.2 ± 1.2	0.220
P4 day hCG (ng/ml)	**14.7 ± 7.3**	**13 ± 4.7**	**<0.001**

In bold are statistically significant values. All values are mean ± SD.
BMI, body mass index; PGT-A, preimplantation genetic testing for aneuploidies; P4, progesterone.

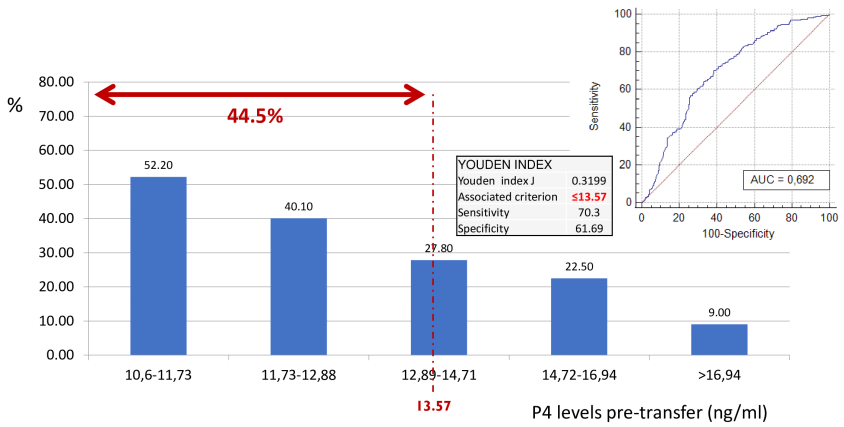

FIGURE 2
Probability of having progesterone levels < 10.6 on day of pregnancy test according to pre-transfer progesterone levels. ROC curve. Classification of low P on the day of hCG. Considering the P4 value on the day before ET. As a result of this ROC curve, a cutoff point of 13.57 ng/ml was selected with specific sensitivity and specificity values. Therefore, 44.5% of the cycles with progesterone ≤ 1 3.57 ng/ml, despite being "high", have a low progesterone value on the day of hCG. ROC, receiver operating characteristic; hCG, human chorionic gonadotropin; P4, progesterone; ET, embryo transfer.

inadequate level of P4 on the day of the pregnancy test, 75 (64.7%) were supplemented with a higher dose of vaginal P4 (1,000 mg/day), while 41 out of 116 (35.3%) were maintained on the same dose of vaginal micronized P4. Eventually, clinical pregnancy and LBR were compared between the three groups: groups A, B, and C.

Clinical pregnancy was significantly lower for group C, compared to groups B and A (46 *vs.* 100 *vs.* 87%), and the same was shown for live birth also significantly lower in group C (mean LBR 7.3%) compared to group A (mean LBR 71.9%) and group B (mean LBR 96%), regardless of the FET-IVF technique used (recipients of oocytes, PGT-A, or FET with own oocytes, Table 4; Figures 3, 4).

P levels on the day of pregnancy test with possible P4 supplementation and clinical outcomes (for hCG ≥ 100 IU)

Due to the significantly lower values of hCG in group C and the arbitral decision to supplement P4 when necessary, and in the intent to better understand the real effect of P4 supplementation, in the third part of the analysis, only the good prognosis pregnancies were considered (according to hCG levels ≥ 100 IU) (Table 5). By excluding all the pregnancies with hCG < 100 IU (first hCG assessment, 10 days after ET), the three groups became smaller: A with N = 214, B with N = 64, and C with N = 19. T he mean (SD) hCG values were respectively 316.5 (251.1) IU, 372.8 (277.8) IU, and 349.1 (377.6) IU (p = 0.318) for groups A, B, and C.

Clinical pregnancy was respectively 74%, 100%, and 96% for groups C, B, and A, while the live birth rates were 15.8%, 96.9%, and 82.2%, respectively, for groups C, B, and A.

The effect of P4 supplementation for patients with low P4 values on the day of pregnancy test

Within the cohort of cycles with low P4 values on the day of the pregnancy test, 75 cycles were supplemented and 41 were not supplemented with extra P4. Better outcomes were found in the

TABLE 3 Only hCG- positive cycles (hCG ≥ 10 IU); group division is based on the levels of P4 on the day of pregnancy test and supplementation or no supplementation.

	A (N: 295)	B (N: 75)	C (N: 41)	p
Age oocyte (years)	33.1 ± 6.1	32.7 ± 5.5	32.5 ± 6.6	0.797
BMI	**24.6 ± 4.1**	**25.9 ± 5.1**	**26.7 ± 5.3**	**0.021**
PGT-A	82/256 (27.8%)	27/75 (36%)	9/19 (22%)	0.215
P4 day before transfer (ng/ml)	**15.7 ± 5.1**	**13.9 ± 5.9**	**12.6 ± 1.6**	**<0.001**
Endometrial thickness (mm)	10.6 ± 1.9	10.6 ± 1.8	10.4 ± 2.1	0.740
Number of embryos transferred	1.08 ± 0.3	1.03 ± 0.2	1.05 ± 0.2	0.251
Good-quality embryo transferred	0.7 ± 0.5	0.7 ± 0.5	0.6 ± 0.5	0.315
Score transfer	9.3 ± 1.2	9.6 ± 0.9	8.9 ± 1.3	0.014
P4 day hCG (ng/ml)	**16.9 ± 7.5**	**9.3 ± 1.3**	**8.5 ± 1.7**	**<0.001**
hCG	**245.3 ± 243.6**	**327.8 ± 278.7**	**185.8 ± 296.9**	**0.009**

Group A, P4 ≥ 10.6 ng/ml (no supplementation); group B, P4 < 10.6 ng/ml (supplemented); group C, P4 < 10.6 ng/ml (no supplementation). All values are mean ± SD, and p- values refer to a three-group comparison.
hCG, human chorionic gonadotropin; BMI, body mass index; PGT-A, preimplantation genetic testing for aneuploidies; P4, progesterone.
In bold are statistically significant values.

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TABLE 4 Clinical outcomes per group of progesterone and supplementation or not analyzed for all the pregnancies considered with hCG ≥ 10 IU.

	A (N = 295)	B (N = 75)	C (N = 41)	p-Value
Mean hCG	245.3 ± 243.6	327.8 ± 278.7	185.8 ± 296.9	0.009
Clinical pregnancy				
Recipients of oocytes (%)	69/81 (85.2)	23/23 (100)	7/16 (43.8)	<0.001
PGT-A (%)	72/82 (87.8)	27/27 (100)	5/9 (55.6)	0.002
FET with own oocytes (%)	115/132 (87.1)	25/25 (100)	7/16 (43.8)	<0.001
Total (%)	256/295 (86.8)	75/75 (100)	19/41 (46.3)	<0.001
Live birth				
Recipients of oocytes (%)	61/81 (75.3)	22/23 (95.7)	1/16 (6.3)	<0.001
PGT-A (%)	65/82 (79.3)	27/27 (100)	1/9 (11.1)	<0.001
FET with own oocytes (%)	86/132 (65.2)	23/25 (92)	1/16 (6.3)	<0.001
Total (%)	212/295 (71.9)	72/75 (96)	3/41 (7.3)	<0.001
Miscarriage				
Recipients of oocytes (%)	7/69 (10.1)	1/23 (4.3)	5/7 (71.4)	<0.001
PGT-A (%)	5/72 (6.9)	0/27 (0)	4/5 (80)	<0.001
FET with own oocytes (%)	28/115 (24.3)	2/25 (8)	5/7 (71.4)	<0.001
Total (%)	40/256 (15.6)	3/75 (4)	14/19 (73.7)	<0.001

Group A, P4 ≥ 10.6 ng/ml (no supplementation); group B, P4 < 10.6 ng/ml (supplemented); group C, P4 < 10.6 ng/ml (no supplementation). Values are reported as crude numbers and (%). p- values refer to a three-group comparison.
hCG, human chorionic gonadotropin; PGT-A, preimplantation genetic testing for aneuploidies; FET, frozen embryo transfer.

group that received P4 supplementation, in terms of CPR and LBR of 100%*vs.* 46.7%, respectively, for supplemented *vs.* not supplemented, and LBR was 96% *vs.* 7.3%, respectively, for supplemented *vs.* not supplemented (p > 0.001).

Discussion

To our knowledge, this is the first study demonstrating that P4 levels should be assessed more than once during the luteal phase. Based on our findings, performing a second assessment on those cycles with initially normal P4 values shows that approximately one-third of them resulted in abnormally low P4 values. Furthermore, we showed that the higher the P4 levels on the day before ET, the lower the likelihood of inadequate serum levels of P4 on the day of pregnancy test assessment. The clinical relevance of such a finding is that a second P4 assessment permits a better follow-up of the serum P4 and allows the "rescue protocol" at different time points of the luteal phase, thus increasing the live birth rate.

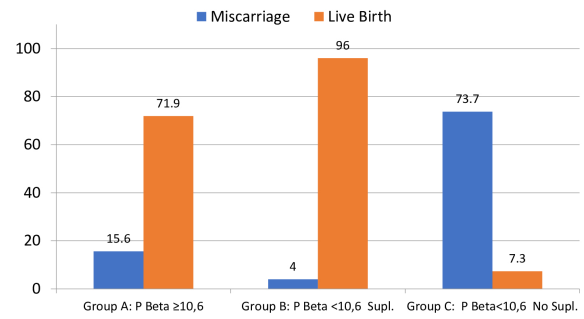

FIGURE 3
Percentage of miscarriage and live birth per positive hCG (≥10 IU). Note that groups A and C do not reach 100% because group A has three VIG (>12 weeks) and 1 still birth (malformation) and group C has two VIG (>12 weeks). VIG, voluntary interruption of gestation; hCG, human chorionic gonadotropin. Miscarriage p < 0.0001; live birth p < 0.0001.

In fact, when looking at the ability of the first P4 assessment to predict the second P4 assessment, an area under the ROC curve of 0.69 resulted, indicating that the lowest level can be statistically defined as acceptable discrimination (25). However, from a clinical perspective, a second assessment may be always preferable, given the relatively low costs and invasiveness of the procedure.

Focusing on the secondary outcome, the results of the present study demonstrate a strong association between serum P4 on the day of pregnancy tests and live birth rates. In fact, in those FET-HRT cycles where P4 could be kept above 10.6 ng/ml throughout the entire luteal phase, the live birth rate was higher. A significantly lower LBR was found when no progesterone supplementation was performed in case P4-hCG < 10.6 ng/ml, independently on the first hCG value. Because hCG was significantly higher in group B compared to groups A and C, the specific analysis of the cycle outcomes was performed considering two groups (the entire population of positive hCG (≥10 IU) and only the hCG ≥ 100 IU). One could postulate that the arbitral decision of adding P4 was taken only for good prognosis patients, thus biasing the results of the study. However, when considering only the good prognostic pregnancies, the results remained the same, confirming that the

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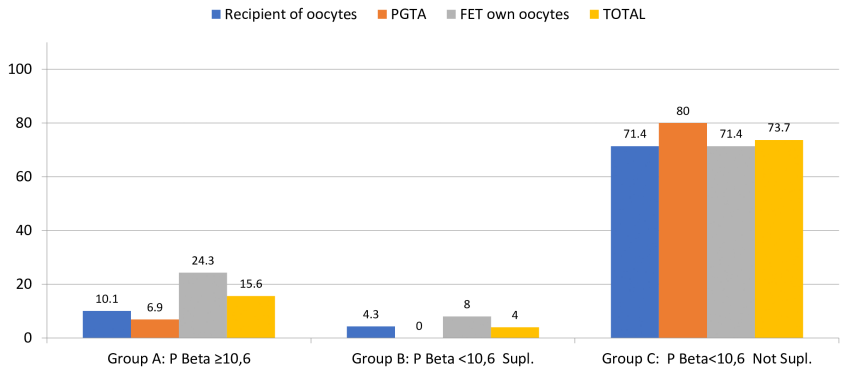

FIGURE 4
Miscarriage/pregnancy in the different groups of patients (per progesterone).

biggest influence on the outcome (LBR) is driven by adequate values of P4 for the whole luteal phase.

To summarize, the clinical "take-home message" is that we cannot rely on a single assessment of P4 in the early luteal phase. Therefore, assessing P4 also on the day of the pregnancy test is of utmost importance to establish the adequacy of the P4 value in the late luteal phase and possibly enhance the supplementation, with the result of increasing live birth rate. In fact, the luteal phase can be seen as a "window of opportunity" where repeating the assessment of P4 on the day of the hCG gives us the possibility to still revert to a deficient cycle and be on time.

The current results are in line with the outcomes of the research of the last years, where the main message is the importance of luteal P4 evaluation in artificially prepared endometrium cycles (10, 16, 18, 21, 22). Although having different timing in the P4 assessment, quite impressively, all the previous studies nearly showed the same outcome, having higher values of luteal P4 (>9 ng/ml) related to higher outcomes rate. More specifically, the consistency of these results goes beyond the timing in which progesterone was evaluated (day of FET, day before FET, or 11 days after FET, respectively, for 10, 16, 18, 19, 22). In fact, the common feature of all the results was that whenever P4 was in the lower limit, the cycle outcomes were hindered. Although nowadays many clinics are performing FET in the natural or modified natural cycle due to the evidence of the latest years supporting the role of the corpus luteum (von 26), a great fraction of FET cycles are performed in an artificial cycle, especially due to the advantages of least cycle monitoring, easy scheduling, and

TABLE 5 Clinical outcomes per group of progesterone and supplementation or not for all the pregnancies with first hCG value ≥ 100 IU.

	A (N = 214)	B (N = 64)	C (N = 19)	p-value
Mean hCG	316.5 ± 251.1	372.8 ± 277.8	349.1 ± 377.6	0.318
Clinical pregnancy				
Recipients of oocytes (%)	57/60 (95)	19/19 (100)	6/8 (75)	0.035
PGT-A (%)	59/61 (96.7)	26/26 (100)	2/2 (100)	0.625
FET with own oocytes (%)	89/93 (95.7)	19/19 (100)	6/9 (66.7)	0.001
Total (%)	205/214 (95.8)	64/64 (100)	14/19 (73.7)	<0.001
Live birth				
Recipients of oocytes (%)	51/60 (85)	19/19 (100)	1/8 (12.5)	<0.001
PGT-A (%)	54/61 (88.5)	26/26 (100)	1/2 (50)	0.028
FET with own oocytes (%)	71/93 (76.3)	17/19 (89.5)	1/9 (11.1)	<0.001
Total (%)	176/214 (82.2)	62/64 (96.9)	3/19 (15.8)	<0.001
Miscarriage				
Recipients of oocytes (%)	6/57 (10.5)	0/19 (0)	5/6 (83.3)	<0.001
PGT-A (%)	4/59 (6.8)	0/26 (0)	1/2 (50)	0.051
FET with own oocytes (%)	17/89 (19.1)	2/19 (10.5)	4/6 (66.7)	0.001
Total (%)	27/205 (13.2)	2/64 (3.1)	10/14 (71.4)	<0.001

Group A, P4 ≥ 10.6 ng/ml (no supplementation); group B, P4 < 10.6 ng/ml (supplemented); group C, P4 < 10.6 ng/ml (no supplementation). Values are reported as crude numbers and (%). p- Values refer to three-group comparisons.
hCG, human chorionic gonadotropin; PGT-A, preimplantation genetic testing for aneuploidies; FET, frozen embryo transfer.

wider applicability even to women without ovarian cycle. The artificial cycle is widely used and mostly with the scheme of 10–2 days of oral estrogens followed by vaginal P4 administered with a common dose of 200 mg three to four times a day (4). As determined by the literature on this issue, with such a pattern of P4 intake, up to 30% of the women have inadequate levels of P4 when measured before the ET (10, 18, 27, 28). With the objective to increase serum P4 levels, Cédrin-Durnerin et al. (27) investigated the strategy to increase (twofold) the vaginal micronized P4 intake in women with low P4 values on the day of FET. In this specific study, increasing the dosage from 600 to 1,200 mg/day of micronized P4 did not improve the serum P4 concentration. Indeed, still, 30% of the women had P4 < 10 ng/ml, showing that there is probably a limit/problem in the absorption from the vaginal mucosa or a possible effect on the microbiome. Other authors did not manage to show an advantage of increasing the vaginal dosage of P4 (29, 30). However, previously, Alsbjerg et al. (31) described, in patients undergoing a FET in artificial cycles, better outcomes when vaginal P4 was doubled (from 90 mg vaginal gel once a day to twice a day).

To date, evidence on the most efficient way for P4 supplementation remains controversial. Subcutaneous P4 has been proven efficient for endometrial preparation and luteal phase support in both ART and FET cycles (18, 21). More specifically, subcutaneous (SC) P4 has been demonstrated to be associated with higher serum P4 levels than the vaginal route (30, 32), well accepted, and easily used by patients (33).

However, the unresolved question stays unanswered: is one assessment of serum P4 level enough to detect P4 deficiency in the luteal phase, given the very high variability of serum P4, and given the high correlation found between p- values and pregnancy rates? The current analysis shows that one serum P4 assessment is not enough to figure out the P4 trend within the luteal phase. According to the ROC analysis, we cannot be confident with a good serum P4 around the FET. Despite P4 ≥ 10.6 ng/ml the day before FET, the chance for P4-hCG < 10.6 ng/ml remains high and according to this study with a negative impact on LBR.

The retrospective nature of the study represents one of the most relevant limitations due to the unknown population bias. Furthermore, the decision of whether to supplement or not was arbitrarily taken by the clinicians. However, the feasibility of a randomized trial on this topic may have introduced ethical issues, given the proven beneficial effects of P4 supplementation. Furthermore, the sample size was not a particular limitation, except for subgroup C (low P4-hCG, not supplemented); the P4 assessment was performed in the morning for the entire population included. H owever, it is important to keep in mind the P4 variation, and LBR was calculated as the total number of live births on the number of positive hCG (hCG ≥ 10 IU) because only clinical pregnancy was included in the second part of the analysis. T herefore, the percentage should be regarded cautiously. Lastly, only cycles supplemented with vaginal P4 were included in the final analysis, and P4 was not assessed after the supplementation.

However, this is a single-center study. T herefore, the major strength is that it was conducted under the same laboratory and clinical conditions. The double determination of P4 the day before FET and on the day of the pregnancy test allows an excellent opportunity for developing a "rescue protocol" even in those cases with adequate serum P4 around the FET, providing an individualized strategy based on each patient's situation. Lastly, a rigorous analysis was performed to better understand the causes of the influence of P4 on LBR by considering the hCG values and their possible impact on LBR.

Conclusions

The results of our studies point consistently in the same direction, i.e., that P4 should be measured repeatedly in the luteal phase of an artificial cycle and, in case of inadequate levels, supplemented.

If measuring P4 at one timepoint could already be seen as personalization of care, repeated P4 assessment represents a step forward to account for the variability of the clinical situation in that specific critical period. We should not be 100% confident about adequate P4 levels around the ET, as this is not a guarantee of adequate P4 levels on the day of the pregnancy test and can compromise the pregnancy results. Finally, larger and more robust confirmatory studies are warranted to validate the present findings.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by institutional Review board of Dexeus Mujer (approval number: 072020102604). The patients/participants provided their written informed consent to participate in this study.

Author contributions

CB conceived the original idea and the overall design of the study. GS and IR performed the statistical analysis. RA, AM, PN, and CB made substantial contributions to the acquisition and interpretation of data and critically assessed the results in the context of scientific literature. RA wrote the article. G-FI and PN revised the final draft of the manuscript. All the authors substantially revised the manuscript, have approved the submitted version, and have agreed both to be personally accountable for the authors' own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the authors were not personally involved, were appropriately investigated and resolved and that the resolution is documented in the literature.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Identification of biallelic variations of *CEP70* in patients with male infertility

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Introduction: Male infertility is a severe health issue caused by complex and multifactorial pathological conditions. Genetic factors are a major cause of male infertility. CEP70, a centrosomal protein, has been reported to play an important role in male reproduction in mice. However, the role of CEP70 in human male infertility is limited.

Methods: Whole exome sequencing and Sanger sequencing were used to identify the genetic cause of the infertile patients. Papanicolaou staining, scanning electron microscopy and transmission electron microscopy were further conducted to explore morphological and ultrastructural defects in spermatozoa from the patient. Immunofluorescence staining was used to detect the pathogenicity of the identified variants and the particular expression of CEP70 in testis.

Results: In this study, we identified biallelic mutations of *CEP70* in two unrelated infertile male individuals with oligoasthenoteratozoospermia that followed a recessive inheritance pattern. Papanicolaou staining, scanning electron microscopy and transmission electron microscopy showed that morphological and ultrastructural defects in the acrosome and flagellum of sperm from the patient in a pattern strikingly similar to that in *Cep70*$^{-/-}$ male mice. The results of immunofluorescence staining suggested that CEP70 was normally expressed in the acrosome and flagellum of control sperm but was hardly detected in the sperm of patient carrying *CEP70* variation. We also explored the particular expression pattern of CEP70 during spermatogenesis in humans and mice.

Conclusions: Biallelic mutations of *CEP70* might be a novel genetic cause of human male infertility, which could potentially serve as a basis for genetic counseling and diagnosis of male infertility.

KEYWORDS

male infertility, oligoasthenoteratozoospermia, recessive inheritance, *CEP70*, WES

Introduction

Infertility is a major health problem worldwide. More than 186 million people suffer from infertility, accounting for 8-12% of couples of reproductive age (1, 2). Among them, male infertility causes more than half of the childless cases worldwide (3). Male infertility is a complex multifactorial pathological state with a highly heterogeneous phenotype, including azoospermia or oligozoospermia, asthenozoospermia and teratozoospermia (4). The causes of male infertility can be related to abnormal hypothalamic-pituitary-axis function, disrupted spermatogenesis in quantity and quality, and obstruction or dysfunction of reproductive ducts (4). Importantly, genetic factors are suggested to be the major causes of male infertility, accounting for 15% of all cases (5). Identifying the genetic causes of male infertility has obvious clinical significance, as it can guide individualized treatment that may have an impact on the reproductive health of patients and their children (6). In recent decades, modern genomics tools, especially exome sequencing, have led to further developments in the study of genetic factors in male infertility (7). However, the genetic etiology remains unknown in approximately 50% of cases (5). Therefore, it is important to explore more genes associated with human infertility in future studies.

Centrosomal proteins are the active components of the centrosome and are essential to the centrosome function (8). The centrosome mediates mitosis and meiosis in the early stage of spermatogenesis, connects the sperm head and tail, forms the sperm tail, controls the movement of the sperm tail, and organizes the cytoskeleton of the zygote (9). Due to the vital role of the centrosome in spermatogenesis, studies have revealed that centrosome abnormalities lead to male infertility, such as oligozoospermia, asthenozoospermia and teratozoospermia (9, 10). Centrosomal protein 70 (CEP70) belongs to the centrosomal protein family and is highly expressed in human sperm according to single-cell transcriptome data from germ cells (11). CEP70 was first found in proteomics studies of the centrosome and was suggested to express in the sperm tail (12, 13). However, only one study has suggested that loss of CEP70 function is involved in male infertility (14). In that study, the authors found that CEP70 deficiency leads to male infertility in mice, which is associated with abnormalities in sperm flagellum, head and acrosome, and heterozygous mutations of *CEP70* in four azoospermia patients were detected (14). Therefore, the role of *CEP70* in human fertility needs to be further explored.

In the present study, we identified two novel biallelic variants in *CEP70* that are responsible for human oligoasthenoteratozoospermia. Specifically, the patient carrying the *CEP70* mutation exhibited various abnormalities in sperm flagella as well as a lack of acrosomes, which was consistent with *Cep70*-/- male mice. Our findings revealed an undiscovered recessive inheritance pattern of *CEP70* mutations in human male infertility, which may provide new genetic evidence for the diagnosis and treatment of male infertility cases.

Materials and methods

Study participants

A 28-year-old Han Chinese male (Patient 1) and a 32-year-old Han Chinese male (Patient 2) from two different nonconsanguineous families with primary infertility were recruited for this study. The probands' parents and controls with normal fertility were also included in the study. The inclusion criteria for controls with normal fertility were as follows: they must have at least one offspring; the semen quality of normal controls must reach the reference values of human semen characteristics provided by the World Health Organization (WHO), including total sperm count ≥ 39 million per ejaculation, sperm concentration ≥ 15 million/ml, progressive sperm motility ≥ 32% and morphologically normal sperm ≥ 4% (1). According to human semen characteristics, male infertility is divided into azoospermia or oligozoospermia, asthenozoospermia and teratozoospermia. Oligoasthenoteratozoospermia was defined as total number of spermatozoa, and percentages of both progressively motile and morphologically normal spermatozoa, less than the reference limits provided by WHO. This study was approved by the Ethics Committee of the Second West China Hospital of Sichuan University (reference number: 202053). All participants in this study signed an informed consent form.

Genetic studies

Genomic DNA was isolated from peripheral blood samples of the probands for whole-exome sequencing (WES) using the FitAmp Plasma/Serum DNA Isolation Kit (Axygen Scientific, Union City, San Francisco, CA, USA). Then, exome capture was conducted with an Agilent SureSelect Human All Exon V6 Kit and Illumina HiSeq 2500 platform. ANNOVAR was used for functional annotation. The data filtering of gene variations was screened by a variety of databases, including the 1000 Genomes Project, ExAC, and HGMD databases. Furthermore, the pathogenicity of nonsynonymous variations was analyzed by SIFT, Mutation Taster, PolyPhen-2, and CADD software. Finally, the variations were selected according to the literature and public databases, including the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov), Human Protein Atlas (http://www.proteinatlas.org), and Mouse Genome Informatics (MGI, http://www.informatics.jax.org).

The candidate pathogenic variants were further validated in the patients and their healthy parents by Sanger sequencing. The PCR primers were as follows: Proband 1-F 5'-ATCAGATGCAAGAA CCCAAAGTT- 3', Proband 1-R5'-CTGCACATAAGACTGGT CACAA- 3'; Proband 2-F 5'-TTTTCCCAGCATTTCAGGCA- 3', Proband 2-R 5'- GCATGGACAGAATGATGCCA- 3'.

Papanicolaou staining

Semen samples fixed in 4% paraformaldehyde were coated on slides and air-dried. The slides were rehydrated with 80%, 70%, 50% ethanol and distilled water and stained with Lea's hematoxylin. Then, the slides were rinsed with distilled water and stained with G-6 orange stain and EA-50. Finally, the slides were dehydrated with ethanol and mounted.

Electron microscopy

The spermatozoa from the patient and control were imaged with scanning electron microscopy (SEM) and transmission electron

microscopy (TEM). For SEM, the spermatozoa were prefixed in 2.5% glutaraldehyde at 4°C overnight. After washing in 1x phosphate-buffered saline (PBS) three times, the spermatozoa were dehydrated in progressive concentrations of ethanol (35, 50, 75, 90, 95, and 100%, 10 min each) and dried by a CO2 critical-point dryer (Eiko HCP-2, Hitachi). Finally, the spermatozoa were imaged using SEM (Hitachi S3400) at an accelerating voltage of 15 kV.

For TEM, the spermatozoa were fixed in electron microscopy fixative and postfixed with 1% OsO4. After dehydration with gradient acetone solutions, the spermatozoa were embedded in Epon 812. Ultrathin sections were obtained by an Em UC6 Ultramicrotome (Leica) and double stained with lead citrate and uranyl acetate. The sections were imaged using TEM (TECNAI G2 F20, Philips) at 120 kV.

Immunofluorescence staining

For spermatozoa staining, the sperm of humans and mice were fixed with 4% paraformaldehyde solution and coated on slides. The spermatozoa were permeabilized with 0.3% Triton X-100 and blocked with 5% bovine serum albumin (BSA) solution. In regard to peanut agglutinin (PNA) immunofluorescence staining, 30% donkey serum was used for blocking instead of 5% BSA. Then, the spermatozoa were incubated with primary antibodies, including anti-CEP70 (1:50, 16280-1-AP, Proteintech) and anti-alpha-tubulin (1:1000, ab7291, Abcam), overnight at 4°C. After washing in 1x PBS (3 × 10 min), the spermatozoa were incubated with Alexa Fluor 488 (1:1000, 2309139, Thermo Fisher Scientific), Alexa Fluor 594 (1:1000, 2160431, Thermo Fisher Scientific), 4,6-diamidino-2-phenylindole (DAPI, 28718-90-3, Sigma–Aldrich) and PNA (1:50, 2328948, Thermo Fisher Scientific) for 2 h at room temperature. The spermatozoa were imaged with a laser scanning confocal microscope (Olympus, FV3000).

For testicular tissue staining, the testicular tissue of the adult mice was fixed with 4% paraformaldehyde solution. Then, the tissue was embedded in paraffin and cut into sections. After deparaffinization and rehydration, the tissue sections were boiled with 20 mM sodium citrate for 15 min at 95°C. Next, the tissue sections were incubated with the primary antibody anti-CEP70 (1:50, 16280-1-AP, Proteintech) overnight at 4°C, followed by incubation with Alexa Fluor 594 and DAPI for 2 h at room temperature.

RNA isolation and quantitative real-time PCR

Total RNA of mouse tissues was extracted using TRIzol reagent (Invitrogen) and was reverse transcribed into cDNA using the PrimeScript RT reagent Kit (Takara). qRT–PCR was conducted using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) on an iCycler RT–PCR Detection System (Bio-Rad Laboratories). Each test for each sample was performed in triplicate. In addition, actin was used as an internal reference, and the qRT–PCR data were normalized using the 2−ΔΔCt method. The primer sequences for real-time PCR were as follows: *Actin*-F 5'-CCTAGGCACCAGGG TGTGAT- 3', *Actin*-R 5' -TCACGGTTGGCCTTAGGGTT- 3'; *Cep70*-F 5' -GCCCCAAACGGCAATAAAGA- 3', *Cep70*-R 5' -CTCCGACTTTGACACCTTCCT- 3'.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 9.0.0 (GraphPad Software Inc, USA) and SPSS 17.0 (IBM Statistics, USA). Statistical significance between two groups was determined using an unpaired two-tailed Student's *t* test. The P value of less than 0.05 was considered statistically significant.

Results

Identification of biallelic *CEP70* variants in two infertile males with oligoasthenoteratozoospermia

Two infertile males were recruited for this study (Figure 1). The results of their semen analysis are shown in Table 1. The sperm count and motility were reduced significantly, and the malformed sperm morphology was considerably obvious. WES further revealed two biallelic mutations in *CEP70* in the two affected individuals (Supplementary Data 1 and 2). Noticeably, a homozygous frameshift variant of c.1842dupT (p. Pro615Thrfs*14) was identified in patient 1. This variant was absent or rare in most human populations according to the 1000 Genomes Project (0), ExAC Browser (0) and gnomAD databases (0.028%). For patient 2, compound heterozygous variants of c.1058C>G (p.Gly353Ala) and c.1059_1063del (p.Trp354Thrfs*14) were identified. These variants are both absent in the 1000 Genomes Project and ExAC Browser databases and are rare in the gnomAD database (0.025% and 0.025%, respectively).

To further clarify the contribution of these variants, we verified these variants in the two families by Sanger sequencing. For patient 1, his healthy parents each carried a heterozygous variant of c.1842dupT (Figure 1). For patient 2, his fertile mother carried a heterozygous variant of c.1059_1063del, and we could not obtain a DNA sample from his father who was dead. Our findings suggest that *CEP70* mutations might be related to male infertility in a recessive inheritance pattern.

The spermatozoa phenotype involved in CEP70-mutated men

We next investigated the abnormal morphology of spermatozoa in patient 1. Regrettably, patient 2 refused to provide his semen for further research. Using the analysis of Papanicolaou staining and SEM, we observed that most of the spermatozoa had round, pyriform, tapered, or amorphous heads and a mosaic of flagellar morphological abnormalities, including absent, short, bent, coiled, and irregular tails (Figures 2A, B). In addition, TEM was used to analyze the ultrastructure of the sperm from patient 1 and the normal control. The sperm nucleus contained large vacuoles with membranous structures; the acrosome showed irregularly shaped deformations or was absent (Figure 2C). Remarkably, we found ultrastructure defects in the flagella of sperm from patient 1. Compared with the normal control, the cross section of flagella showed a severely fuzzy or incomplete '9+2' microtubule structure, including characteristics of

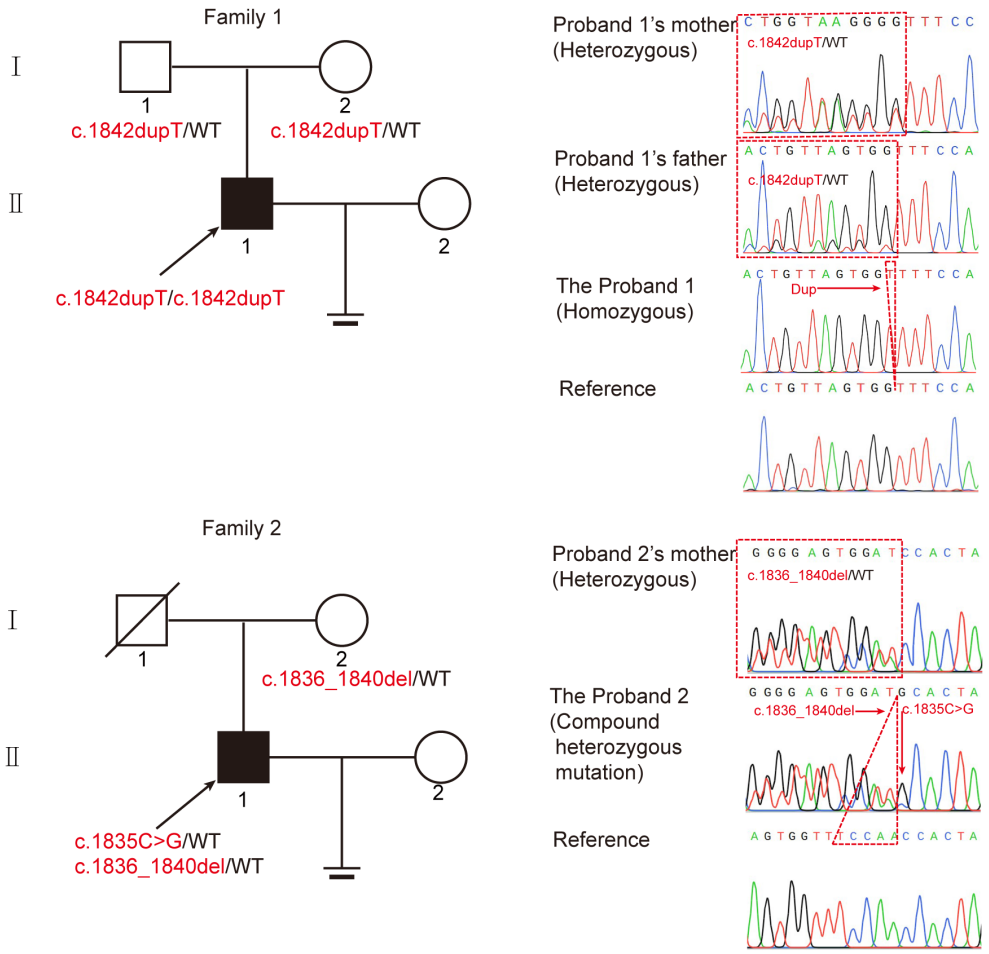

FIGURE 1
Identification of biallelic *CEP70* variants in two infertile males with oligoasthenoteratozoospermia from different families. The pedigree of two families with biallelic *CEP70* variants. The black arrow points to the probands. Sanger sequencing analysis from the two families. The red arrow points to the location of the mutation site. The red dotted triangle represents a duplicated or deleted base sequence.

missed central-pair microtubules, partially absent peripheral microtubule doublets, and incomplete and disorganized outer dense fibers (Figure 2C).

We further assessed the effect of the c.1842dupT variant in *CEP70* on patient sperm by immunofluorescence staining. CEP70 mainly located in the sperm neck and acrosome in the normal control (Figure 3A). However, the CEP70 signal in patient sperm could not be detected (Figure 3A). The western blot using the sperm lysate of the patient also showed the CEP70 protein degradation (Supplementary Figure 1). In addition, PNA staining showed that patient 1 had severe defects in sperm acrosome formation compared to the normal control (Figure 3B). These findings suggest that CEP70 plays an important role in acrosomal and flagellar development during human spermatogenesis.

TABLE 1 Results of the semen analysis and sperm morphology examination of the patients.

Parameter	Patient 1 (n=2)	Patient 2 (n=3)	Reference
Semen volume, ml	4.4 ± 0.57	4.3 ± 0.5	≥1.5
Sperm concentration, million/ml	7.4 ± 0.1	10.4 ± 6.7	≥15
Vitality, %	39.5 ± 29.0	49 ± 25	≥58
Motility, %	9 ± 1.4	3.7 ± 3.1	≥32
Abnormal head (%)	99.6 ± 0.6	99.1 ± 0.1	–
Abnormal head-tail conjunction (%)	9.4 ± 6.2	5.9 ± 5.7	–
Abnormal flagella (%)	82.2 ± 3.3	81.3 ± 2.8	–

Data are presented as mean ± SD.

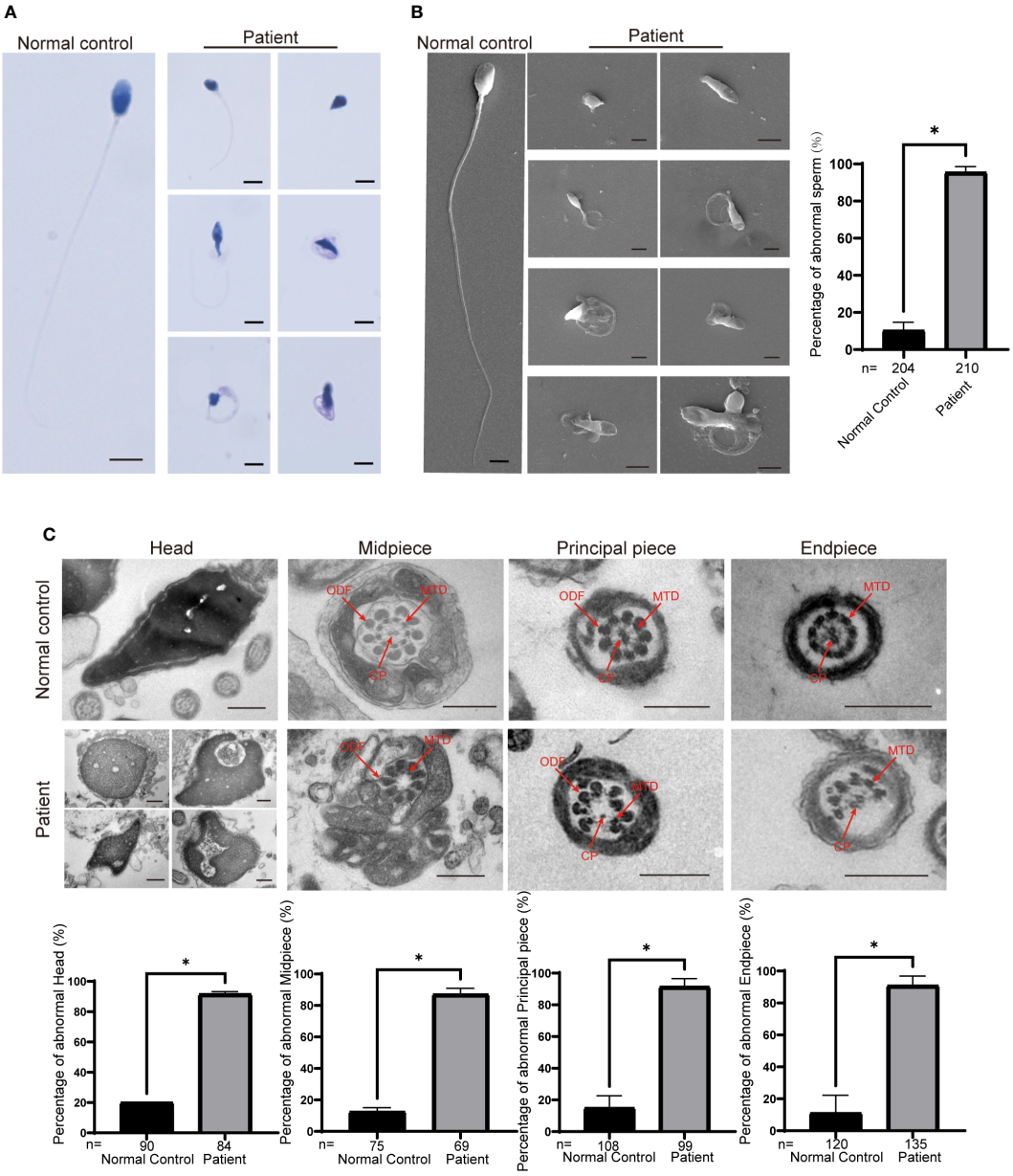

FIGURE 2
Morphological and ultrastructural defects in spermatozoa from the patient with *CEP70* mutation. **(A, B)** Morphological abnormalities in spermatozoa were observed from patient 1 by Papanicolaou staining **(A)** and SEM **(B)** (scale bars, 2.5 μm). Significant increases in the percentage of abnormal morphology in sperm from patient 1 (two-sided Student's t test; *P < 0.05; error bars, s.e.m.). n, the number of sperm analyzed. **(C)** The deformed ultrastructure of spermatozoa was analyzed by TEM. The spermatozoa head of patient 1 was irregular and contained large vacuoles. The acrosome was deformed or even absent. The CP, MTD and ODFs were incomplete, disorganized and/or absent in the flagella of spermatozoa. CP, central-pair microtubules; MTD, peripheral microtubule doublets; ODF, outer dense fiber (scale bars, 500 nm). The percentage of ultrastructural defects in the head, midpiece, principal piece and endpiece in normal control and the patient sperm (two-sided Student's t test; *P < 0.05; error bars, s.e.m.). n, the number of cross sections analyzed.

The expression pattern of CEP70 in the testes of mice and humans

We explored the expression levels of *Cep70* mRNA in various organs of adult mice using qRT–PCR to further identify the role of CEP70 in male reproduction. The qRT–PCR results showed that *Cep70* was mainly expressed in the mouse testis compared to other mouse organs, including the heart, liver, spleen, lung, kidney, stomach, bowel, eye, brain, epididymis, womb, and ovary (Figure 4A). In addition, we investigated the expression levels of *Cep70* in testicular tissues of mice at different postnatal days. The results showed that *Cep70* began to express obviously on postnatal Day 25, reached its highest level on postnatal Day 30, and then showed a stable expression pattern (Figure 4B). In addition, to better understand the roles of CEP70 during mouse spermatogenesis, we performed immunofluorescence staining of mouse testicular tissue. The results showed that CEP70 primarily located in spermatocytes, round spermatids, and elongated spermatids in testicular tissue (Figure 4C). Furthermore, we isolated various germ cells from mouse testes. The staining results showed that CEP70 mainly

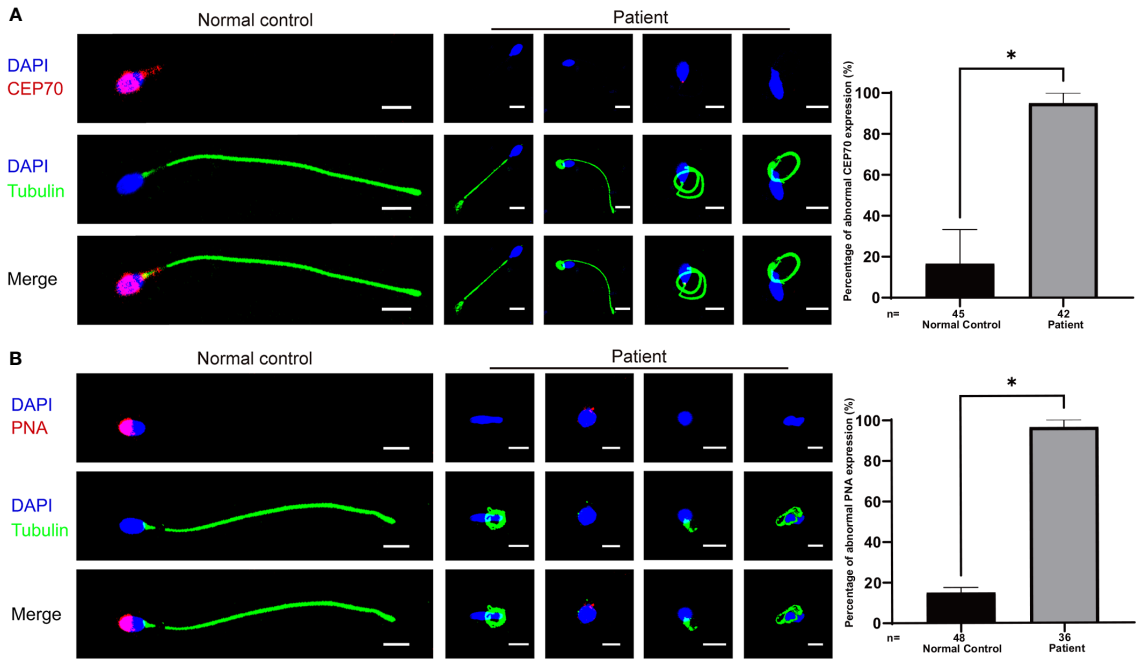

FIGURE 3
Immunofluorescence staining of CEP70 and PNA in patient sperm. **(A)** The immunofluorescence staining showed CEP70 protein localized in the acrosome and neck in the control sperm and was drastically decreased in spermatozoa from the patient (blue, DAPI; green, alpha-tubulin; red, CEP70; scale bars, 5 μm) Significantly abnormal expression of CEP70 in sperm from the *CEP70* mutation patient (two-sided Student's t test; *P < 0.05; error bars, s.e.m.). **(B)** The PNA signal was defective in the sperm from patient 1 compared to the control sperm (blue, DAPI; green, alpha-tubulin; red, PNA; scale bars, 5 μm). Significantly abnormal expression of PNA in sperm from the *CEP70* mutation patient (two-sided Student's t test; *P < 0.05; error bars, s.e.m.). n, the number of sperm analyzed.

located in the nuclei and cytoplasm of spermatogonia, spermatocytes and round spermatids and was also expressed in the head and flagellum of late spermatids and epididymal spermatozoa (Figure 4D).

We also investigated the expression of *CEP70* in the process of spermatogenesis in humans. The results of the MeDas database (https://das.chenlulab.com) analysis showed that *CEP70* is mainly expressed in testicular tissue of adolescents and adults (Figure 5A). In addition, the results of single-cell sequencing in the Human Protein Atlas database showed that *CEP70* was mainly expressed in spermatocytes, early spermatids and late spermatids (Figure 5B). Moreover, we employed immunofluorescence staining to confirm the results provided in the above databases. The expression of CEP70 in spermatogonia was relatively low and was evident in spermatocytes. During spermiogenesis, CEP70 was present in the acrosome and flagella regions in spermatids, which was the same location as CEP70 in mice (Figure 5C). Overall, the current results suggest that CEP70 might play an important role in human male reproduction and influence acrosomal and flagellar formation.

Discussion

Centrosomal proteins are closely associated with reproductive processes (15). However, evidence of a relationship between male infertility and centrosome protein functional defects is limited in humans. *CEP112* and *CEP135* deficiencies in humans lead to acephalic spermatozoa and multiple morphological abnormalities of the sperm flagella, respectively (16, 17). In addition, our recent findings suggest that CEP128 and CEP78 is involved in spermatogenesis in both humans and mice (10, 18). Intriguingly, in this study, biallelic mutations of *CEP70* in two infertile men followed a recessive inheritance pattern. Regrettably, patient 2 was unwilling to provide semen for further study, thus we only obtained the semen from patient 1 to explore the effect of *CEP70* mutations on sperm morphology. The infertile patient with *CEP70* mutations showed amorphous heads, abolished acrosomes, and anomalous flagella morphology in sperm. Our clinical report showed that CEP70 might play a vital role in human male reproduction.

Currently, there is limited information on the function of CEP70. CEP70 acts as a centrosomal protein that increases microtubule length and promotes microtubule stability through interaction with HDAC6 and regulation of microtubule protein acetylation (19, 20). Previous data showed that CEP70 is involved in cilia formation and determines the length of the axoneme in zebrafish embryos (21). A recent study suggested that *Cep70*$^{-/-}$ in mice caused male infertility, which resulted in abnormal acrosome structure and abnormal flagella (14). Furthermore, mice lacking CEP70 also exhibited disturbed spermiogenesis and increased germ-cell apoptosis, which led to a decrease in sperm count (14). Mechanistically, the previous study revealed that the expression of sperm flagellum development related proteins, including AKAP4, Tekt4, ODF1, CABYR, ROPN1 and TXNDC2 were decreased in *Cep70*$^{-/-}$ male mice by proteomics analysis (14). In addition, the reduced expression of the proteins involved in sperm acrosome formation was also detected in *Cep70*$^{-/-}$ mice, including AKAP3, ZP3R, SPACA1, ACRBP (14). Therefore, in our study, we also observed the abnormalities in sperm acrosomal and

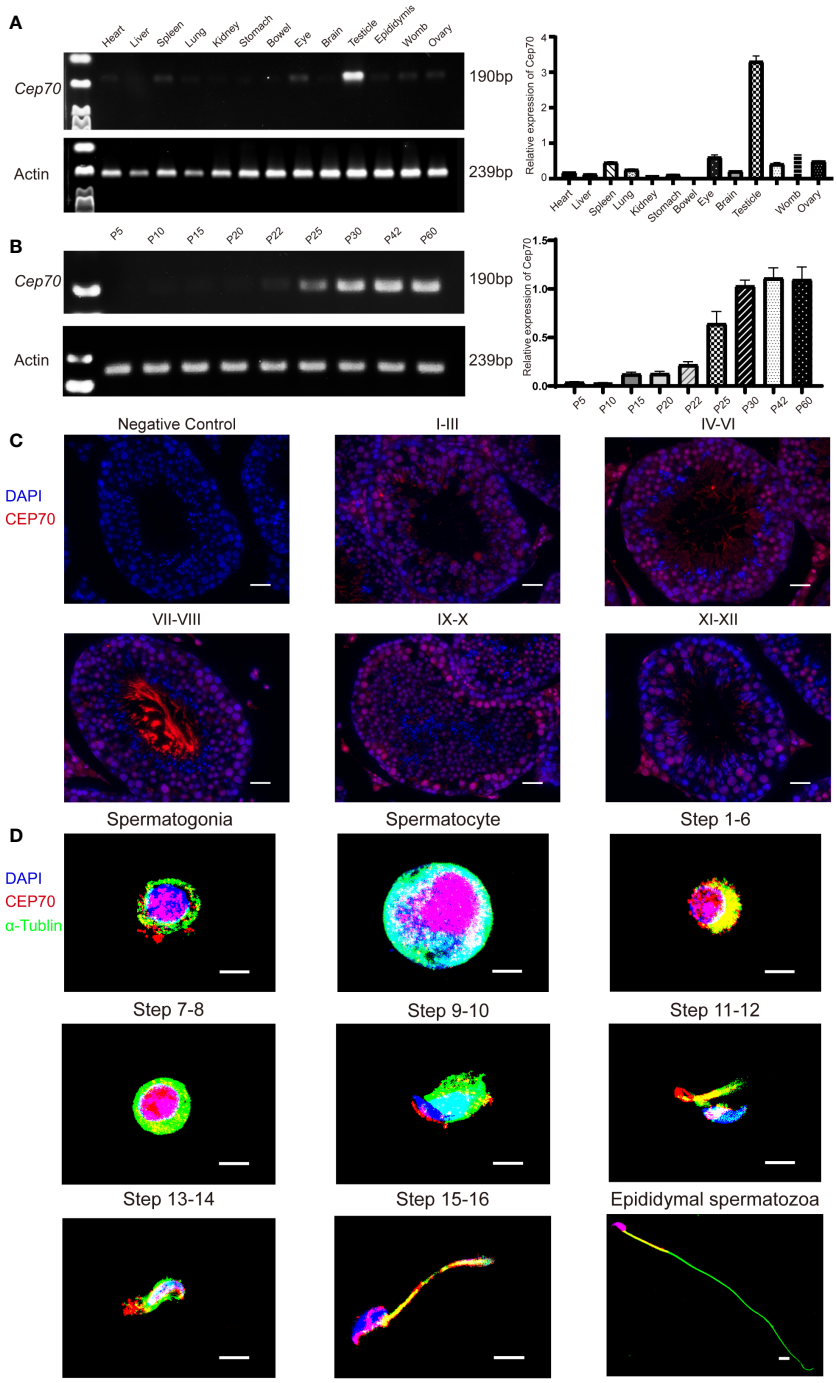

FIGURE 4
The expression pattern of CEP70 in the testes of mice. **(A)** *Cep70* dominantly expressed in the testes, as shown by qRT–PCR analysis. **(B)** qRT–PCR showed that *Cep70* began to express significantly on P25, reached its highest level on P30 and then showed a stable expression pattern (P= Postnatal day). **(C)** Immunofluorescence staining of CEP70 in different stages of mouse spermatogenesis (blue, DAPI; red, CEP70; scale bars, 5 μm). **(D)** Immunofluorescence staining of CEP70 in isolated germ cells (blue, DAPI; green, alpha-tubulin; red, CEP70; scale bars, 5 μm). Three independent experiments were performed.

flagellar formation in the patient with biallelic *CEP70* mutations, which defects might be caused by the diminished expression of the acrosome and flagellum development related proteins suggested in $Cep70^{-/-}$ mice. However, Liu et al. reported a heterozygous mutation in *CEP70* in four azoospermia patients (14). Noticeably, $Cep70^{+/-}$ heterozygous mice have normal fertility to produce $Cep70^{-/-}$ mice. Moreover, our study showed that the heterozygous *CEP70* mutation was detected in the fertile father. In addition, the CEP70 protein is highly conserved in humans and mice. Therefore, it is plausible that the variants of CEP70 are recessively inherited to produce pathogenicity in male infertility.

In conclusion, the current study is the report of *CEP70* mutation-related male infertility in humans with a recessive inheritance pattern, similar to the mouse model. Future cases are needed to corroborate

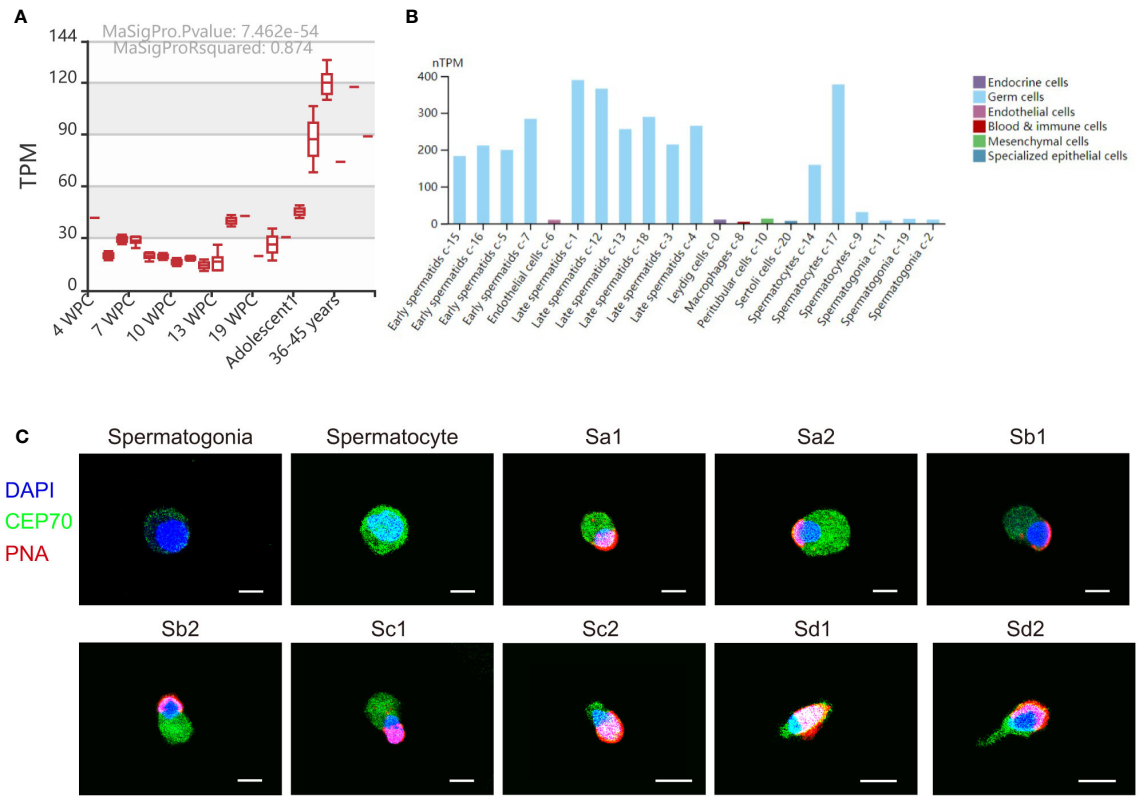

FIGURE 5
The expression pattern of CEP70 in human testes. **(A)** The RNA expression levels of *CEP70* at different developmental stages in human testes (TPM = transcripts per million; WPC = weeks postconception). **(B)** The expression levels of *CEP70* in different types of cells in human testes (https://www.proteinatlas.org/ENSG00000114107-CEP70/single+cell+type/testis). **(C)** Immunofluorescence staining of CEP70 in different stages of germ cells from human testis (blue, DAPI; green, CEP70; red, PNA; scale bars, 5 μm). CEP70 locates at the acrosome and flagella regions in sperm. Sa, Sa spermatid; Sb, Sb spermatid; Sc, Sc spermatid; Sd, Sd spermatid.

the proposed link of *CEP70* mutations with male infertility in order to use it as a target for genetic counseling and diagnosis of male infertility. We believe that this study will expand our understanding of the role of centrosomal proteins in human male infertility.

Data availability statement

The datasets presented in the study are deposited in the MeDas online repository, https://das.chenlulab.com.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Second West China Hospital of Sichuan University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

All authors contributed to the study conception and design. Sample collection whole-exome sequencing, and screening for the mutations were performed by YY and DL. TR, CJ, and GS performed the experiments and collected the data. The first draft of the manuscript was written by TR and YS revised the manuscript, and all authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1133222/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The absence of CEP70 expression in patient's sperm lyste. Western blotting detected loss of CEP70 protein in the sperm lysate from the patient compared to the normal control.

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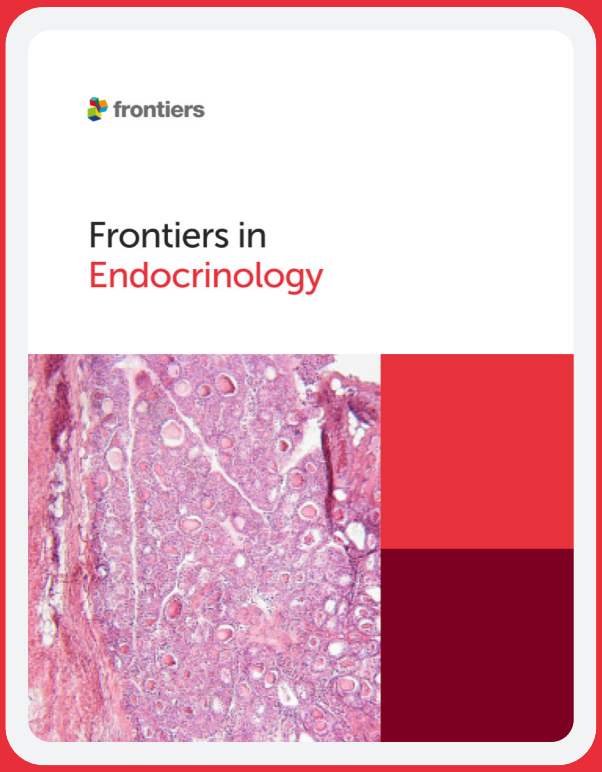

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