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Male reproduction and oxidative stress

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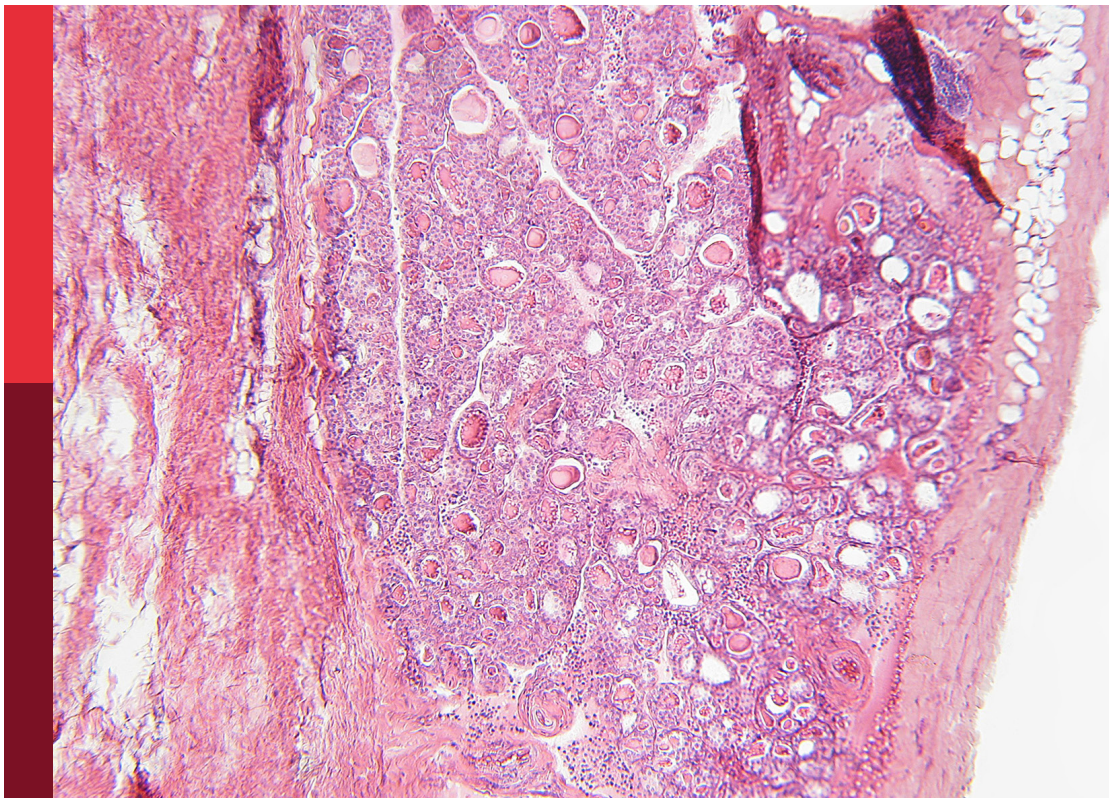

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Male reproduction and oxidative stress

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Association of paternal MTHFR polymorphisms (C677T) with clinical outcomes in ICSI treatment

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Purpose: This study aims to investigate the association between paternal methylenetetrahydrofolate reductase (MTHFR) polymorphisms (C677T) and embryonic development, pregnancy, and neonatal outcomes in intracytoplasmic sperm injection (ICSI) treatment.

Methods: A total of 191 infertile men undergoing ICSI treatment at the Reproductive and Genetic Hospital, The First Affiliated Hospital of USTC, were recruited between January 2020 and June 2021. The MTHFR C677T polymorphism genotyping was evaluated in these male patients, and they were stratified into three groups according to genotyping results: Control (CC), heterozygote mutated (CT), and mutated homozygote (TT). In addition, we conducted a comparative analysis of embryonic development, pregnancy, and neonatal outcomes among these three groups.

Results: The embryonic development (including normal fertilization rate (80.14% vs. 83.06% vs. 85.10%; p = 0.37), high-quality embryo rate (45.26% vs. 43.69% vs. 46.04%; p = 0.72), blastocyst formation rate (42.47% vs. 43.18% vs. 39.38%; p = 0.62), implantation rate (42.47% vs. 36.25% vs. 41.22%; p = 0.62), and clinical pregnancy rate (64.71% vs. 58.75% vs. 66.67%; p = 0.59) were not comparable among these three groups. Moreover, no significant difference was observed in terms of pregnancy outcomes (including miscarriage rate (24.24% vs. 12.77% vs. 22.5%; p = 0.35) and live birth rate (49.02% vs. 51.25% vs. 51.66%; p = 0.96)). Additionally, no marked difference was observed in terms of neonatal outcome (including, preterm delivery rate (24% vs. 14.63% vs. 9.67%; p = 0.35), birth height (p = 0.75), birth weight (p = 0.35), neonatal sex (p = 0.48), gestational age at delivery (p = 0.24), Apgar score (p = 0.34), and birth defects (0% vs. 2% vs. 9%; p = 0.23) among the study groups.

Conclusion: The paternal MTHFR C677T polymorphism is not associated with embryo quality, pregnancy, or neonatal outcomes in ICSI treatment. Therefore, in our population, MTHFR polymorphisms do not provide helpful information in explaining ICSI failure.

KEYWORDS

MTHFR (C677T), intracytoplasmic sperm injection (ICSI), sperm DFI, male infertility, assisted reproductive technologies

Introduction

Assisted reproductive technologies (ART) have become widely accepted as a proven and routine treatment for infertility (1). However, despite recent advancements in *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), only about one-third of these procedures result in live births (2). Understanding the factors that affect the outcome of IVF/ICSI pregnancies is crucial to designing safer and more effective infertility treatments (2). ICSI procedure failures are likely caused by several factors, including the woman's age, sperm and oocyte quality, as well as poor uterine receptivity (3). Previous literatures have suggested that abnormal semen parameters, chromosomal anomalies, sperm DNA fragmentation index (DFI), and genetic–epigenetic aberrations adversely affect pregnancy outcomes following ICSI procedures (4–6). According to a number of studies, methylenetetrahydrofolate reductase (MTHFR) polymorphisms are associated with oxidative stress, sperm parameters, and sperm DFI (7), implying that MTHFR polymorphisms may serve as a genetic risk factor for ICSI.

The human MTHFR gene is located on the short arm of chromosome 1 and encodes one of the regulatory enzymes controlling folate metabolism (8, 9). MTHFR catalyzes 5,10-methylenetetrahydrofolate to turn into 5-methylenetetrahydrofolate (5-MTHF), which is necessary for homocysteine's (Hcy) conversion into methionine *via* the methionine synthesis pathway (10). There are three commonly known polymorphisms of the MTHFR gene, namely MTHFR C677T (rs1801133), MTHFR A1298C (rs1801131), and MTHFR G1793A (rs2274976) (11). The MTHFR C677T polymorphism alters an alanine (Ala) to a valine (Val), which decreases the thermal stability of the enzyme (8). The enzyme activity in homozygous TT mutant individuals is around 30% of that in homozygous CC genotype individuals, whereas enzyme activity in heterozygous genotype (CT) individuals is approximately 65% of that in homozygous CC genotype individuals (12). The MTHFR polymorphism (C667T) was significantly associated with a higher risk of male infertility in the Chinese population, while the MTHFR polymorphism (A1298C) was not considered a risk factor for male infertility, according to numerous studies (8, 13, 14). Some studies have also examined the association between MTHFR polymorphisms, recurrent spontaneous abortions, and recurrent implantation failures (15–17). Although most current reports have focused on women-related factors (18–20), sperm quality may also play an important function. As a result, it is necessary to investigate the correlation between the paternal MTHFR C677T polymorphism and the clinical outcomes of ICSI treatment.

Materials and methods

Study population

In this study, 191 patients undergoing ICSI treatment at the Reproductive and Genetic Hospital, The First Affiliated Hospital of USTC, were recruited between January 2020 and June 2021. Male participants were enrolled from infertile couples due to a male factor.

There were no initial exclusion criteria. As part of their initial prenatal counseling visit, all females were advised to take a prenatal vitamin containing at least 400 micrograms of folic acid.

Semen analysis

Semen samples from the male patients were obtained after 2–7 days of abstinence and assessed after liquefaction at 37°C for 30 min. Semen parameters were determined according to the World Health Organization (WHO) guidelines for semen analysis (fifth edition, 2010). Sperm concentration, progressive motility, and total motility were assessed by computer-assisted sperm analysis (CASA) under a phase-contrast microscope (CX43, Olympus Corporation, Tokyo, Japan) equipped with a SAS-II system (SAS Medical, Beijing, China). Sperm morphology was evaluated through Diff-Quick staining (Ankebio, Hefei, China) at ×100 magnification under a light microscope (UB100i, UOP, Chongqing, China). Leukocytes

were stained with benzidine for the peroxidase test (Ankebio). Antisperm antibody (AsA) levels were measured by the mixed antiglobulin reaction (MAR) method (Ankebio). Sperm DNA fragmentation was evaluated by flow cytometry according to the manufacturer's protocol (Cellpro, Ningbo, China).

Genotype detection

The whole genome DNA was extracted using QIAquick PCR purification kits (QIAGEN, Germany) from the blood samples of the male patients. The MTHFR C677T genotype was analyzed using a fluorescence PCR detection kit (PCR-fluorescence probe) produced by Tailored Medical (Shenzhen, Guangdong, China). Fluorescence PCR detection was conducted under the following conditions: a 4-μl whole genome DNA and a 10-μl PCR reaction system. Next, the manufacturer recommends 95°C denaturation for 15 s and 60°C annealing/extension for 60 s, with 45-cycle reaction conditions. After the PCR reaction, the endpoint fluorescence in each sample well was measured using the ABI 7500 fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA), and the MTHFR genotyping results were accurately determined using the ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Intracytoplasmic sperm injection

ICSI was conducted as previously described (21). The women were subjected to controlled ovarian hyperstimulation using a standard long protocol. First, oocyte retrieval was performed 36 h after 250 μg recombinant hCG (Ovitrelle, Merck Serono, Switzerland) administration. Next, oocytes were treated with a G-morpholinepropanesulfonic acid (MOPS) medium (Vitrolife, Kungsbacka, Sweden). The embryos were then denuded with hyaluronidase using G-*in vitro* fertilization (IVF) PLUS medium (Vitrolife). Embryos were cultured in a G-1 medium (Vitrolife) in a desktop incubator (COOK Medical, Bloomington, IN, USA) for at least 3 days. Progesterone was used for luteal support.

Determination of pregnancy

Clinical pregnancy was confirmed *via* two blood hCG tests conducted 14 days after transplantation. An ultrasound screening was performed 30 days after transplantation to detect visible sacs and to evaluate their development.

Statistical analysis

Quantitative variables of normal distribution were represented using mean ± standard deviation (SD). If not, medians (interquartile range (IQR)) are used. Qualitative variables were described as frequency and percentage; for comparative analysis among the three groups, analysis of variance (ANOVA) was employed for quantitative variables with normal distribution, and the Kruskal–Wallis test was used for the nonnormal distribution. Pearson's chi-square test or Fisher's exact test was employed for qualitative variables. Observed frequencies of different genotypes were separately tested for deviation from the Hardy–Weinberg equilibrium using the exact test. All significance tests were two-sided, and a $p < 0.05$ was regarded as statistically significant. Statistical analyses were performed using R (version 3.5.3).

Result

Baseline characteristics

A total of 191 men from infertile couples were recruited for this study. MTHFR genotyping analysis for locus 677 was conducted in these patients. In total, we observed homozygosity for the C allele in 26.70% ($n = 51$), heterozygosity in 41.88% ($n = 80$), and homozygosity for the T allele in 31.42% ($n = 60$) of patients. According to published studies, our infertile male patients had a greater frequency of the MTHFR 677T allele than the general population, and the allele frequency for C677T was in Hardy–Weinberg equilibrium (Table 1).

Based on the locus 677 genotype evaluation, the patients were divided into three groups: Control (homozygosity for the C allele), heterozygote mutated (heterozygosity), and mutated homozygote (homozygosity for the T allele). Age ($p = 0.79$), clinical conditions (BMI ($p = 0.12$), duration of infertility ($p = 0.22$), type of infertility ($p = 0.96$), and maternal baseline FSH concentration ($p = 0.10$) were not statistically different between the three groups (Table 2).

There was also no statistically significant difference among the three groups in terms of semen parameters (including sperm

TABLE 1 Genotype frequencies according to C677T in patients.

Gene parameters	Genotype			Allele		*p*-value
	CC	CT	TT	C	T	0.08
Frequency (%)	26.70	41.88	31.42	47.64	52.36	
Data were presented as frequency. p-values were derived from Pearson's chi-square test.						

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TABLE 2 Characteristics of the patients enrolled in this study.

Clinical characteristics	CC (n = 51)	CT (n = 80)	TT (n = 60)	p-value
Age of male (years; mean ± SD)	33.92 ± 6.49	33.19 ± 5.89	33.40 ± 5.96	0.79
Age of female spouse (years; mean ± SD)	32.56 ± 5.49	31.96 ± 5.58	32.17 ± 5.47	0.83
BMI of male (kg m^{-2}; mean ± SD)	24.26 ± 3.08	23.81 ± 3.26	24.89 ± 2.87	0.12
BMI of female spouse (kg m^{-2}; mean ± SD)	22.47 ± 3.18	22.59 ± 3.82	22.94 ± 2.98	0.73
Duration of infertility (years; mean ± SD)	4.05 ± 3.08	3.19 ± 2.50	3.38 ± 2.40	0.22
Primary infertility (n (%))	29 (56.86)	47 (58.75)	34 (56.67)	0.96
Secondary infertility (n (%))	22 (43.14)	33 (41.25)	26 (43.33)	
Maternal baseline FSH concentration (U/L; mean ± SD)	7.22 ± 1.60	7.11 ± 2.41	7.91 ± 2.52	0.10
Duration of female induction (days; mean ± SD)	9.78 ± 4.08	10.18 ± 3.75	9.97 ± 5.16	0.87
Female endometrial thickness on hCG day (mm; mean ± SD)	11.24 ± 2.62	11.12 ± 2.94	10.21 ± 2.90	0.09

BMI, body mass index; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin. Normally distributed quantitative. Variables were represented using mean ± SD. Qualitative variables were described as a percentage. Pearson's chi-square test and analysis of variance (ANOVA) were employed for qualitative and quantitative variables with normal distribution.

concentration, morphology, motility, sperm DFI, and AsA level) (Table 3).

Correlations of paternal MTHFR polymorphism (C677T) with embryonic quality and pregnancy outcomes

The embryonic quality following ICSI intervention was first compared among the three groups. Results shown in Table 4 revealed no significant differences in the normal fertilization rate (80.14% vs. 83.06% vs. 85.10%; p = 0.37), high-quality embryo rate (45.26% vs. 43.69% vs. 46.04%; (p = 0.72)), blastocyst formation rate (42.47% vs. 43.18% vs.39.38%; p = 0.62), implantation rate (42.47% vs. 36.25% vs. 41.22%; p = 0.62), and clinical pregnancy rate (64.71% vs. 58.75% vs. 66.67%; p = 0.59). We further examined whether any of the genotypes could influence the pregnancy outcomes, and the results showed that there were also no significant differences in terms of miscarriage rate (24.24% vs. 12.77% vs. 22.5%; p = 0.35)) or live birth rate (49.02% vs. 51.25% vs. 51.66%; (p = 0.96)) among the three groups (Table 4).

Association of paternal MTHFR polymorphism (C677T) with neonatal outcome

The neonatal outcome was evaluated based on gestational age at delivery, preterm delivery, birth height, birth weight,

TABLE 3 Semen parameters of the study's male population.

Sperm parameters	CC (n = 51)	CT (n = 80)	TT (n = 60)	p-value
Abstinence time (days; mean ± SD)	5.47 ± 4.77	5.88 ± 7.44	5.40 ± 3.96	0.87
Semen volume (ml; median (Q1, Q3))	2.90 (2.00, 3.80)	2.70 (1.90, 3.70)	2.75 (1.92, 3.87)	0.79
Sperm concentration ($\times 10^6$ ml^{-1}; median (Q1, Q3))	31.63 (10.95, 56.80)	36.75 (11.75, 97.69)	40.84 (8.71, 66.77)	0.39
Progressive motility (%; median (Q1, Q3))	16.84 (9.42, 33.00)	18.82 (12.66, 40.07)	20.92 (8.05, 29.77)	0.19
Total motility (%; median (Q1, Q3))	20.45 (12.42, 37.57)	25.28 (15.25, 44.71)	25.53 (10.43, 35.72)	0.18
Normal morphology (%; median (Q1, Q3))	3 (2, 4.5)	3 (1, 5)	3 (2, 4.75)	0.88
Leukocyte count ($\times 10^6$ ml^{-1}; median (Q1, Q3))	0.24 (0.08, 0.47)	0.16 (0.08, 0.34)	0.19 (0.08, 0.44)	0.50
AsA (%; median (Q1, Q3))	1 (0, 5)	0 (0, 2)	1 (0, 3.75)	0.06
DFI (%; mean ± SD)	22.68 ± 12.03 (n = 19)	22.97 ± 11.92 (n = 45)	21.59 ± 13.18 (n = 27)	0.89
HDS (%; mean ± SD)	11.41 ± 11.22 (n = 19)	9.37 ± 5.17 (n = 45)	8.85 ± 5.32 (n = 27)	0.43

AsA, antisperm antibody; DFI, DNA fragmentation index; HDS, high DNA stainability; Q1, 25th percentile; Q3, 75th percentile. p-values derived from analysis of variance (ANOVA) or the Kruskal–Wallis test for continuous variables.

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TABLE 4 Embryo quality and pregnancy outcome in association with MTHFR polymorphism.

Embryo and pregnancy parameters	CC (*n* = 51)	CT (*n* = 80)	TT (*n* = 60)	*p*-value
Number of MII oocytes (mean ± SD)	9.04 ± 4.97	9.70 ± 6.08	8.17 ± 6.10	0.31
Cleavage rate (*n* (%))	327/342 (95.61)	652/674 (96.74)	417/434 (96.08)	0.65
Normal fertilization rate (*n* (%))	327/408 (80.14)	652/785 (83.06)	417/490 (85.10)	0.37
High-quality embryo rate (*n* (%))	148/327 (45.26)	343/785 (43.69)	192/417 (46.04)	0.72
Blastocyst formation rate (*n* (%))	79/186 (42.47)	171/396 (43.18)	102/259 (39.38)	0.62
Implantation rate (*n* (%))	44/107 (42.47)	58/160 (36.25)	47/114 (41.22)	0.62
Clinical pregnancy rate (*n* (%))	33/51 (64.71)	47/80 (58.75)	40/60 (66.67)	0.59
Miscarriage rate (*n* (%))	8/33 (24.24)	6/47 (12.77)	9/40 (22.5)	0.35
Live birth rate (*n* (%))	25/51 (49.02)	41/80 (51.25)	31/60 (51.66)	0.96
Singleton birth rate (*n* (%))	19/25 (76)	38/41 (92.68)	28/31 (90.32)	0.17
Multiple-birth rate (*n* (%))	6/25 (24)	3/41 (7.32)	3/31 (9.68)	
p-values derived from Pearson's chi-square test or Fisher's exact test and ANOVA.				

neonatal sex, and Apgar score among the three groups (Table 5). Our results showed that paternal MTHFR polymorphism may not contribute to the neonatal outcome, with no significant difference found in terms of preterm delivery rate (24% vs. 14.63% vs. 9.67%; $p = 0.35$), birth height ($p = 0.75$), birth weight ($p = 0.35$), neonatal sex ($p = 0.48$), gestational age at delivery ($p = 0.24$), and Apgar score ($p = 0.34$). In addition, no statistically significant differences in terms of birth defects (0% vs. 2% vs. 9%; $p = 0.23$) were observed among the three MTHFR C677T genotype groups. The birth defect of one case in the CT group is congenital heart disease, and the birth defect of three cases in the TT group is of patent foramen ovale of heart, congenital heart disease, and cerebral hemangioma.

Discussion

The MTHFR enzyme is a key enzyme in folate metabolism and is necessary for the homocysteine conversion to methionine (22). The 677 C>T MTHFR polymorphism results in decreased activity of MTHFR, causing an increase in homocysteine (Hcy) concentrations in body fluids (12). DNA damage and incorrect methylation caused by excess Hcy can impact the developing gametes and embryos (23). There has been a long-standing interest in the relationship between MTHFR polymorphism(s) and pregnancy outcomes (16). Likewise, numerous studies have investigated the association between maternal MTHFR genetic polymorphism and adverse health outcomes (11, 12, 19).

TABLE 5 Neonatal outcomes in association with MTHFR polymorphism.

Neonatal birth parameters	CC (*n* = 51)	CT (*n* = 80)	TT (*n* = 60)	*p*-value
Gestational age (weeks; mean ± SD)	38.33 ± 2.26	39.18 ± 2.41	38.23 ± 3.07	0.24
Preterm delivery rate (*n* (%))	6/25 (24)	6/41 (14.63)	3/31 (9.67)	0.35
Cesarean delivery rate (*n* (%))	19/25 (76)	29/41 (71)	23/31 (74)	0.89
Normal delivery rate (*n* (%))	6/25 (24)	12/41 (29)	8/31 (26)	
Female baby birth rate (*n* (%))	19/31 (61.29)	22/44 (50)	16/34 (47.05)	0.48
Male baby birth rate (*n* (%))	12/31 (38.71)	22/44 (50)	18/34 (52.95)	
Apgar score (mean ± SD)	9.90 ± 0.30	9.93 ± 0.26	9.76 ± 0.07	0.34
Birth height (cm; mean ± SD)	48.61 ± 2.73	49.14 ± 2.89	49.18 ± 4.33	0.75
Birth weight (kg; mean ± SD)	3.01 ± 0.69	3.25 ± 0.70	3.16 ± 0.72	0.35
Neonatal birth abnormality rate (*n* (%))	0/31 (0)	1/44 (2)	3/34 (9)	0.23
p-values derived from Pearson's chi-square test or Fisher's exact test and ANOVA.				

According to several reports, MTHFR polymorphisms in 677 C>T and hyperhomocysteinemia lead to decreased global sperm DNA methylation and are considered to be risk factors for semen parameters and human infertility (7, 15). Testing for paternal MTHFR polymorphisms may also be crucial because it is widely acknowledged that the paternal genome has a significant role in embryonic development (24). Nevertheless, the correlation between MTHFR genetic polymorphism variants in men and clinical outcomes has not been well established. The present study is the first to investigate the effects of paternal MTHFR polymorphisms on clinical outcomes following ICSI treatment in terms of embryonic quality, pregnancy, and neonatal outcomes. Based on our findings, the male MTHFR genetic polymorphism (677 C>T) does not affect embryo quality, the frequency of early losses, or neonatal outcomes after ICSI treatment. Dobson et al. previously found that paternal MTHFR C677T was not associated with pregnancy rate, positive pregnancy tests, and clinical pregnancy rate in IVF treatment (20). Meanwhile, Poorang et al. also indicated that no significant difference was observed in the frequency of the methylated MTHFR epigenotype between recurrent pregnancy loss (RPL) and non-RPL men (15). This is somewhat surprising; one possible reason for the lack of association between paternal MTHFR genotype and ICSI outcomes in our study is that oocytes may store folic acid or other methyl donors, such as SAM. It is known to all that according to the Chinese guidelines for the prevention of neural tube defects (NTDs) by periconceptional folic acid supplementation, all women trying to conceive ought to take 400 or 800 μg of folate daily at the time of preparation (25). Another possible reason is that the sample size of ICSI patients ($N = 191$) in our research seems insufficient. This is one of the limitations of our study. It should be emphasized that although there was no statistically significant difference, the proportion of birth abnormalities was higher in the MTHFR 677TT group. Possibly, increasing the sample size could bring about more convincing results.

There has recently been evidence that the MTHFR polymorphism (677 C>T) is associated with male infertility in different populations (8, 13, 14, 26). Indeed, scientists found the 222 Val allele (677CT) and Val–Val (677TT) genotypes were significantly more frequent in azoospermic and oligozoospermic men (27–29). A similar result was found in our study of male patients with severe and very severe oligozoospermia. In our study, however, we found that sperm DFI was not affected by the MTHFR polymorphism, consistent with several reports published by Cornet et al. (7).

In conclusion, we did not find any association between the paternal MTHFR C677T polymorphisms and embryonic quality, pregnancy outcomes, including miscarriage, live birth rate, or neonatal outcomes, such as preterm delivery, birth height, birth weight, or gestational age at delivery. Further studies investigating the combined effect of paternal MTHFR polymorphisms on ICSI outcomes with larger sample sizes are recommended.

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 authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethical Committee of the First Affiliated Hospital of USTC (Approval ID: 2022-RE-261). The patients/participants provided their written informed consent to participate in this study.

Author contributions

JH and YW designed the studies. YW and BX collected the data. XJ, YW, and WL performed the data analysis. JH and XJ wrote the manuscript. BX revised the manuscript. All authors reviewed and approved the final manuscript.

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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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Embryo development and live birth resulted from artificial oocyte activation after microdissection testicular sperm extraction with ICSI in patients with non-obstructive azoospermia

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Introduction: The application of microdissection testicular sperm extraction (micro-TESE) to retrieve the sperm of patients with non-obstructive azoospermia (NOA) has greatly increased. Patients with NOA often have poor quality sperm. Unfortunately, there are few studies on artificial oocyte activation (AOA) performed on patients who successfully retrieved motile and immotile sperm by micro-TESE after intracytoplasmic sperm injection (ICSI). Therefore, this study sought to obtain more comprehensive evidence-based data and embryo development outcomes to aid consultation of patients with NOA who opted to receive assisted reproductive techniques and to determine whether AOA needs to be performed in different motile sperm after ICSI.

Methods: This retrospective study involved 235 patients with NOA who underwent micro-TESE to retrieve adequate sperm for ICSI between January 2018 and December 2020. A total of 331 ICSI cycles were performed in the 235 couples. Embryological, clinical, and neonatal outcomes were demonstrated comprehensively between motile sperm and immotile sperm using AOA and non-AOA treatment.

Results: Motile sperm injection with AOA (group 1) showed significantly higher fertility rate (72.77% *vs.* 67.59%, p=0.005), 2 pronucleus (2PN) fertility rate (64.33% *vs.* 60.22%, p=0.036), and miscarriage rate (17.65% *vs.* 2.44%, p=0.018) compared with motile sperm injection with non-AOA (group 2). Group 1 had comparable available embryo rate (41.29% *vs.* 40.74%, p=0.817), good embryo rate (13.44% *vs.* 15.44%, p=0.265), and without an embryo for transfer rate (10.85% *vs.* 9.90%, p=0.815) compared with group 2. Immotile sperm injection with AOA (group 3) displayed significantly higher fertility rate (78.56% *vs.* 67.59%, p=0.000), 2PN fertility rate (67.36% *vs.* 60.22%, p=0.001), without an embryo for transfer rate

(23.76% *vs.* 9.90%, p=0.008), and miscarriage rate (20.00% *vs.* 2.44%, p=0.014), but significantly lower available embryo rate (26.63% *vs.*40.74%, p=0.000) and good embryo rate (15.44% *vs.* 6.99%, p=0.000) compared with group 2. In groups 1, 2, and 3, the rates of implantation (34.87%, 31.85% and 28.00%, respectively; p=0.408), clinical pregnancy (43.87%, 41.00%, and 34.48%, respectively; p=0.360) and live birth (36.13%, 40.00%, and 27.59%, respectively; p=0.194) were similar.

Discussion: For those patients with NOA from whom adequate sperm were retrieved for ICSI, AOA could improve fertilization rate, but not embryo quality and live birth outcomes. For patients with NOA and only immotile sperm, AOA can help achieve acceptable fertilization rate and live birth outcomes. AOA is recommended for patients with NOA only when immotile sperm are injected.

KEYWORDS

intracytoplasmic sperm injection, microdissection testicular sperm extraction, artificial oocyte activation, non-obstructive azoospermia, fertility rate, live birth rate

1 Introduction

Non-obstructive azoospermia (NOA) is the most severe form of male infertility, characterized by the inability of the testes to produce mature sperm. NOA accounts for 60% of all patients with azoospermia (1). In 1999, Schlegel (2) reported that testicular sperm was obtained by cutting open the testes under magnification of a surgical microscope to search for the curving spermatogenic tubules, which was the first application of microdissection testicular sperm extraction (micro-TESE) in the field of assisted reproductive techniques. This procedure provided a new positive outlook for male patients with NOA.

Micro-TESE has gradually become a popular surgical technique with a high sperm retrieval rate and low tissue loss. Compared with men with normal spermatogenesis, patients with NOA had significantly lower rates of fertilization and pregnancy in testicular sperm undergoing an intracytoplasmic sperm injection (ICSI) cycle. In these patients the sperm retrieved through micro-TESE surgery had poor morphology and motility, increased oxidative stress levels, and higher degrees of DNA fragmentation. In addition, after cryopreservation, the reactive oxygen species significantly increased the DNA fragmentation of testicular sperm, resulting in chromatin damage, aneuploidy, mosaicism, and DNA damage, which contributes to poor embryo quality and increased miscarriage rates. Furthermore, the testicular sperm of men with impaired spermatogenesis may have a reduced rate of fertilization after ICSI due to lower sperm maturity relative to those of men with normal spermatogenesis, or fertilization may fail because the sperm cannot trigger oocyte activation (3, 4). In these cases, assisted oocyte activation (AOA) methods, including mechanical stimuli (5), electrical pulses (6), and chemical stimuli (7, 8) aim to induce artificial calcium (Ca^{2+}) rises in the oocytes cytoplasm. Currently, Ca^{2+} ionophores, such as ionomycin and calcimycin, are most widely used in clinical application to keep intracellular Ca^{2+} levels sufficiently high (9). Over the past 20 years, AOA treatment with Ca^{2+} ionophores has been successfully used in infertile patients with failed or low fertilization (10, 11) and severe male factor infertility (12).

Studies have shown that AOA can improve fertilization rate in patients with ejaculatory sperm head malformation, at least one ICSI fertilization failure, and low fertilization rate (<30%) (13, 14). Nasr-Esfahani et al. (15) used AOA combined with ionemycin to improve the fertilization rate and ovulation rate of patients with teratospermia. Some reproductive centers have also found that AOA could improve the embryo quality of epididymal sperm ICSI in patients with OA when comparing the effect of epididymal sperm combined with testicular sperm in patients with OA and patients with NOA (16). Furthermore, many studies have focused on the relationship of defective sperm with low activation ability and oocyte activation. However, due to few centers being available for micro-TESE surgery and low sperm retrieved, how to select micro-TESE sperm for ICSI and whether AOA is required after injecting testicular sperm with different motility were still unknown.

Here, we performed this retrospective data analysis aiming to determine the AOA treatment on reproductive and neonatal outcomes of NOA couples attempting ICSI with micro-TESE sperm. Updated information of the AOA's impact on pregnancy and newborns' outcomes after ICSI with micro-TESE sperm with different motility will be crucial for embryologist to make proper operation and for physicians to make proper recommendations.

2 Materials and methods

2.1 Study design, setting, and participants

This retrospective study included 235 couples who visited the Reproductive Medicine Center of Third Affiliated Hospital of

Guangzhou Medical University for ICSI between January 2018 and December 2020. The male partners were diagnosed with NOA, and their sperm was retrieved by micro-TESE in our hospital. Diagnosis of NOA was based on previous reports obtained at our center (17). All patients who initiated the assisted reproductive cycle had at least one or more tubes of cryopreserved testicular sperm. Testicular tissue suspension was frozen according to our center's usual methods (18). The female patients underwent ovarian stimulation using recombinant follicle-stimulating hormone or human menopausal gonadotropin combined follicle-stimulating hormone antagonists or follicle-stimulating hormone-agonist (19). On the day of oocyte retrieval, testicular sperm was thawed, and ICSI was performed for 331 cycles. AOA was approved by the Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University and was carried out in accordance with the Helsinki Declaration. Due to the study's retrospective nature, informed consent was not required, and patient data were used anonymously.

2.2 Testicular tissue suspension thawed on oocyte retrieval day

After the oocytes were retrieved, a thawing process was immediately performed. The cryovial was removed from the liquid nitrogen container and placed at 24 ± 2 °C for 10 min. Then, the thawed liquid was transferred to a fresh 15 mL centrifuge tube and 2 mL of SpermRinse washing buffer (Vitrolife, Sweden) was added to the tube dropwise and gently mixed. The mixture was centrifuged at 400 g for 10 min, and the supernatant was subsequently removed. The pellet was then resuspended with 1–2 mL of SpermRinse washing buffer. After the second wash, the sperm pellet was resuspended with 50–100 μL of G-IVF-Plus (Vitrolife, Sweden) fluid and placed in a CO_2 incubator until ready to use.

2.3 ICSI procedure

On the day of oocyte retrieval, the ICSI dish was prepared according to usual laboratory procedures, and the incubator was preheated to 37 °C (without CO_2) for ≥30 min. Before ICSI, the treated testicular sperm was added to G-MOPS-plus (Vitrolife, Sweden) droplets in the dish for incubation and a drop of sperm agonist containing 3.6 mmoL/L Pentoxifylline was added (20). Sperm were then observed under the microscope and transferred to PVP (Vitrolife, Sweden) droplets. After obtaining adequate available sperm, they were sequentially immobilized for ICSI.

During ICSI, motile sperm were first screened for injection; in case of insufficient motile sperm, immotile sperm can also be used for ICSI. If motile sperm and immotile sperm are used in the same oocyte retrieval cycle, the oocytes should be cultured separately according to the motility of injected sperm. Oocytes injected with motile sperm with severe teratospermia, with suspected low fertilization, or immotile sperm were placed into an AOA dish after ICSI. Oocytes injected with motile sperm were transferred directly into the embryo culture dish without AOA.

2.4 Artificial oocyte activation process

According to Nasr-Esfahani (15), after ICSI operation, oocytes were immediately transferred to G-IVF-plus medium with a final concentration of 10 μmol/L ionomycin (Sigma, USA) and placed in an incubator at 37 °C with 6% CO_2 for 15 min. They were washed in G-IVF-plus medium for three successive rounds and then transferred to G1-plus (Vitrolife, Sweden) medium droplets that were balanced overnight in an embryo incubator for routine culture.

2.5 Evaluation criteria for embryo culture

According to laboratory evaluation criteria, which indicate that the standard reference of available embryos on day 3 is the number of pronucleus (PN) on day 1, the number of blastomere of embryos on day 3 was ≥5, the difference of blastomere size was ≤30%, and the proportion of fragments was ≤20%. The high-score embryos were those with 7–9 blastomeres and no difference in size. The fertilization rate is defined as the number of zygotes of all observed pronuclei divided by the number of injected oocytes on day 1. The available embryo rate is the ratio between the number of available embryos and the number of zygotes. The high-score embryo rate is the ratio between the number of high-score embryos and the number of 2PN. The no available embryo cycle rate is the ratio of the number of ICSI cycles of no available embryos to the total number of ICSI cycles. The clinical pregnancy rate is the ratio of the number of clinical pregnancy cycles to the number of total transfer cycles. Finally, the live birth rate is the number of live birth cycles divided by the total number of transfer cycles.

2.6 Statistical analysis

Statistical analyses were performed with SPSS software for Windows, version 26.0 (SPSS, Chicago, IL, USA). Normally-distributed quantitative parameters were expressed as mean ± standard deviation (SD) and compared using Students' *t* test or one-way ANOVA when appropriate. Quantitative parameters which were not normally-distributed were expressed as median (25th and 75th quartiles) and compared using Mann–Whitney U test. Comparisons of frequencies and proportions were made using Chi-squared test. A P value <0.05 was considered to have statistical significance.

3 Results

3.1 Micro-TESE sperm morphology and AOA status of patients with NOA

A total of 331 ICSI cycles were included (Figure 1). The micro-TESE sperm in patients with NOA is mostly small with a pear-shaped or cone-shaped head (Figure 2A) under an inverted microscope. In a few samples, sperm of normal morphology can be observed (Figure 2B). Cycles injected with motile sperm and treated with AOA to prevent low fertilization were labeled as group 1 (129 cycles). Cycles injected with motile sperm without AOA were labeled as group 2 (101 cycles). The other 101 cycles injected with immotile sperm and treated with AOA were labeled as group 3.

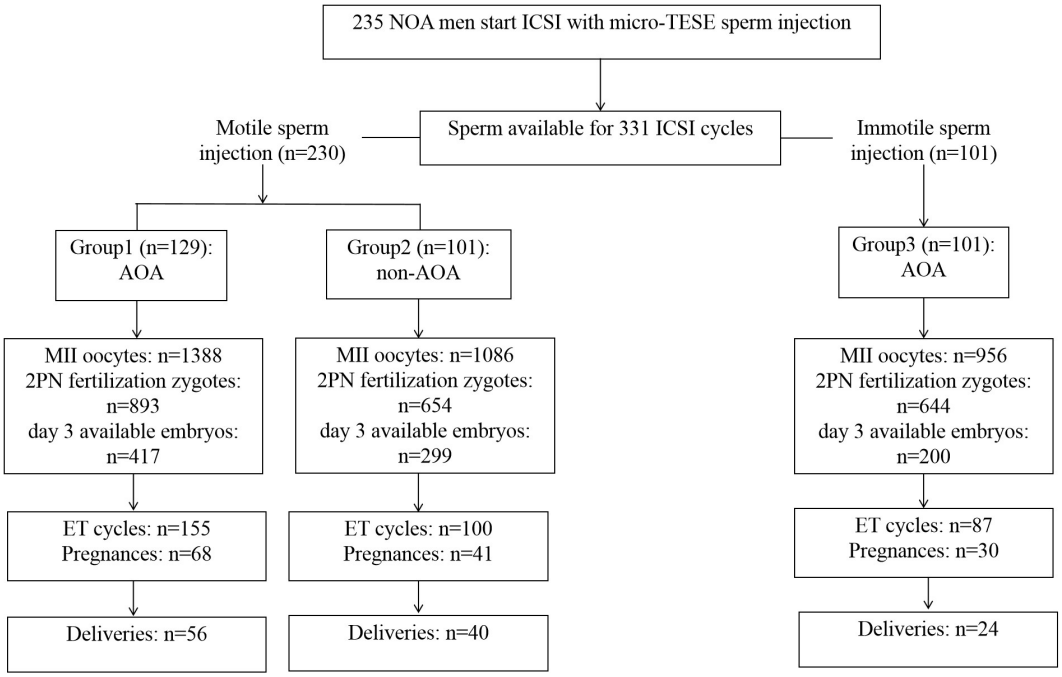

FIGURE 1
Schematic overview of the study.

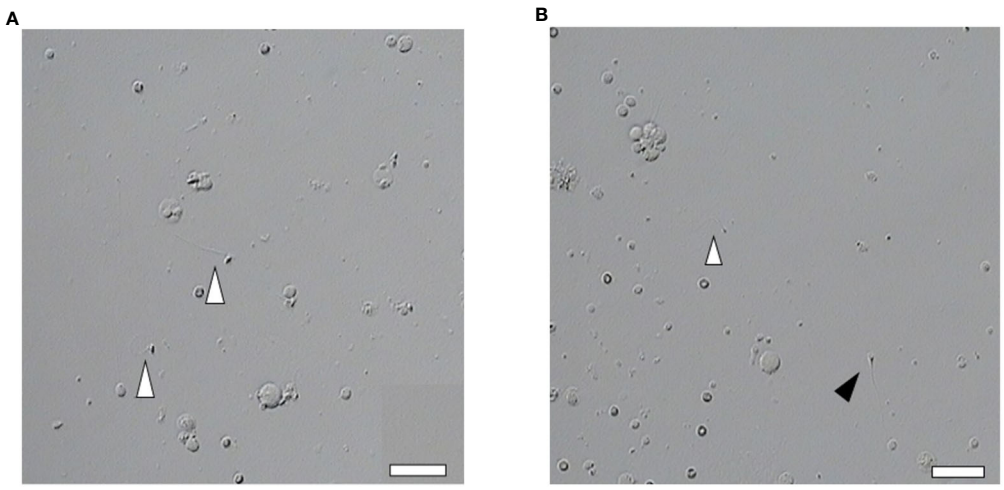

FIGURE 2
Abnormal morphology of sperm retrieved by micro-TESE. **(A, B)** Most of the sperm retrieved by micro-TESE are teratogenic, but occasionally normal sperm can be found. Open arrowhead indicates teratospermia. Solid arrowhead indicates normal morphology sperm (Scale bar, 20 μm).

3.2 Comparisons of basic clinical characteristics among groups

The baseline characteristics were comparable considering male age, female age, anti-Mullerian hormone (AMH), antral follicle counting (AFC), body mass index (BMI), type of infertility, and endometrial thickness among groups (Table 1).

3.3 Comparison of ICSI outcomes after AOA or non-AOA; motile sperm or immotile sperm injection

As demonstrated in Table 2, group 1 (AOA with motile sperm injection) have comparable female age and MII number compared with group 2 (non-AOA group). In group 1, cycles had higher

TABLE 1 Baseline characteristics of the patients enrolled in this study.

	Group 1 (n=129)	Group 2 (n=101)	Group 3 (n=101)	$t/H/\chi^2/$	*P-value*
Male, Age (years)	32.89 (29.00, 35.00)	32.10 (28.00, 35.00)	31.69 (28.00, 34.00)	4.303	0.116
Female,					
Age (years)	29.96 (27.00, 32.50)	30.57 (27.00, 34.00)	29.77 (27.00, 33.00)	1.927	0.382
Infertility duration (years)	4.31 (2.00, 5.50)	4.15 (2.00, 5.00)	4.31 (2.00, 5.00)	0.699	0.705
AMH (ng/mL)	5.39 (2.28, 6.99)	5.13 (2.53, 6.30)	4.31 (2.08, 5.46)	4.264	0.119
Total AFC	20.14 (13.00, 24.00)	21.03 (13.00, 26.00)	19.84 (15.00, 24.00)	0.006	0.997
BMI (kg/m^2)	22.42 (19.82, 23.95)	21.69 (19.20, 23.20)	21.97 (19.30, 23.81)	3.687	0.158
Type of infertility % (n)				0.097	0.953
Primary	83.72% (108)	83.17% (84)	82.18% (83)		
Secondary	16.28% (21)	16.83% (17)	17.82% (18)		
Endometrial thickness (mm)	9.91 ± 2.40	10.38 ± 2.38	10.12 ± 2.34	1.100	0.334
Endometrial type % (n)				3.274	0.195
Type A	42.64% (55)	31.68% (32)	41.58% (42)		
Type B	57.36% (74)	68.32% (69)	58.42% (59)		
Type C and others	0	0	0		

Values are mean ± standard deviation; median (Quartile1, Quartile3) or percent (n).
AMH, anti-mullerian hormone; AFC, antral follicle count; BMI, body mass index.

TABLE 2 Comparison of the ICSI outcomes between AOA and non-AOA after motile or immotile sperm injection.

	Group 1	Group 2	Group 3	*P*-value	*P1*-value	*P2*-value
NO. of ICSI cycles	129	101	101	/	/	/
Age (years)	29.96 (27, 33)	30.57 (27, 34)	29.77 (27, 33)	0.382	0.243	0.244
NO. of MII	10.85 (6, 15)	10.75 (7, 15)	9.47 (6, 12)	0.143	0.848	0.106
Fertilization rate (%)	72.77 (1 010/1 388)[a]	67.59 (734/1 086)[b]	78.56 (751/956)[c]	0.000*	0.005*	0.000*
2PN rate (%)	64.33 (893/1 388)[a,b]	60.22 (654/1 086)[b]	67.36 (644/956)[a]	0.003*	0.036*	0.001*
1PN rate (%)	7.49(104/1 388)[a]	5.89 (64/1 086)[a]	10.77 (103/956)[b]	0.000*	0.117	0.000*
3PN rate (%)	0.94 (13/1 388)[a,b]	1.47 (16/1 086)[b]	0.42 (4/956)[a]	0.051	0.218	0.016*
Rate of available embryos on day 3 (%)[1]	41.29 (417/1 010)[a]	40.74 (299/734)[a]	26.63 (200/751)[b]	0.000*	0.817	0.000*
Rate of high-score embryos on day 3 (%)[2]	13.44 (120/893)[a]	15.44 (101/654)[a]	6.99 (45/644)[b]	0.000*	0.265	0.000*
Rate without an embryo for transfer cycle (%)	10.85 (14/129)[a]	9.90 (10/101)[a]	23.76 (24/101)[b]	0.006*	0.815	0.008*

Values are mean ± standard deviation; median (Quartile1, Quartile3) or percent (n).
Group 1, AOA after motile sperm injection; Group 2, non-AOA after motile sperm injection; Group 3, AOA after immotile sperm injection.
P1, Group 1 compared with group 2; P2, Group 3 compared with group 2. Focusing on P1 and P2, the comparison between group 1 and group 3 was not performed.
[a,b,c] Different superscript letters indicate statistical significance between groups(P<.05).
*Significantly different (P<.05).
[1]Computational formula: number of available embryos on day 3/number of fertilized oocytes.
[2]Computational formula: number of high-score embryos on day 3/number of 2PN.
AOA, assisted oocyte activation; ICSI, intracytoplasmic sperm injection; MII, mature oocyte; PN, primary nucleus.

fertilization rate (72.77% *vs.* 67.59%, *p*=0.005) and 2PN fertilization rate (64.33% *vs.* 60.22%, *p*=0.036) compared with group 2. The 1PN fertilization rate (7.49% *vs.* 5.89%, *p*=0.117), multi-PN fertilization rate (0.94% *vs.* 1.47%, *p*=0.218), available embryo rate (41.29% *vs.* 40.74%, *p*=0.817), high-score embryo rate (13.44% *vs.* 15.44%, *p*=0.265), and no embryo for transfer cycle rate (10.85% *vs.* 9.90%, *p*=0.815) were comparable between the two groups (Table 2).

When compared with group 2, group 3 (immotile sperm injection and AOA) had comparable female age and MII number. Group 3 had higher fertilization rate (78.56% *vs.* 67.59%, *p*=0.000), 2PN fertilization rate (67.36% *vs.* 60.22%, *p*=0.001), and no embryo for transfer cycle rate (23.76% vs. 9.90%, *p*=0.008). However, other outcomes, including available embryo rate (26.63% vs. 40.74%, *p*=0.000) and high-score embryo rate (6.99% vs. 15.44%, *p*=0.000) were lower relative to group 2 (Table 2).

3.4 Comparison of clinical outcomes after AOA or non-AOA; motile sperm or immotile sperm injection

As some ICSI cycles had more than one embryo transfer (ET), there were 155 ET cycles in group 1, 100 ET cycles in group 2, and 87 ET cycles in group 3. As demonstrated in Table 3, group 1 had comparable blastocyst-stage ET rate (24.37%, 24.84%, and 19.20%, *p*=0.464), implantation rate (34.87%, 31.85%, and 28.00% *p*=0.408), clinical pregnancy rate (43.87%, 41.00%, and 34.48%, *p*=0.360), and live birth rate (36.13%, 40.00%, and 27.59%, p=0.194) compared with group 2 and group 3, respectively. However, in group 3, more women had miscarriage compared with group 2 (20.00% *vs.* 2.44%, *p*=0.014). Group 1 had a higher rate of pregnancy with their first ET (67.09%, *p*=0.004) compared with the other groups (Table 3).

TABLE 3 Comparison of clinical outcomes between AOA and non-AOA after motile or immotile sperm injection.

	Group 1	Group 2	Group 3	*P-value*	*P1*-value	*P2*-value
No. of ET cycles	155	100	87		/	/
Age (years)	29.95 (27, 33)	30.25 (27, 34)	29.11 (27, 32)	0.366	0.346	0.107
Embryos per transfer	1.51 (1, 2)	1.56 (1, 2)	1.41 (1, 2)	0.131	0.433	0.057
Blastocyst-stage ET rate (%)	24.37 (58/238)	24.84 (39/157)	19.20 (24/125)	0.464	0.915	0.259
Implantation rate (%)	34.87 (83/238)	31.85 (50/157)	28.00 (35/125)	0.408	0.533	0.484
Clinical pregnancy rate (%)	43.87 (68/155)	41.00 (41/100)	34.48 (30/87)	0.360	0.651	0.360
Miscarriage rate (%)	17.65 (12/68)[a,b]	2.44 (1/41)[b]	20.00 (6/30)[a]	0.043*	0.018*	0.014*
Pregnancy rate of first ET (%)	67.09 (53/79)[a]	50.00 (32/64)[a,b]	40.28 (29/72)[b]	0.004*	0.039*	0.255
Live birth rate (%)	36.13 (56/155)	40.00 (40/100)	27.59 (24/87)	0.194	0.533	0.074
Neonatal outcomes of embryo transfer				0.369	0.634	0.307
Singletons (%)	78.57 (44/56)	82.50 (33/40)	91.67 (22/24)			
Twins (%)	21.43 (12/56)	17.50 (7/40)	8.33 (2/24)			
Baby's sex				0.030*	0.011*	0.606
Birth babies	68	47	26			
Male (%)	39.71 (27/68)[a]	63.83 (30/47)[b]	57.69 (15/26)[a,b]			
Female (%)	60.29 (41/68)	36.17 (17/47)	42.31 (11/26)			
Birth weight (g)						
Singletons	3177.84 ± 435.03	3175.76 ± 444.40	3200.45 ± 424.29	0.975	0.795	0.970
Twins	2220.83 ± 646.29	2156.64 ± 671.03	2358.75 ± 130.28	0.849	0.850	0.110
Body length (cm)						
Singletons	49.34 (48.00, 50.75)	50.06 (49.00, 51.00)	50.00 (50.00, 50.25)	0.708	0.607	0.661
Twins	46.00 (45.25, 48.75)	44.29 (43.00, 48.00)	47.00 (46.00, 48.00)	0.712	0.455	0.518
Early neonatal death rate (%)	0	0	0			
Birth defect rate (%)	0	0	0			

Values are mean ± standard deviation; median (Quartile1, Quartile3) or percent (n).
Group 1, AOA after motile sperm injection; Group 2, non-AOA after motile sperm injection; Group 3, AOA after immotile sperm injection.
P1, Group 1 compared with group 2; P2, Group 3 compared with group 2. Focusing on P1 and P2, the comparison between group 1 and group 3 was not performed.
[a,b] Different superscript letters indicate statistical significance between groups (P<.05).
*Significantly different (P<.05).
AOA, assisted oocyte activation; ET, embryo transfer.

Neonatal outcomes, including singleton and twin births rate, baby's birth weight, and baby's body length were comparable among the three groups. In group 2, women had more male newborn birth compared with group 1 (63.83% *vs.* 39.71%, p=0.011).

4 Discussion

The clinical indications for the use of AOA in assisted reproductive techniques mainly include complete fertilization failure or fertilization rate ≤30% in the patient's previous ICSI (10), and severe teratospermia (such as round head sperm and acrosome defects) (13, 15). AOA methods include mechanical stimulation and chemical activation. In the mechanical stimulation method, activation of oocytes was assisted by the suction of injection pipette directly during ICSI to improve fertilization rate (5). The chemical activation method mainly uses calcium ionophore of a certain concentration (calcimycin A23187 or ionomycin) to incubate with oocytes for 10 to 15 min after ICSI (15, 21). These outcomes suggested that AOA could effectively improve ICSI fertilization rate; however, Meerschaut et al. (21) believed that not all patients with low ICSI fertilization rate could benefit from AOA.

Due to the poor spermatogenic function of patients with NOA, sperms obtained by micro-TESE often have extremely poor motility, acrosome defects, and other morphological abnormalities, that often lead to low fertilization rate, poor embryo quality, and low clinical pregnancy rate due to the inability to activate oocytes and initiate the second meiosis after ICSI (22). To improve the ICSI and clinical outcomes with severely abnormal sperm, AOA was applied on the oocytes of some patients whose partners had produced motile sperm and for partners of all patients who produced only immotile sperm after micro-TESE.

The main strength of this study is the comprehensive analysis of clinical outcomes for patients with NOA with different motile sperm retrieved by micro-TESE who underwent ICSI cycles, which is rarely reported in previous literature.

4.1 Motile sperm improves embryo and live birth outcomes in patients with NOA

NOA is caused by Y-chromosome microdeletions and chromosomal abnormalities, as well as non-genetic etiologies, such as cryptorchidism, heat exposure, infections, and chemoradiotherapy (23). The sperm of patients with NOA for ICSI can be retrieved through micro-TESE surgery. Usually, the selection of motile sperm is given priority, but immotile sperm with normal morphology can also be chosen when the number of motile sperm is insufficient. However, during micro-TESE surgery, testicular spermatozoa DNA fragmentation could increase significantly after cryopreservation in cryotubes, possibly due to the formation of reactive oxygen species that cause chromatin damage (24–26). To improve laboratory and clinical outcomes in this extremely abnormal sperm condition, we performed AOA treatment on the oocytes of some patients with motile or immotile sperm obtained through micro-TESE surgery after ICSI. The laboratory and clinical outcomes of ICSI treated with or without AOA were compared and analyzed.

Our results showed that after ICSI, patients with motile sperm had greater availability of embryos, high-score embryos, and lower possibility of no embryo for transfer compared with patients with immotile sperm. This suggests that patients with NOA with motile sperm are more likely to become biological fathers than those with immotile sperm. Thus, when micro-TESE sperm is needed for ICSI, efforts should be made to retrieve motile sperm.

4.2 Motile sperm and non-AOA treatment benefits live birth outcomes in patients with NOA

We compared the fertilization, embryo development, pregnancy outcomes, and live birth rate of AOA and non-AOA treatment in patients injected with motile sperm. AOA improved the rates of fertilization and pregnancy obtained from the first ET of patients with NOA, but miscarriage rate increased. Furthermore, our study showed that AOA treatment did not improve the quality of embryos after fertilization. This is consistent with a study reported that AOA can effectively improve fertilization from ICSI, but not all patients with low fertilization rates can benefit (21).

AOA used in this study used Ca^{2+} ion carriers to assist oocyte activation. It has been reported that specific Ca^{2+} signatures would likely impact cellular events during oocyte activation (27) and subsequent embryonic development (28). Moreover, researchers found that a physiological or artificial lack of Ca^{2+} signaling during oocyte activation in mice and humans could impair preimplantation development, blastocyst quality, and gene expression profiling (29, 30). Therefore, patients with low Ca^{2+} signaling patterns may benefit from AOA treatment for improving embryological and clinical outcomes. In our study, there were no significant differences in available embryos on day 3, high-score embryos on day 3, blastocyst-stage ET, clinical pregnancy, implantation, and live birth rates between patients with AOA and non-AOA injected with motile sperm, which indicated that NOA patients with motile sperm may have normal Ca^{2+} signaling patterns and normal embryonic development activation levels. Under these conditions, an additional AOA operation did not improve embryo quality. Conversely, the excessive artificial Ca^{2+} oscillations created by AOA might affect other downstream sequences, such as mitochondrial metabolism, or other critical developmental pathways (31, 32). Notably, current studies have focused on congenital abnormalities, birth weight, and neurodevelopmental outcomes of AOA-born children; however, long-term follow-up data were absent and need to be investigated in future studies. Therefore, we recommend that patients with NOA do not need AOA operation after ICSI using motile sperm.

4.3 Motile sperm is not a determinant of live birth outcomes in patients with NOA

Since AOA did not improve embryo quality after motile sperm ICSI, we analyzed whether AOA could improve live birth outcomes of immotile sperm injection, by comparing patients who retrieved motile sperm after AOA with those who retrieved motile sperm without AOA. In patients with no motile sperm, the 2PN fertilization rate was significantly higher than that of the patients receiving motile sperm injection without AOA. However, the available embryos on day 3 and the high-score embryos on day 3 rates were significantly lower than those of the patients ICSI with motile sperm. In addition, the proportion of no embryos for transfer was still high. Although the embryo quality of patients with AOA injected with immotile sperm was poor than that of patients without AOA injected with motile sperm, the rates of pregnancy and live birth was comparable between them. This is consistent with previous studies in which the clinical pregnancy rates of patients with AOA and immotile sperm injection were similar to those with motile sperm injection alone (33). Although some studies have reported that immotile sperm after thawing may still lead to normal fertilization (34, 35), ICSI with immotile sperm after thawing usually presents lower fertilization rates than when using motile sperm. Furthermore, several studies have reported that the addition of AOA with ionomycin after ICSI could significantly increase the fertilization rate in patients with severe teratozoospermia (4, 36). For example, Ebner et al. (37) showed that AOA with a Ca^{2+} ionophore could enhance fertilization in patients with cryptozoospermia. In our study, we applied AOA to some patients with NOA using thawed motile or immotile micro-TESE sperm, which significantly improved fertilization rate and live birth rate, regardless of sperm motility status. Considering the rarity of sperm in patients with NOA, the uncertainty of the survival status of immotile sperm, and the acceptable live birth rate of immotile sperm brought by AOA, it is recommended that patients without motile sperm use immotile sperm for ICSI, followed by AOA with a Ca^{2+} ionophore.

4.4 Patients with NOA have higher miscarriage rate after AOA

In this study, the miscarriage rates in patients with AOA with both motile and immotile sperm were significantly higher compared with patients without AOA, and higher than the overall ART spontaneous abortions (10–15%) reported in China (38). The high miscarriage rate was probably related to the fact AOA operation cannot improve embryo quality. Furthermore, the motile micro-TESE sperm injected can be fertilized normally without AOA, indicating the motile micro-TESE sperm may have normal Ca^{2+} signaling patterns and normal activation levels for fertilization and subsequent embryonic development. Under this condition, an additional AOA operation did not improve the embryo quality. Conversely, the excessive artificial Ca^{2+} oscillations created by AOA might affect other critical developmental pathways. The high miscarriage rate of immotile sperm injection may be related to the poor sperm quality in patients with NOA. These patients retrieved sperm through micro-TESE with poor morphology and motility, increased oxidative stress levels, and higher DNA fragmentation degrees. In addition, after cryopreservation, the formation of reactive oxygen species significantly increases DNA fragmentation in testicular sperm, resulting in chromatin damage, aneuploidy, mosaicism, and DNA damage that contribute to poor embryo quality and increased miscarriage rates (32, 33).

4.5 Limitations

This study has some limitations, including the small sample size of the group of embryo transfer patients injected with immotile sperm, the need for more follow-up data on live births, and patient selection bias. In future, further focus should be given to congenital abnormalities, birth weight, and neurodevelopmental outcomes of AOA-born children, and long-term follow-up data need to be collected.

5 Conclusions

In summary, patients with NOA do not need AOA after injection of motile micro-TESE sperm; the fertilization rate of 2PN can reach 60.22%, while live birth rates can reach 40%. It is suggested that immotile sperm with normal morphology is used for ICSI in patients without motile sperm, who then undergo AOA; in this way, the live birth rate of immotile sperm injection can reach 27.59%, which is not significantly different from that of motile sperm injection, and the 2PN fertilization rate can reach 67.36%. It should be noted that there was a significant increase in miscarriage rates after AOA with both motile and immotile sperm.

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 Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

The contributions of all authors were as follows: conceptualization and data curation: LeL and HD Writing-

original draft: XZ. Statistical analysis: LiL. Data collection: WZ, YL, and YM. 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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Management of male erectile dysfunction: From the past to the future

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Erectile dysfunction is a common disease of the male reproductive system, which seriously affects the life quality of patients and their partners. At present, erectile dysfunction is considered as a social-psychological-physiological disease with complex etiology and various treatment methods. Oral PDE5I is the first-line treatment for erectile dysfunction with the advantages of high safety, good effect and non-invasiveness. But intracavernosal injection, hormonal replacement therapy, vacuum erection device, penile prosthesis implantation can also be alternative treatments for patients have organic erectile dysfunction or tolerance to PDE5I. With the rapid development of technologies, some new methods, such as low-intensity extracorporeal shock wave and stem cell injection therapy can even repair the organic damage of the corpora cavernosa. These are important directions for the treatment of male erectile dysfunction in the future. In this mini-review, we will introduce these therapies in detail.

KEYWORDS

erectile dysfunction, phosphodiesterase 5 inhibitor, intracavernosal injection, hormonal replacement therapy, vacuum erection device, penile prosthesis implantation, low-intensity extracorporeal shock wave, stem cell injection therapy

Introduction

Erectile dysfunction (ED) is defined as the consistent inability to attain and maintain an erection sufficient to perform satisfactory sexual intercourse (1). ED is a common male problem at all ages that has a great impact on the quality of life of sufferers and their partners. More than 150 million men worldwide are reported to have ED in different extent (2). Due to racial and regional differences and different definitions of ED, there is a large gap in the existing epidemiological data of ED. In the United States, the incidence of ED is 25.9 cases per 1000 people, and it increases with age with more than 70% of men over 70 years old affected by ED. It is predicted that by 2025, 322 million men worldwide will have ED (3–5). Studies have shown that the occurrence of ED is associated with many

comorbidities and risk factors, such as aging, smoking, obesity, decreased androgen levels, cardiovascular disease, depression, prostate surgery, and penile trauma (6–8).

Normal penile erection is a neurovascular phenomenon controlled by psychological factors and coordinated by endocrine, vascular, and nervous system (9). The first step in management of ED is often making lifestyle changes, such as losing weight, reducing alcohol intake, and avoiding smoking. These psychosocial methods are effective when ED is mainly caused by emotional or psychological factors (10). Current therapies to treat ED mainly include oral phosphodiesterase 5 inhibitor (PDE5I), intracavernosal injection, hormonal replacement therapy, vacuum erection device, penile prosthesis, low-intensity extracorporeal shock wave (Li-ESW), and stem cell injection therapy (11) (Figure 1). Under different conditions, any option can be the first line of treatment. To data, PDE5I is still the most popular treatment option due to its good efficacy, safety, and non-invasiveness. But, the growing number of patients with no or low response to PDE5I, and the potential of adverse reactions have prompted the development of safer and more effective treatments (12). In this paper, we will introduce various treatment methods for ED in detail based on current research progress.

Drug therapy: Phosphodiesterase 5 inhibitors

Phosphodiesterase 5 (PDE5) is highly expressed in vascular smooth muscle and it is the most common PDE subtype in penile smooth muscle. Penile erection mainly depends on the activation of NO/cGMP signal pathway and the function of PDE5 is to block the decomposition of cyclic guanosine monophosphate (cGMP) (13, 14). the NO produced by non-adrenergic/non-cholinergic neurons and endothelial cells is released into the corpora cavernosa, resulting in an increase in the concentration of cGMP, thus promoting the relaxation of smooth muscle in the corpora cavernosa and the expansion of penile blood vessels, finally leading to vascular filling and penile erection (15). Therefore, PDE5I can enhance erectile response and treat erectile dysfunction by enhancing the downstream cGMP effect caused by NO.

PDE5Is are the first-line treatment for erectile dysfunction (16). At present, four PDE5I drugs have been approved by the FDA, namely sildenafil, tadalafil, vardenafil, and avanafil. These PDE5Is have different pharmacokinetic properties but have similar efficacy, safety, and tolerability (17). Among them, sildenafil is the first approved, safe, and effective oral drug for the treatment of ED. In a randomized, double-blind study, Goldstein et al. (18) included 532 male patients diagnosed with ED at least 6 months and randomly assigned 316 patients to the sildenafil group (25, 50 or 100ng) and 216 patients to the placebo control group. After 24 weeks, the erectile function of the oral sildenafil treatment group improved significantly, and men taking 100 mg of sildenafil showed a better therapeutic effect compared to the placebo control group. Sildenafil can also be used in combination with other drugs. In another randomized controlled study, 59 patients with organic ED were included in the study (19). One group received oral sildenafil 50 mg, the other group received oral sildenafil 50 mg and L-arginine 1g, after 8 weeks of treatment, patients in the combined treatment group showed better erectile function. These studies have proved the effectiveness of sildenafil in the treatment of ED and its potential in combination with other therapies. As a powerful and highly selective PDE5I, avanafil has been reported to have better effects and fewer adverse reactions. Kumar et al. (20) recently reported a randomized, controlled, double-blind clinical trial in which 220 patients with ED were randomly divided into two groups in a 1:1 ratio, they were given orally 100 mg of avanafil and 50 mg of sildenafil respectively. International index of erectile function (IIEF)

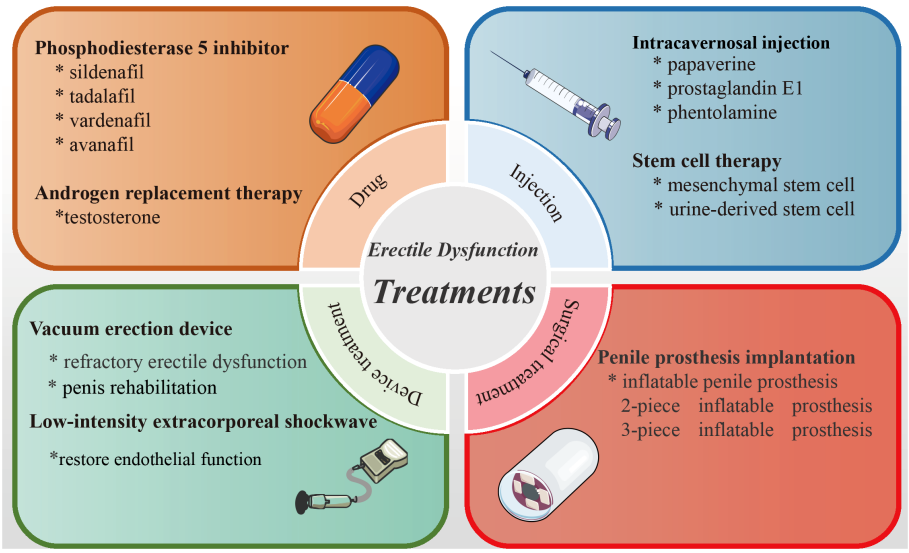

FIGURE 1
Summary diagram of the therapies of erectile dysfunction.

score, sexual encounter profile (SEF), and adverse drug events were evaluated, and the results demonstrated that avanafil took effect quickly, and most people showed good erectile function after 15 minutes of medication.

In addition to acting on PDE5 of cavernous smooth muscle, PDE5I also inhibits PDE5 and its isozymes in blood vessels, viscera, skeletal muscle, platelets, and other tissues, causing reactions in multiple systems. During treatment, adverse reactions such as headache, blush, dyspepsia, and visual disturbance can occur (21). In addition, after taking tadalafil for 6 months, the weight of testis, sperm quantity and sperm activity of the aged male rats were significantly reduced (22), after 12 weeks of oral administration of sildenafil, tadalafil, and vardenafil in male rabbits, the number of sperm in sperm cells and testis were also decreased (23). In general, PDE5I is a safe, effective, and well-tolerated first-line treatment for ED, for most patients, 50 mg sildenafil is the preferred treatment, after drug tolerance, 10 mg tadalafil or 100 mg udenafil can be used instead (24). Not only that, but current studies also found new molecular mechanisms of ED beyond the 'NO/cGMP' pathway. For example, hyperglycemia and increased oxidative stress are main contributors to endothelial dysfunction in ED patients complicated with diabetes mellitus (25). The major regulatory unit of myosin light chain phosphatase MYPT1 regulated ED by G-protein couple receptor pathway, and the lotusine could recover the level of MYPT1 and improve the function of injured penile smooth muscles. These studies provide novel therapeutic targets for the treatment of ED in the future (26).

Intracavernosal injection

Intracavernosal injection (ICI) of vasoactive drugs such as papaverine and prostaglandin E1 to induce penis erection is a breakthrough in the treatment of ED and can also be used as a diagnostic method (27). ICI is an effective local drug therapy for ED, individualized treatment plans can be formulated according to the individual conditions and needs of the patients (28). The combination of different vasoactive drugs and different injection doses can significantly improve the treatment effect and reduce complications. The study showed that the patients injected with papaverine and prostaglandin E1 could achieve satisfactory erectile function, which was better than patients injected with prostaglandin E1 alone (29). In a retrospective study, the researcher included 105 middle-aged and elderly patients and found that after ICI treatment, the patient's penis hardness increased, erectile function improved, and there were no obvious complications, this means that ICI therapy is safe and feasible (30). However, with the use of PDE5Is, the clinical application of ICI has gradually decreased, because it has a high dropout rate and is related to priapism, ecchymoses, hematoma, and penile fibrosis (31). At present, the combination of ICI and Doppler ultrasound is mainly used in the diagnosis of ED and the evaluation of penile hemodynamics (32).

Hormonal replacement therapy

Androgen plays an important role in promoting the normal growth of the penis and stimulating the secondary sexual characteristics of men. Androgen is mainly secreted by the testis, androgen deficiency will lead to a series of pathophysiological conditions, which will damage the sexual function and overall health of the body (33). Researchers found that serum total testosterone, especially free testosterone and bioavailable testosterone levels of men will gradually decrease with age. A study showed that 64% of men over 40 years of age will be diagnosed with moderate, severe and very serious ED and older men over 60 years of age are more likely to suffer from more serious ED (34). Similarly, Rabijewski et al. found that 53% of the elderly men over 65 years old had lower testosterone levels, and ED was more serious in these men. They also found that there was significant negative correlation between age and testosterone ($r=-0.3328$, $p<0.05$), IIEF score and testosterone ($r=-0.3149$, $p<0.05$), and age and IIEF score ($r=-0.3463$, $p<0.05$) (35).

In clinic, androgen replacement therapy can restore the serum testosterone level to normal, and improve the sexual desire of patients with hypogonadism. In addition, compared with the placebo group, after receiving androgen replacement therapy, the patients would get better mood and the depression was relieved (36). Another study showed that in older men with low testosterone levels older than 65 years, the frequency of sexual activity increased significantly and the sexual desire improved after one year of androgen replacement treatment (37). More importantly, androgen replacement therapy combined with PDE5I can effectively treat ED, and patients' erectile function can even be maintained well after drugs withdrawal (38).

Vacuum erection device

Vacuum erection device (VED) is a mechanical device that can increase the blood flowing into the corpora cavernosa by creating a vacuum environment of up to 250 mmHg and there is a restraining ring at the root of the penis to maintain congestion, promote erection (39). VED is mainly used to treat patients with organic ED and it has high success rate and small side effects (40). In a recent study, 56 middle-aged and elderly patients with ED were treated with VED, 96% of the patients believed that the device could promote the ability of erection and 94% of the patients and their partners thought that they regained satisfactory sexual activities after VED treatment. Nevertheless, nearly 28.6% of the patients reported physical discomfort when using the device, usually due to the pain caused by using the restraining ring (41).

In addition to treating ED, VED can also treat penis atrophy after radical prostatectomy by enhancing oxygen saturation in the corpora cavernosa (42). Rats with penile atrophy and decreased erectile function were created by bilateral cavernous compression injury (BCNI). After 6 weeks of treatment with VED for BCNI rats, the penis diameter of the rats was increased, the degree of atrophy decreased, and the oxygen saturation in the corpora cavernosa increased. These results show that VED treatment has therapeutic effect by increasing the anti-hypoxia ability of corpora cavernosa (43). PDE5I tolerance is a common outcome of oral medication in ED patients and about 30% of ED patients have no obvious response to PDE5I treatment eventually (44). After using VED

treatment for these patients, their erectile function improved and their sexual desire increased. VED is the second-line treatment for ED, but it should be considered as the first-line treatment for some men who have tolerance to PDE5I or need penis rehabilitation (45).

Penile prosthesis implantation

Penile prosthesis implantation (PPI) is currently the third-line treatment for ED. Because it can cause irreparable damage to the smooth muscle of the corpora cavernosa, it is usually considered when oral PDE5I drugs, intracavernosal injection, and VED therapy are ineffective (46). 3-piece inflatable prosthesis is the most common implant at present, and is also the most satisfactory (47). The 3-piece inflatable prosthesis can manually adjust the thickness, length, and hardness of the penis, and simulate the natural erection process. Therefore, it should be recommended for patients who choose PPI.

An early multi-center study reported that more than 90% of patients with ED and their partners can achieve normal sexual activity after receiving penis prosthesis implantation (48). Recent study has shown that penis prosthesis implantation is particularly suitable for ED patients secondary to Peyronie's disease (49). Nevertheless, PPI is costly, traumatic, and prone to complications, such as prosthetic infection, pump migration, automatic inflation, secondary surgery, etc. This is the main reason why it cannot become a first-line treatment (50).

Low-intensity extracorporeal shock wave

Low-intensity extracorporeal shock wave (Li-ESW) is a physical shock wave that emits energy density lower than 0.1mj/mm^2. As a non-invasive treatment technology, Li-ESW focuses on the target tissue area through the sound wave passing through the tissue structure (51). Studies have shown that one of the causes of ED is the decreased blood circulation in the corpora cavernosa, Li-ESW can stimulate the expression of eNOS, VEGF and other vascular growth factors in the corpora cavernosa, expand blood vessels, induce neovascularization, promote blood flow, and improve erectile function (52, 53).

Vardi et al. (54) treated 20 middle-aged patients with vascular ED with Li-ESW, the patients received 12 Li-ESW treatments within 6 weeks. The results showed that the erectile function of 75% of patients was significantly improved, and the IIEF score was obviously increased, the penile blood flow, erection duration and penile hardness of the patients were also increased. Li-ESW therapy has also been proved to be a safe and effective method for those patients with poor effect of PDE5I (55). Another study found that the use of Li-ESW can reverse the PDE5I tolerance, and more than 50% of the patients can achieve sufficient erectile stiffness (56).

Li-ESW is a promising treatment for refractory ED. It can restore the endothelial function of the penis and increase the blood flow of the corpora cavernosa. However, the therapeutic mechanism of Li-ESW is not yet completely clear, and more research and exploration are still needed.

Stem cell injection therapy

Stem cells can differentiate into different types of cells under the stimulation of complicated external environments and cytokines. They can be divided into totipotent stem cells, pluripotent stem cells, multipotent stem cells, and unipotent stem cells (57). Studies have shown that stem cells can also promote angiogenesis, tissue healing, and anti-apoptosis through paracrine action, which is also the theoretical basis of the therapy for ED (58). In the current scheme of stem cell therapy, it mainly includes mesenchymal stem cell (MSC), adipose tissue-derived stem cell (ADSC), urine-derived stem cell (UDSC), and muscle-derived stem cell (MDSC) (58, 59). In 2004, Bochinski et al. first discovered that injection of neural embryonic stem cells into the corpus cavernosa of male rats with neurogenic impotence could improve the erectile function (60). In the BCNI rat model, injection of autologous ADSC cells into the corpora cavernosa of rats can effectively prevent erectile dysfunction caused by cavernous nerve injury, enhance the ratio of smooth muscle over collagen content, and promote the neuronal nitric oxide synthase-positive nerve regeneration (61, 62). In rat model of erectile dysfunction, intracavernous injection of ADSCs can ameliorate ultrastructural damage and systemic oxidative stress states caused by chronic tobacco exposure or hyperlipidemia (63, 64). In addition, intracavernous injection of ADSCs-derived microtissues improves erectile function in STZ-induced diabetic rats *via* expressing vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and tumor necrosis factor-stimulated gene-6 (TSG-6) (65). Furthermore, the exosomes secreted by stem cells have also been proved to have an effect on improving erectile dysfunction in rats. The study found that the injection of ADSC-derived exosomes into the cavernous body of rats can promote the growth of endothelial cells and smooth muscle cells, inhibit cell apoptosis, alleviate tissue hypoxia, and promote the recovery of erectile function in rats (66, 67).

Although many animal experiments have confirmed the effectiveness of stem cell therapy in treating ED, there are only a few clinical trials on stem cell therapy. In an open-label phase 1 clinical trial, 17 male patients with ED after radical prostatectomy were insensitive to PDE5I and ICI. The researchers injected autologous adipose-derived regenerative cells (ADRCs) into the corpora cavernosa of patients, the results showed that 73% of the patients recovered their erectile function within 3 months after treatment (68). According to animal experiments and a small number of clinical studies on stem cell therapy for ED, stem cell therapy is safe and reliable. Stem cell therapy has a broad prospect in the treatment of ED, but more clinical trials are still needed before clinical application.

Conclusions and outlook

ED is a common disease in men and seriously affects the life quality of patients and their partners. Currently, oral PDE5I is the first-line treatment of ED which has the advantages of high safety and good effect. For patients with low response to PDE5I, other treatments include intracavernosal injection, hormonal treatment, vacuum erection device, and penile prosthesis implantation can also be

alternative methods. In recent years, some new therapies like low-intensity extracorporeal shock wave and stem cell injection therapy proved to have exciting effect and can even reverse the organic damage of the corpora cavernosa. In fact, there are other advanced therapies, such as gene therapy (69), 3D-printed hydrogel scaffolds (70), and gene edited stem cells (71), they have all been shown to improve erectile function in animal experiments.

Despite these promising therapies are important directions for treatment of ED in the future, but only impressive animal studies proving the benefit. Large-scale, randomized, placebo-controlled studies are desperately needed for these novel therapeutics. In summary, ED is a complex disease associated with multiple risk factors, and effective therapy should be taken according to the etiology and individual conditions. The safety and efficacy of the promising therapies still need to be evaluated through a number of clinical trials with ethical support and fully informed consent.

Author contributions

This mini review is contributed by all authors. XH and JX conceived and designed the study. CW and BW wrote the manuscript. CW, BW, PX, JX, and XH reviewed and edited 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.

Publisher's note

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Relationship of the levels of reactive oxygen species in the fertilization medium with the outcome of *in vitro* fertilization following brief incubation

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Embryo selection in *in vitro* fertilization–embryo transfer (IVF-ET) mostly relies on morphological assessment using a conventional microscope or the time-lapse monitoring system, which is not comprehensive. Inappropriate levels of reactive oxygen species (ROS) in the fertilization medium may cause damage to gametes, eventually leading to adverse IVF outcomes. The present study aimed to identify the optimal oxidation–reduction level in the fertilization medium for IVF outcomes by measuring the static oxidation–reduction potential (sORP) using a highly accurate and sensitive MiOXSYS system. A total of 136 patients undergoing IVF following brief incubation were divided equally into 4 groups in this prospective cohort study. The sORP value in the fertilization medium was detected using the MiOXSYS system, and its relationship with IVF outcomes was analyzed. The primary outcome was pregnancy outcomes, including live birth rate (LBR), clinical pregnancy rate (CPR), biochemical pregnancy rate (BPR), and implantation rate (IR). The secondary outcome was embryo quality, including fertilization rate (FR), cleavage rate (CR), available embryo rate (AER), and good-quality embryo rate (GQER). Group II (sORP: 228.7–235.3 mV) showed a higher LBR, CPR, BPR, and IR compared with Group III (sORP: 235.4–242.7 mV), presented as follows: LBR (32.0% for Group II vs 3.6% for Group III, $P = 0.033$), CPR (32.0% for Group II vs 3.6% for Group III, $P = 0.033$), BPR (36.0% for Group II vs 3.6% for Group III, $P = 0.019$), and IR (31.3% for Group II vs 2.7% for Group III, $P = 0.003$). The FR in Groups I and II had lower significant differences compared with that in Groups III and IV (71.7% and 70.3% for Groups I and II vs 83.5% and 80.4% for Groups III and IV, $P = 0.000$). The GQER in Group I to Group IV was 32.7%, 37.4%, 26.5%, and 33.3%, respectively ($P = 0.056$). This study indicated that the sORP value in the fertilization medium might be a potential indicator of embryo quality and pregnancy outcome.

KEYWORDS

Fertilization medium, IVF outcome, MiOXSYS system, reactive oxygen species, sperm

1 Introduction

Reactive oxygen species (ROS), including superoxide (O_2^-), hydroxyl radical (OH^-), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2), are produced by various biochemical cellular reactions, especially in the mitochondrial respiratory chain (1). Numerous mitochondria are retained during spermatogenesis to maintain various sperm functions while producing some ROS (2). A moderate level of ROS in spermatozoa regulates essential functional characteristics, such as motility, capacitation, and sperm–oocyte fusion (3). Oxidative stress occurs when the production of ROS exceeds the antioxidant scavenging ability, which has detrimental effects on sperm function, eventually affecting the fertility of an individual (4). In *in vitro* fertilization (IVF), the retrieved cumulus–oocyte complexes (COCs) are co-incubated with capacitated spermatozoa, which is a more physiological approach to selecting spermatozoa. Long-time spermatozoa–oocyte co-incubation results in an increase in the level of ROS in fertilization droplets because sperm may continue to produce ROS. A brief incubation of gametes is used to prevent the influence of excessive ROS on fertilization and embryo development. Some studies reported that a shorter co-incubation time was beneficial to improving embryo quality and clinical pregnancy (5, 6). However, other studies reported contradictory results (7, 8). Higher ROS levels in a blastocyst culture medium have been reported to lead to a lower blastocyst formation rate (BFR) (9).

The quality of embryos in IVF is mainly assessed by embryologists under an inverted microscope (10). Although clinical studies suggest that embryos with an appropriate number of blastomeres, good blastomere homogeneity, and less fragmentation may have a higher clinical pregnancy rate (CPR), identifying more objective indicators to optimize embryo selection is urgently required. One study reported that the ROS levels in day 3 embryo culture medium were negatively correlated with BFR and CPR (11). Another study reported that embryo quality, BFR, or CPR were not significantly influenced by the ROS levels in the culture medium (12). Considering that ROS detection in the fertilization medium does not affect day 3 fresh embryo transfer (ET), exploring the relationship between ROS levels in the fertilization medium and IVF outcomes is meaningful. Currently, the ROS levels in the medium are measured using the chemiluminescence assay, which is time-sensitive, time-consuming, and requires complex instrumentation and large sample volumes, complicating its application in routine diagnosis. The MiOXSYS system, which is based on a galvanic measure of electrons, has been used to measure the static oxidation–reduction potential (sORP) in human semen. ORP is described as an integrated measure of the balance between total oxidant activity (i.e., ROS, oxidized thiols, superoxide radicals, hydroxyl radicals, hydrogen peroxides, nitric oxides, peroxynitrites, transition metal ions, and so forth) and total reductant activity (i.e., free thiols, ascorbates, α-tocopherols, β-carotenes, uric acids, and so forth) (13). A high sORP value indicates an excess of oxidizing agent relative to the reducing agent, that is, an imbalance in the oxidation–reduction (REDOX) state. This method is time-saving, requires fewer samples, and can be monitored in real time (14, 15). This study aimed to investigate the correlation between REDOX level in the fertilization medium and IVF outcomes by detecting the sORP of the fertilization medium through a brief incubation of gametes.

2 Material and Methods

2.1 Study design, patients, and sample selection

This study was conducted between October 2019 and October 2020 at the Center of Reproductive Medicine of Shanghai First Maternity and Infant Hospital, Tong Ji University. A total of 136 patients attending the Center for brief IVF were screened and recruited. The inclusion criteria were as follows: female patients aged ≤35 years and participants undertaking *in vitro* fertilization–ET (IVF-ET) treatment for tubal factor, endometriosis, or mild male factor. The exclusion criteria were as follows: endometriosis stage III and IV; history of ovarian cystectomy; and polycystic ovary syndrome (PCOS). This study was approved by the ethics committee of Shanghai First Maternity and Infant Hospital, and written informed consent was obtained from each patient.

All participants included in this study underwent personalized controlled ovarian stimulation (COS). The fertilization medium was collected soon after the zygotes were removed, and sORP was subsequently evaluated using the MiOXSYS system.

2.2 Controlled ovarian stimulation

The patients underwent COS treatment by standard routines at the Center. The COS treatment included gonadotropin-releasing hormone (GnRH)-antagonist (GnRH-ant) protocol, GnRH-agonist (GnRH-a) protocol, and other protocols including mild stimulation protocol or progestin-primed ovarian stimulation (PPOS) protocol, as decided by clinicians. In the GnRH-ant protocol, GnRH-ant (cetrotide or ganirelix) was started at a dose of 0.25 mg on day 6 of stimulation or when at least one follicle reached a diameter of 12 mm until the trigger day. For the long GnRH-a protocol, GnRH-a (intramuscular triptorelin 1.25–1.88 mg single dose) was initiated in the mid-luteal phase of the prestimulation cycle for pituitary downregulation. Then, gonadotropin treatment was commenced on day 2 of the menstrual cycle. For the mild stimulation protocol or PPOS protocol, the participants received triptorelin (subcutaneous injection 0.1 mg/d), oral clomiphene citrate (50–100 mg q.d.), or oral dydrogesterone (10 mg b.i.d) on day 2 or 3 of the menstrual cycle, and gonadotropin was used at the same time. When at least one follicle with a diameter ≥18 mm was detected, recombinant human chorionic gonadotropin was administered as a trigger and then transvaginal ultrasound-guided oocyte retrieval was performed after 36 h.

2.3 IVF procedures

Semen specimens were analyzed according to the fifth edition of the World Health Organization guidelines, and sperm were

collected by the swim-up method. The COCs were washed twice after 2–3 h of oocyte retrieval and then placed in 100 μL of G-IVF medium (Vitrolife, Sweden) to achieve a final concentration of 1–2 × 10^6 spermatozoa/mL. Then, the fertilization dish containing oocytes and sperm was incubated in a 5% CO_2 humidified incubator at 37°C for 4 h. After the co-incubation of oocytes and sperm for 4 h, the oocytes were removed and placed in new drops of the G-IVF medium. After the oocytes were removed, 30 μL of the fertilization medium was collected and used for the sORP detection by the MiOXSYS system.

2.4 Measurement of sORP

The sORP was measured using a galvanostat-based MiOXSYS system (Aytubio Science, USA). Briefly, 30 μL of the fertilization medium was added to the sample port of the MiOXSYS sensor. The sensor was then inserted into the MiOXSYS analyzer for 2 min for analysis. Next, the sORP values were automatically displayed on the analyzer screen in millivolts (mV).

2.5 Embryo evaluation

Normal fertilization was confirmed 18–20 h after insemination by the presence of two pronuclei and two polar bodies. The cleavage-stage embryos were graded as Grade 1 to Grade 6 according to the number and symmetry of blastomeres, as well as the proportion of cytoplasmic fragments (10). Grade 1 embryos implied symmetrical blastomeres and no cytoplasmic fragments; Grade 2 embryos denoted symmetrical blastomeres and less than 25% fragmentation; Grade 3 embryos indicated asymmetrical blastomeres and no cytoplasmic fragments; Grade 4 embryos implied asymmetrical blastomeres and less than 25% fragmentation; and Grades 5 and 6 embryos denoted asymmetrical blastomeres and more than 25% and 50% fragmentation, respectively. Grade 1 or 2 embryos with six to nine cells were regarded as good-quality embryos, and all frozen and transferred embryos were defined as available embryos.

2.6 ET, cryopreservation, and frozen-thawed ET

In the fresh ET cycle, fresh embryo was transferred under transabdominal ultrasound guidance on day 3 after fertilization. On the day of ET, surplus Grades 1 and 2 embryos were cryopreserved using the vitrification kit (Kato Corp., Shizuoka, Japan) following the manufacturer's protocols. The patients who did not undergo a fresh ET cycle received frozen ET (FET) 2 months after the fresh ET cycle if they had at least one frozen embryo. Embryo thawing was also performed using the vitrification kit (Kato Corp.) following the manufacturer's protocols. The patients who underwent fresh ET cycle or FET received either vaginal or intravenous luteal support. The patients who confirmed a biochemical pregnancy 2 weeks after transplantation received a transvaginal ultrasound scan after 4 weeks to identify the presence of a gestation sac with a fetal heart, signifying clinical pregnancy.

2.7 Definition of primary and secondary outcomes

The primary outcome was pregnancy outcomes, including live birth rate (LBR), clinical pregnancy rate CPR, biochemical pregnancy rate (BPR), and implantation rate (IR). The secondary outcome was embryo quality, including fertilization rate (FR), cleavage rate (CR), available embryo rate (AER), and good-quality embryo rate (GQER).

2.8 Statistical analysis

All continuous data were expressed as means ± standard deviation if the normal distribution was verified using the Kolmogorov–Smirnov test; otherwise, the median (interquartile range) was used for data description. The categorical variables were expressed as frequencies [percentage (%)]. The subgroup characteristics were compared using the analysis of variance or the Kruskal–Wallis test for continuous variables, and the chi-square test or Fisher's exact test was used for comparing categorical variables.

The Spearman correlation analysis was conducted to investigate the linear correlation between sORP value and clinical outcomes. The embryo quality assessment and the clinical outcomes of total transfer cycles among the four sORP groups were compared using the chi-square test, and *post hoc* pairwise comparisons were conducted using the Bonferroni test. Statistical analysis was performed using SPSS software (v22; IL, USA). A two-sided P value <0.05 indicated a statistically significant difference.

3 Results

3.1 Demographic and clinical characteristics of patients

The Spearman correlation analysis was performed for the relationship between sORP and embryo quality as well as clinical outcomes, and no linear correlation was found (Table S1). A total of 136 patients were divided equally into 4 groups to explore an exact range of sORP value with a guiding or predicting value for IVF outcomes, arranged in sORP order as follows: Group I (n = 34, sORP: 191.5–228.4 mV), Group II (n = 34, sORP: 228.7–235.3 mV), Group III (n = 34, sORP: 235.4–242.7 mV), and Group IV (n = 34, sORP = 242.8–295.3 mV).

The demographic and clinical features of the patients are presented in Table 1. The demographic and clinical characteristics were homogeneous among the four groups, including age, body mass index, infertility duration, and stimulation protocol. The ovarian reserve in female patients included basic FSH, antral follicle count, and anti-Mullerian hormone. The semen quality assessment in male patients included progressive motility, forward movement sperm number, and DNA fragmentation index.

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TABLE 1 Demographic and characteristics of patients.

Characteristic	Group I (N=34)		Group II (N=34)		Group III (N=34)		Group IV (N=34)		P-Value
	No.	Value	No.	Value	No.	Value	No.	Value	
Age (yr)									
Male		31.38 ± 0.48		32.68 ± 0.71		31.88 ± 0.56		31.91 ± 0.73	0.541
Female		30.97 ± 0.34		31.24 ± 0.34		31.03 ± 0.47		31.03 ± 0.55	0.975
BMI		21.53 ± 0.36		21.54 ± 0.55		21.58 ± 0.46		20.80 ± 0.41	0.576
Infertility status									
Duration (yr)		2.53 ± 0.34		2.69 ± 0.32		2.72 ± 0.29		2.47 ± 0.26	0.922
Primary (No.%)		20 (58.8)[d]		22 (64.7)[d]		16 (47.1)		9 (26.5)[a,b]	**0.009**
Secondary (No.%)		14 (41.2)		12 (35.3)		18 (52.9)		25 (73.5)	
Stimulation protocol (No.%)									
GnRH agonist		13 (38.2)		16 (47.1)		16 (47.1)		13 (38.2)	0.880
GnRH antagonist		9 (26.5)		11 (32.4)		7 (20.6)		13 (38.2)	
Mild-stimulation protocol		5 (14.7)		3 (8.8)		5 (14.7)		3 (8.8)	
PPOS		7 (20.6)		4 (11.8)		6 (17.6)		5 (14.7)	
Laboratory test									
Female:									
FSH level on 3rd day (mIU/ml)		6.97 ± 0.58		6.93 ± 0.60		7.02 ± 0.61		7.01 ± 0.40	0.999
AFC	21	13.00 ± 1.21	18	13.72 ± 1.21	25	13.12 ± 1.29	20	14.80 ± 1.12	0.723
AMH (ng/ml)	21	3.81 ± 0.41	29	3.38 ± 0.36	22	4.55 ± 0.97	18	3.74 ± 0.65	0.564
Male:									
PR (%)		41.66 ± 2.69		42.63 ± 2.41		43.68 ± 2.90		41.46 ± 2.23	0.925
FMSN		3154.96 ± 475.15		4302.58 ± 713.85		4284.48 ± 692.49		4878.43 ± 680.76	0.303
DFI (%)		8.02 ± 0.55		9.83 ± 1.08		8.98 ± 1.10		9.75 ± 1.07	0.515
Sex hormone level on hCG day									
LH (IU/ml)	31	2.30 ± 0.38	34	3.85 ± 1.39	33	2.92 ± 0.48	33	4.37 ± 2.05	0.680
Estradiol (pg/ml)	31	2826.73 ± 350.22	34	1893.14 ± 240.61[c]	33	4108.22 ± 658.70[b,d]	33	2444.89 ± 292.22[c]	**0.002**
Progesterone (ng/l)	31	1.88 ± 0.52	34	0.91 ± 0.10	33	1.10 ± 0.11	33	1.01 ± 0.11	**0.045**

Values are mean ± SD or No.(percentage)
BMI, Body mass index; FSH, follicle-stimulation hormone; AFC, Antral follicle count; AMH, Anti-mullerian hormone; PR, Forward motile sperm percentage; FMSN, Forward movement sperm number; DFI, DNA fragmentation index; LH, Luteinizing Hormone.
Post-hoc comparison was conducted using the Bonferroni test.
P-value considered significant was in bold.
[a]p<0.05 compared with group I; [b]p<0.05 compared with group II; [c]p<0.05 compared with group III; [d]p<0.05 compared with group IV.
If the data was partly missing in the analysis, the available number of patients was presented.

3.2 Embryo quality assessment

The retrieved oocytes, FR, CR, AER, and GQER are shown in Table 2. The FR had lower significant differences in Groups I and II than in Groups III and IV (71.7% and 70.3% for Groups I and II vs 83.5% and 80.4% for Groups III and IV, $P = 0.000$). The GQER in Group I to Group IV was 32.7%, 37.4%, 26.5%, and 33.3%, respectively ($P = 0.056$) (Table 2).

3.3 Assessment of clinical outcomes

A total of 105 patients underwent ET, and only the first transfer cycle was enrolled in Table 3. Group II delivered a higher LBR, CPR, BPR, and IR compared with Group III, presented as follows: LBR (32.0% for Group II vs 3.6% for Group III, $P = 0.033$), CPR (32.0% for Group II vs 3.6% for Group III, $P = 0.033$), BPR (36.0% for Group II vs 3.6% for Group III, $P = 0.019$), and IR (31.3% for Group II vs 2.7% for Group III, $P = 0.003$).

TABLE 2 Embryo quality assessment among 4 groups.

	Group I	Group II	Group III	Group IV	Total	P-Value
Oocytes retrieved (No.)	357	263	425	397	1442	
Fertilization rate (No.%)	256 (71.7)[c,d]	185 (70.3)[c,d]	355 (83.5)[a,b]	319 (80.4)[a,b]	1115 (77.3)	**0.000**
Cleavage rate (No.%)	251 (98.0)	182 (98.4)	344 (96.9)	309 (96.9)	1086 (97.4)	0.639
Available embryo rate (No.%)	124 (49.4)	94 (51.6)	150 (43.6)	164 (53.1)	532 (49.0)	0.086
Good-quality embryos rate (No.%)	82 (32.7)	68 (37.4)	91 (26.5)	103 (33.3)	344 (31.7)	0.056

Values are No.(percentage).
Post-hoc comparison was conducted by Bonferroni test and Fisher's exact probability was conducted while the sample size was less than 40 or theoretical frequencies were less than 5.
P-value considered significant was in bold.
[a]p<0.05 compared with group I; [b]p<0.05 compared with group II; [c]p<0.05 compared with group III; [d]p<0.05 compared with group IV.

4 Discussion

This study evaluated the REDOX status in the fertilization medium using the MiOXSYS system to explore the relationship between ROS levels in the fertilization medium and IVF outcomes. The results revealed that the sORP of the fertilization medium (228.7–235.3 mV) was an optimal sORP range for IVF outcomes, which presented the highest LBR, CPR, BPR, and IR.

Most conventional assays for Oxidative Stress (OS)evaluation were designed only to detect a single oxidant and lacked a full-scale view of the REDOX status. sORP detection provided a snapshot of the current balance of overall oxidation or reduction capacity, which was convincing, rapid, and accurate. Therefore, we used the MiOXSYS system on the fertilization medium to get a reliable and convincing REDOX level. As expected, an optimal sORP range was located in Group II for achieving a better clinical outcome.

Nowadays, single-embryo ET is promoted for the safety of pregnant women, and an accurate and rapid method for selecting the best-quality embryo is urgently needed. The embryo scoring is based on the morphological system, which is not comprehensive. In recent years, researchers have been trying to determine the potential of embryonic development by detecting related molecular markers in the embryo culture medium, which is invasive and sensitive. Huo et al. showed that the difference in amino acids in the D3 embryo culture medium had a high accuracy in predicting the embryo implantation potential (16). Abreu et al. found that the D5 culture medium of morphologically inviable embryos had higher concentrations of interleukin 8 and tumor necrosis factor-alpha, which was associated with abnormal cell division and cell death (17). In addition, the ROS level in the D3 culture medium might serve as a biochemical marker for blastulation and clinical pregnancy (11). This study was the first to explore the relationship between ROS levels in the fertilization medium and

TABLE 3 Clinical outcomes of transfer cycles.

Characteristic	Group I (N=23)	Group II (N=25)	Group III (N=28)	Group IV (N=29)	P-Value
One/two embryos (No./No.)	16/7	18/7	19/9	23/6	0.796
Biochemical pregnancy (No.%)	3 (13.0)	9[b] (36.0)	1[a] (3.6)	5 (17.2)	**0.019**
Implantation outcome					
Implantation (No.%)	2 (6.7)	10[b] (31.3)	1[a] (2.7)	4 (11.8)	**0.003**
One/two embryos implanted (No./No.)	2/0	6/2	1/0	4/0	0.066
Pregnancy outcome					
Clinical pregnancy (No.%)	2 (8.7)	8[b] (32.0)	1[a] (3.6)	4 (13.8)	**0.033**
Singleton/Multiple Pregnancy (No./No.)	2/0	6/2	1/0	4/0	0.066
Delivery outcome					
Live birth (No.%)	2 (8.7)	8[b] (32.0)	1[a] (3.6)	4 (13.8)	**0.033**
Singleton/Multiple birth (No./No.)	2/0	7/1	1/0	4/0	0.059

Values are mean±SD, No.(percentage) or No./No.
In each sORP group, variables were analyzed in total transfer cycles by Chi-square test.
Post-hoc comparison was conducted by Bonferroni test and Fisher's exact probability was conducted while the sample size was less than 40 or theoretical frequencies were less than 5.
P-value considered significant was in bold.
[a]p<0.05 compared with group II; [b]p<0.05 compared with group III.

IVF outcomes. If the ROS level in the fertilization medium can be one of the indicators to predict the clinical pregnancy outcomes, we can select embryos for transplantation by combining the ROS value of the fertilization medium and the morphological score of embryos. In this study, we found that Group II (sORP: 228.7–235.3 mV) had a higher GQER than other groups, but it was not statistically significant ($P = 0.056$). We speculated that it could be attributable to the small sample size of the study ($n = 34$ in each group). Group II had higher LBR, CPR, BPR, and IR values, suggesting that the ROS level in the fertilization medium should be maintained at an appropriate range, and extremely high or low levels may affect clinical outcomes. A similar study reported that ROS in media induced lipid peroxidation in sperm membranes, which enhanced spermatozoa binding to the zona pellucida (18). In addition, the FR in Groups I and II was significantly lower than that in Groups III and IV. Considering that the total number of samples was relatively small, we speculated that the fertilization rates in Groups I and II were significantly lower than those in Groups III and IV due to no-and low-fertilization in Groups I and II.

In the traditional IVF method, sperm and oocytes are incubated for 16–18 h. Long exposure of oocytes to spermatozoa can increase the damage caused by ROS, leading to short-term IVF (4–6 h co-incubation) (19). In this study, ROS was detected after 4 h. We next aimed to set more groups, including 4 h, 8 h, and 16 h, to observe the relationship between ROS levels at different time points and IVF outcomes.

This study had some limitations. First, short-term fertilization was not a routine treatment protocol in our Center, and was only used for patients with unexplained infertility. So, the sample size was still relatively small in this study. Second, sperm, oocytes, and granulosa cells were present in the fertilization medium, and the total number of sperm for one oocyte was imprecise (approximately 10,000–20,000). We failed to identify the relative contribution of ROS from these cells.

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 Ethics Committee of Shanghai First Maternity and Infant Hospital. The patients/participants provided their written informed consent to participate in this study

Author contributions

Y.G. and X.T. conceived and designed the study. Q.C. and J.L. contributed to the sample collection. J.X. analyzed the data. Q.C., Y.G. and J.N performed the experiments and wrote the manuscript. X.T., X.J. and W.L critically revised the manuscript. All authors provided a critical review and approved the final manuscript.

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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.2023.1133566/full#supplementary-material

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MKRN2 knockout causes male infertility through decreasing STAT1, SIX4, and TNC expression

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Makorin-2 (*Mkrn2*) is an evolutionarily conserved gene whose biological functions are not fully known. Although recent studies have shed insights on the potential causes of male infertility, its underlining mechanisms still remain to be elucidated. We developed a *Mrkn2* knockout mice model to study this gene and found that deletion of *Mkrn2* in mice led to male infertility. Interestingly, the expression level of signal transducer and activator of the transcription (STAT)1 was significantly decreased in MKRN2 knockout testis and MEF cells. Co-IP assay showed an interaction between MKRN2 and STAT1. Moreover, our results further indicated that MKRN2 regulated the expression level of SIX4 and tenascin C (TNC) *via* the EBF transcription factor 2 (EBF2) in mice. The results of our study will provide insights into a new mechanism of male infertility.

KEYWORDS

MKRN2, STAT1, SIX4, TNC, male infertility

Introduction

Makorin-2 (MKRN2; HSPC070) belongs to the *MKRN* gene family with the ribonucleoproteins characterized by a variety of zinc-finger motifs, which contains four C3H zinc fingers and a C3HC4 really interesting new gene (RING) zinc finger domain (1, 2). MKRN2 was first identified in human CD34+stem/progenitor cells, as well as in some leukemic cell lines (1, 3, 4). Previous studies reported that mkrn2 in *Xenopus laevis* acted upstream of glycogen synthase kinase-3b in the phosphatidylinositol 3-kinase/Akt pathway. The third C3H zinc finger and the RING motif are required for the antineurogenesis activity (5, 6). Recent studies also investigated the role of MKRN2 in tumorigenesis, such as lung cancer and melanoma (7, 8). Though MKRN2 is a highly conserved gene, however, its function remains largely unknown.

The signal transducer and activator of the transcription (STAT) protein family mediate the transcription of several genes, such as cytokine-inducible genes and growth factors (9–12). So far, seven STAT proteins have been identified: STAT1–6 and STAT5b

(13, 14). When the receptor bounds to STATs, a conserved tyrosine residue in the C-terminal domain will be phosphorylated by the Janus kinase (JAK), then two STATs assemble through reciprocal phosphotyrosine/SH2 domain interactions leading to dimerization. Once dimerized, STATs are translocated into the nucleus and regulate the transcription of several different genes. Truchet et al. reported that JAK/STAT pathway is functional during early embryonic development, and STAT1 is present in mouse oocytes and in preimplantation embryos (15). STAT1 was reported to be phosphorylated in response to capacitation and the acrosomal reaction (16). Moreover, human samples with varicocele conveyed a significant negative correlation between the phosphorylated levels of STAT1 and sperm head morphological defects (17). Moreover, The *Six4* genes belong to the mammalian homolog of the *Drosophila sine oculis homeobox* (*Six*) family, and studies have reported that SIX4 is required for genital primordium formation and testicular differentiation of male gonads (18). SIX4 and tenascin C (TNC) have been reported to be functioning in a productive system. Thus, we wonder how to evaluate the regulatory mechanism between MKRN2 and STAT1/SIX4/TNC to figure out the novel mechanism of MKRN2 in male infertility.

In this study, we plan to address: (a) the role of MKRN2 in male infertility; (b) whether MKRN2 regulates the expression level of STAT1; and (c) how MKRN2 induces the expression levels of SIX4 and TNC by the transcription factor EBF transcription factor 2 (EBF2). The results of our study will provide insights into a new mechanism of MKRN2 in regulating male infertility.

Materials and methods

Generation of Mkrn2-knockout mice

To generate *Mkrn2*-heterozygous and *Mkrn2*-knockout mice, *Mkrn2*-floxed mice have been crossed with transgenic EIIa-cre mice with a C57BL/6 J background as we previously described (19) to obtain mosaic mice with the $Mkrn2^{+/flox(-)}$·EIIa-cre genotype. The resulting mice were crossed with C57BL/6 J to obtain *Mkrn2* heterozygotes. *Mkrn2* knockout mice were generated by sibmating *Mkrn2* heterozygotes. Genomic DNAs isolated from the tails were genotyped with indicated primers by PCR as described (19). All the animals were housed in specific pathogen-free conditions, and all the experiments were approved by the Committee of Laboratory Animal Experimentation of Zhengzhou University.

Cell culture

Mkrn2-WT and *Mkrn2*-KO primary mouse embryonic fibroblasts (MEFs) were derived from 13.5-day embryos and cultured as described (19), and 293T cells were cultured in the DMEM medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml), and were maintained at 37°C.

H&E staining

The H&E staining procedure used here is as follows: (a) Deparaffinize all sections in xylene two times for 10 min each. (b) Gradually rehydrate sections with graded alcohol: wash in absolute alcohol two times for 5 min each, then 95% alcohol for 2 min, and 70% alcohol for 2 min. (c) Briefly wash in distilled water. (d) Stain in Harris hematoxylin solution for 8 min, then wash for 5 min under running water. (e) Differentiate in 1% acid alcohol for 30 s, then wash under running water for 1 min. (f) Blue in 0.2% ammonia water for 30 s, then wash under running water for 5 min. (g) Rinse in 95% alcohol at 10 dips. (h) Counterstain in an eosin–phloxine solution for 1 min. (i) Dehydrate with 95% alcohol and wash with absolute alcohol two times at 5 min each. (j) Clear in xylene two times at 5 min each. (k) Lastly, mount with a xylene-based mounting medium.

Western blot analysis

Cells were harvested, washed with cold PBS twice, and then suspended in 200 μl of cold cell lysed buffer with protease inhibitor. Tissues were homogenized and suspended in 500 μl of cold cell lysed buffer with protease inhibitor. All of the lysates were incubated on ice for 30 min and centrifuged for 10 min at 4°C; the supernatants were collected, and the protein concentration was quantified using the Bradford method (20). The cell lysates were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. After being incubated in the blocking buffer (5% BSA in TBST) for 2 h at room temperature, the membranes were incubated with primer antibodies overnight at 4°C, washed three times with TBST, and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, then washed three times more with TBST. The protein–antibody complex was detected by a chemiluminescence detection system *via* an ECL chemiluminescence detection kit.

Quantitative real-time PCR

Quantitative real-time RT-PCR was used to determine the gene expression levels. RNAs were extracted using a Trizol reagent, reverse transcribed using SYBR Premix Dimer Eraser, and real-time PCR was performed using QuautStudio-5 Real-Time Thermal Cycler (ABI, Los Angeles, CA, USA). The following primer sequences were used for Q-PCR: Six4 forward primer: 5′-CTCCTGTCTCAGTAGCAGCTTC-3′; reverse primer: 5′-GGAACGGTGTATACCACTGCAC-3′; Ebf2 forward primer: 5′-GAGCAAGAAGGCTTGACCCATC-3′; reverse primer: 5′-CCAAACACAACCTGGAGACCATC-3′; Tnc forward primer: 5′-GAGACCTGACACGGAGTATGAG-3′; reverse primer: 5′-CTCCAAGGTGATGCTGTTGTCTG-3′; and GAPDH forward primer: 5′-ATGGGTGTGAACCATGAGAAGTATG-3′; reverse primer: 5′-GGTGCAGGAGGCATTGCT-3′. The gene expression levels were normalized to the value of GAPDH, and fold changes were calculated by relative quantification ($2^{-\Delta\Delta Ct}$).

Co-immunoprecipitation

HEK293T cells were cultured in a 10-cm plate and transfected with the indicated plasmids for 48–72 h. Cells were then collected and lysed in immunoprecipitation (IP) buffer (1 M Tris, 0.5 M EDTA, 5 M NaCl, 100% glycerol, 100% Np40) and treated with antibodies at 4°C overnight. The lysis was then incubated with agarose beads (Roche, Indianapolis, IN, USA) for 4–6 h at 4°C. IP samples were separated by 8%–10% SDS-PAGE gels, and protein bands were detected by the chemiluminescent substrate.

Protein quantification and differently expressed protein analysis

Total proteins were extracted from *Mkrn2*-WT and *Mkrn2*-KO MEFs, with four biological repetitions in each group. The proteins of different samples were respectively subjected to trypsin enzymatic hydrolysis, and peptide fractionation as described (21). The samples were further analyzed on a Thermo HFX MS (Thermo Fisher Scientific, Waltham, MA, USA) interfaced with an EASY-nLC1200 LC system (Thermo Fisher Scientific). Raw files were searched against the mouse refseq protein database (27,414 proteins, version 04/07/2013) with Proteome Discoverer (Thermo Fisher Scientific, version 1.4). The levels of proteins were estimated with a label-free, intensity-based absolute quantification (iBAQ) approach (22). The iBAQ of each protein was normalized by dividing the sum of all identified proteins. We replaced the extremely small values with 10^{-8}. The matrix file integrated with all samples was subjected to different protein expression between *Mkrn2*-WT MEFs and *Mkrn2*-KO MEFs by using the limma package in R 3.6.1 software (23). Different proteins were filtered by |Log2(Fold Change)|>1 and p-value < 0.05. Heatmap shows 977 differently expressed proteins.

Pathway enrichment analysis

The pathway enrichment analysis of differently expressed proteins was performed with the Reactome database, using the ReactomePA package in R software. Default parameters (OrgDb, org.Mm.eg.db; p-value cutoff, 0.05; q-value cutoff, 0.2; p-value adjust method, BH; readable, T) were set for pathway enrichment analysis. Enriched pathways with FDR < 0.2 were considered statistically significant.

Differently expressed gene analysis

The matrix data from GEO (GSE6872) is an expression profiling array, and the analysis of differently expressed genes was performed by using the limma package in R software (24). The threshold for differently expressed genes is |Log2(Fold Change)| > 1 and p-value < 0.05. The volcano plot shows analysis results including upregulated genes (red), downregulated genes (blue), and nonsignificant genes (black).

Statistical analysis

All results have been obtained from at least three independent experiments, and data were analyzed *via* GraphPad Prism 8 software. The statistical evaluation for data analysis was determined by a t-test. The differences were considered to be statistically significant at $p < 0.05$.

Results

MKRN2 is related to male spermatogenesis

In order to investigate the effects of MKRN2 on male spermatogenesis, we detected the macroscopic appearance of the testis of *Mkrn2* knockout and wild-type (WT) mice *via* HE staining. We selected the testis at phase VIII of testicular development and epididymis, and the results showed that compared to the wild-type group, there were no sperm in the testis of *Mkrn2* knockout mice (Figures 1A, B). In our previous study, we showed that *Mkrn2* knockout induced spermiation failure, but we also found that a few *Mkrn2* knockout mice had another new phenotype in which there is no sperm generated. We then analyzed the MKRN2 expression level of four normal spermatogenesis individuals (normal) and 27 nonobstructive azoospermia samples (patients) from the GEO dataset GSE45885. The results showed that the expression level of MKRN2 was significantly lower than the normal individuals (Figure 1C). These results suggested that MKRN2 played a key role in testicular development and male spermatogenesis.

MKRN2 expression levels are highly correlated with the receptor tyrosine kinase signaling pathway

To investigate the role of MKRN2 in testicular development and male spermatogenesis further, we performed a proteomics analysis of *Mkrn2*-WT MEFs and *Mkrn2*-KO MEFs (Figure 2A), as well as an analysis of differential expression genes (DEGs) in normospermic and teratozoospermic groups from the GEO dataset GSE6872 (Figure 2B). The signaling pathways represented among overlapping genes and ranked according to the number of enriched genes were screened for overlapping genes between differentially expressed proteins from proteomics and DEGs from GSE6872. The results showed that the primary signaling pathway (Figures 2C, D) was receptor tyrosine kinase signaling. We performed a transcription factor assay to compare the different transcription factors between Mkrn2-WT and Mkrn2-KO MEFS, and the overlapping genes of the transcription factor array and genes of the receptor tyrosine kinase signaling pathway were analyzed. STAT1 was the only gene that overlapped (Figure 2E). We then analyzed the expression levels of STAT family members in

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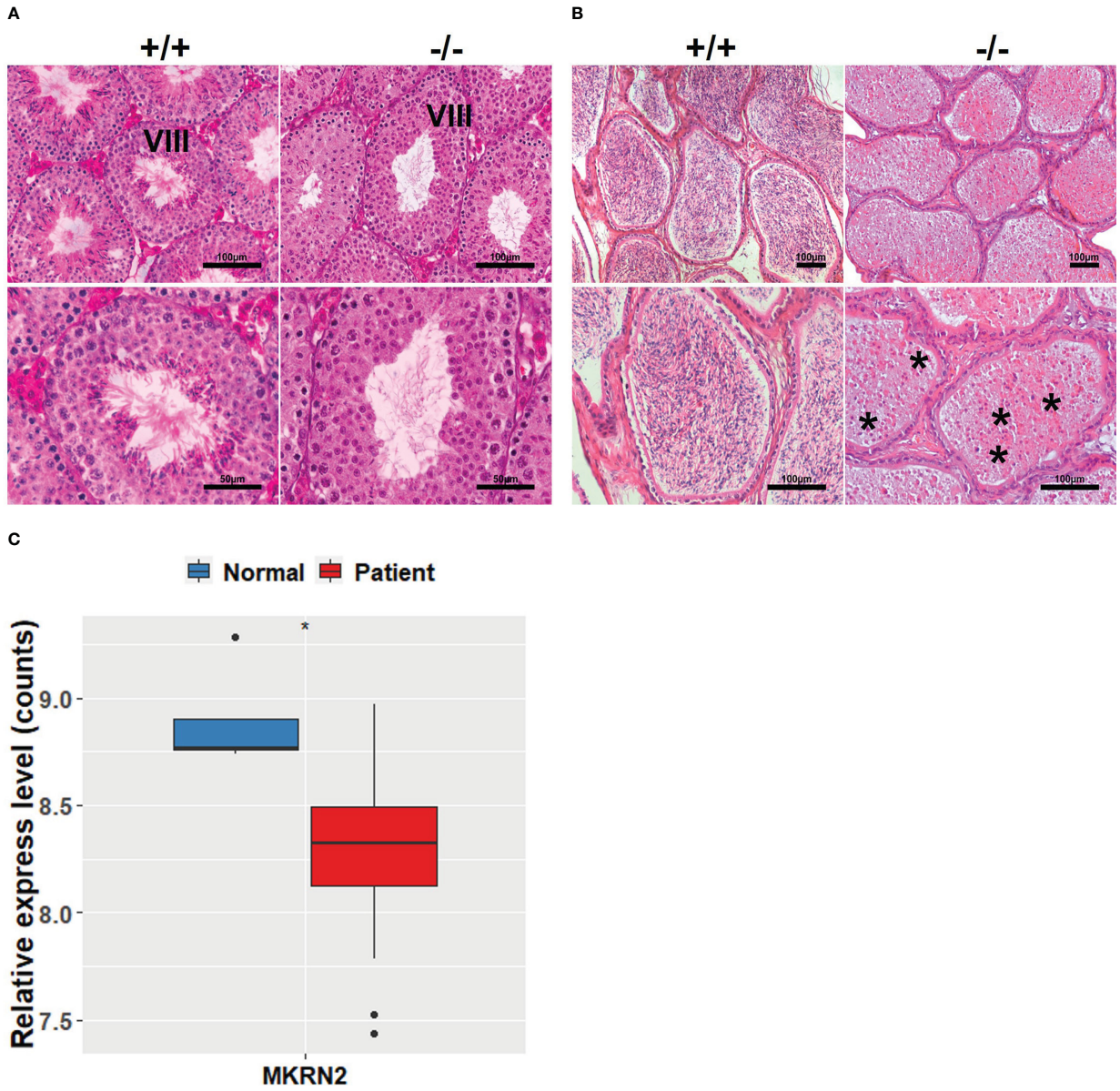

FIGURE 1
MKRN2 is related to male spermatogenesis. **(A, B)** Hematoxylin and eosin (H&E) staining sections of *Mkrn2*-WT (+/+) and *Mkrn2*-KO (–/–) mice testes in the phase VIII of sperm development and epididymis. Representative pictures are shown, Asterisk represents no sperm. **(C)** Expression levels of MKRN2 in four normal spermatogenesis individuals (normal) and 27 nonobstructive azoospermia samples (patients) from GEO dataset GSE45885. Data are analyzed by two-tailed, unpaired Student's *t*-test. *$p < 0.05$, a significant difference.

Mkrn2-WT MEFs and *Mkrn2*-KO MEFs according to the transcription factor array results, and STAT1 was the most downregulated gene in the *Mkrn2*-KO MEFs (Figure 2F). All of these results indicated that MKRN2-regulated STAT1 is involved in the process of male infertility.

MKRN2 regulates the expression level of STAT1

To further investigate the regulatory effect of MKRN2 on STAT1, we measured the protein and mRNA expression levels of MKRN2 and STAT1 in *Mkrn2*-WT MEFs and *Mkrn2*-KO MEFs, and the results showed that the expression level of STAT1 was significantly downregulated in *Mkrn2*-KO MEFs (Figure 3A). We then overexpressed MKRN2 in the 293T cells and measured the protein and mRNA expression levels of STAT1. The results showed that the expression level of STAT1 was significantly upregulated in MKRN2 overexpression cells (Figure 3B). We further found that MKRN2 could interact with STAT1 using a co-immunoprecipitation assay (Figure 3C). All of these results indicated that MKRN2 could interact with STAT1 and regulate its expression level.

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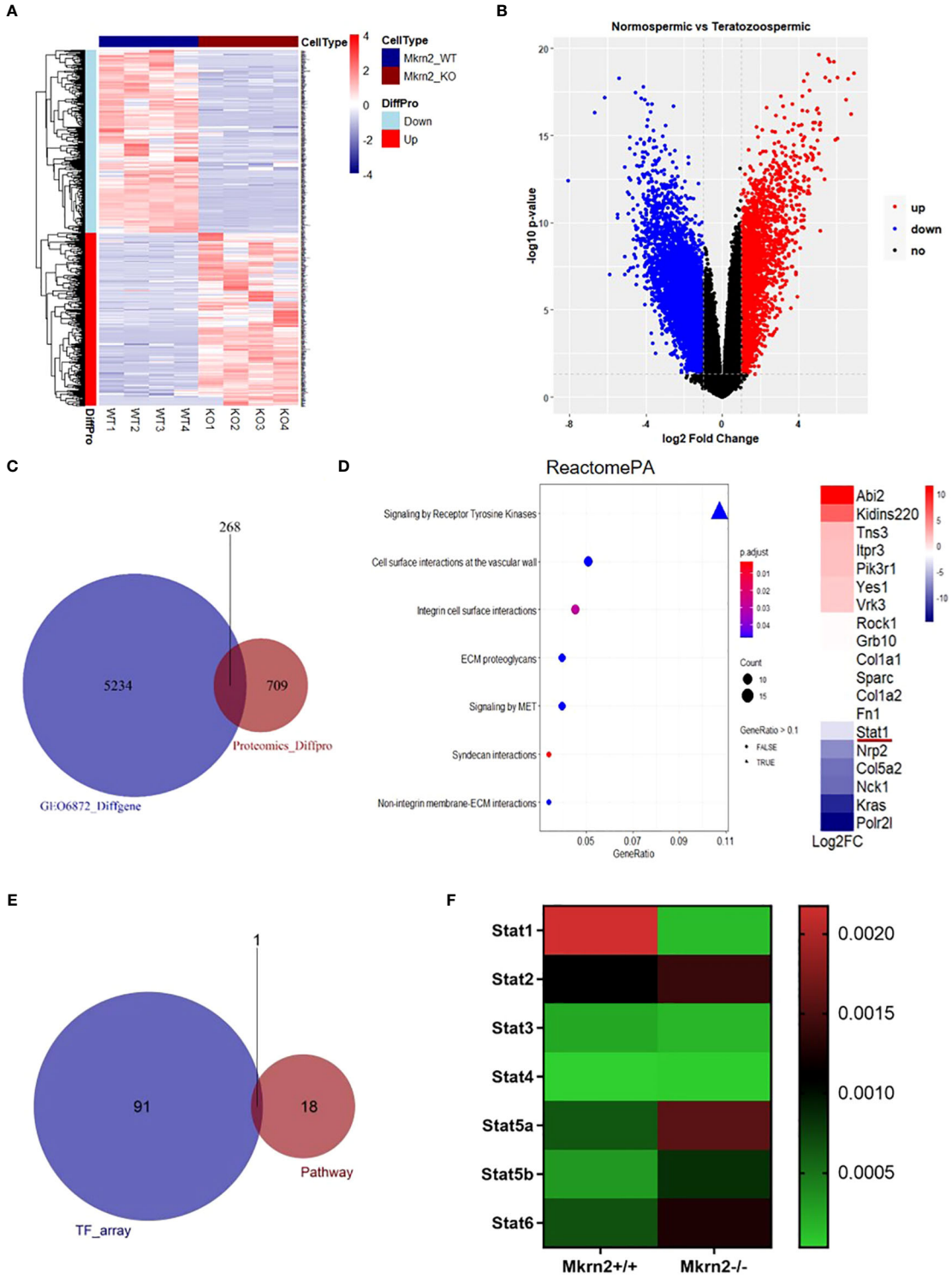

FIGURE 2
MKRN2 expression levels are highly correlated with the receptor tyrosine kinase signaling pathway. **(A)** Heatmap plot of all differentially expressed proteins in *Mkrn2*-WT MEFs and *Mkrn2*-KO MEFs. The proteins and samples are respectively clustered by Euclidean distance, and the color block shows the classification. Upregulated proteins are coded in red color and downregulated proteins are coded in blue. **(B)** Volcano plot of DEGs in normospermic group and teratozoospermic group from GEO dataset GSE6872. According to the values of logFC and *p*-value (logFC ≥ 1 or ≤ −1, $p < 0.05$), all genes were classified into upregulated genes (red), downregulated genes (blue), and no changed genes (black). **(C)** The overlapping between differentially expressed proteins from proteomics (Proteomics_Diffpro) and DEGs from GSE6872 (GSE6872_Diffgene). **(D)** The signaling pathways are represented among overlapping genes and ranked according to the number of enriched genes. The color of the dots represents the *p*-value; the size of the dot represents the gene count, and the shape of the dots represents the gene ratio. The primary signaling pathway is signaling by receptor tyrosine kinases, including 18 genes, ranked by log2FC. **(E)** The overlapping of the results of the transcription factors array (TF_array) and genes of the signaling by receptor tyrosine kinase pathway. **(F)** Expression levels of the STAT family members in *Mkrn2*-WT MEFs and *Mkrn2*-KO MEFs, according to TF array results.

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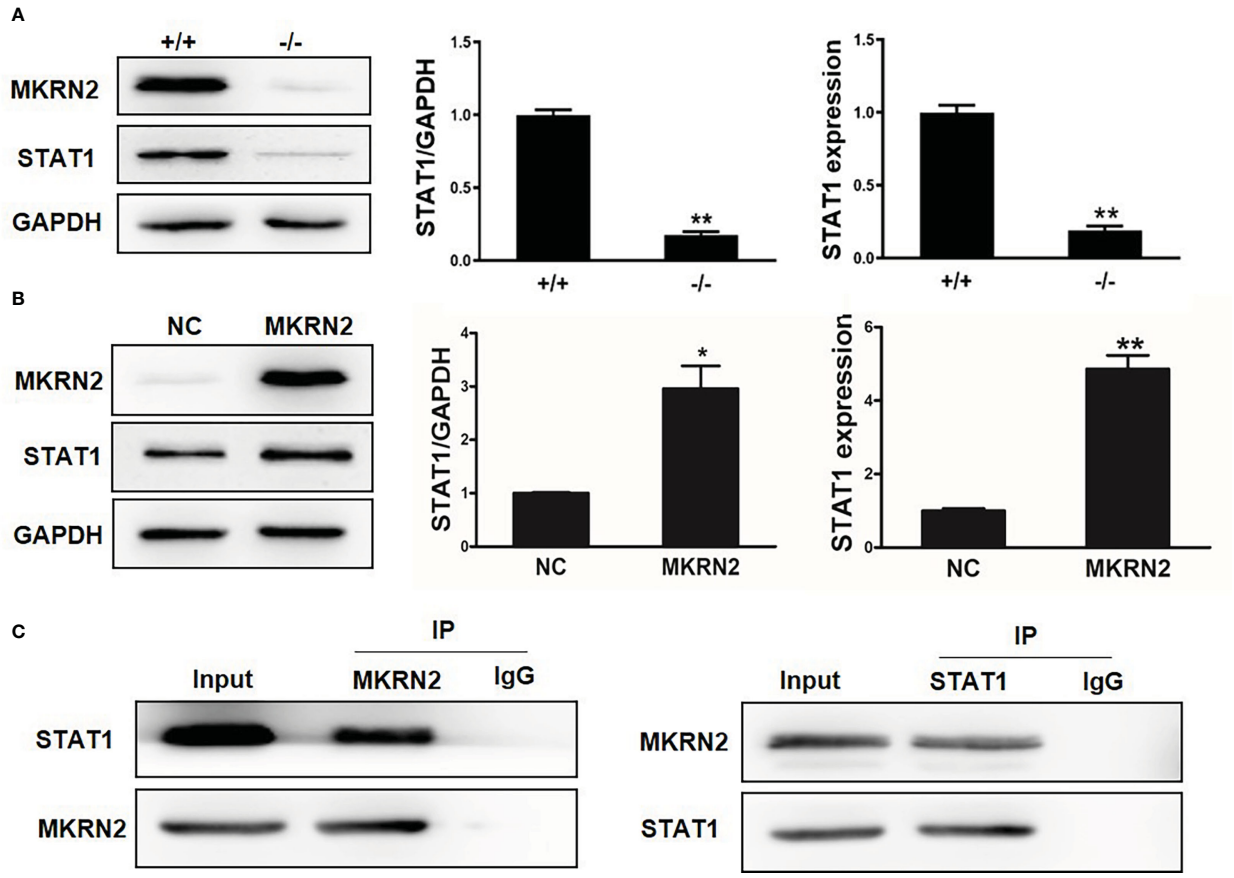

FIGURE 3
MKRN2 regulates the expression level of STAT1. **(A)** The protein and mRNA expression levels of MKRN2 and STAT1 in *Mkrn2*-WT MEFs and *Mkrn2*-KO MEFs. **(B)** Overexpressed MKRN2 in the 293T cells and the protein and mRNA expression levels of MKRN2 and STAT1 are shown. **(C)** Overexpressed MKRN2 or STAT1 in the 293T cells; the co-immunoprecipitation assay was preformed to detect the interaction of MKRN2 and STAT1, and the representative pictures are shown. *$p < 0.05$ and **$p < 0.01$—significant difference.

MKRN2 mediates male teratozoospermia by regulating the expression level of STAT1

To verify the role of MKRN2 in the male teratozoospermia, we analyzed the expression levels of MKRN2 and STAT1 in 13 normally fertile male patients and eight infertile individuals with a severe and consistent heterogeneous teratozoospermia based on the GEO dataset GSE6872. The results showed that the expression levels of MKRN2 and STAT1 were both significantly downregulated in the infertile patients (Figure 4A). We then detected the protein and mRNA expression levels of MKRN2 and STAT1 in the testis of *Mkrn2* knockout and wild-type (WT) mice and found that the expression levels of MKRN2 and STAT1were both significantly downregulated in the testis tissues of *Mkrn2* knockout mice (Figures 4B–D). These results indicated that MKRN2 mediated male teratozoospermia by regulating the expression level of STAT1.

MKRN2 knockout results in the reproductive pathway disorder

To further investigate the effects of Mkrn2 on other reproductive pathways, we obtained a dataset of all genes related to reproduction from the Mouse Genome Informatics (MGI) website (http://www.informatics.jax.org/), overlapped the differentially expressed proteins of *Mkrn2*-WT MEFs and *Mkrn2*-KO MEFs and the reproduction-related genes, and then obtained 68 overlapped genes (Figure 5A). We then looked into the overlap of differentially expressed proteins from proteomics, genes from reproduction pathway, and genes from related molecular function sets of male infertility. There were nine overlapping genes with male gonad development (Figure 5B), 18 with spermatogenesis (Figure 5C), 18 with male gamete generation (Figure 5D), and two with prostate gland development (Figure 5E). All of the above findings suggested that MKRN2 knockout cause a disruption in the reproductive pathway.

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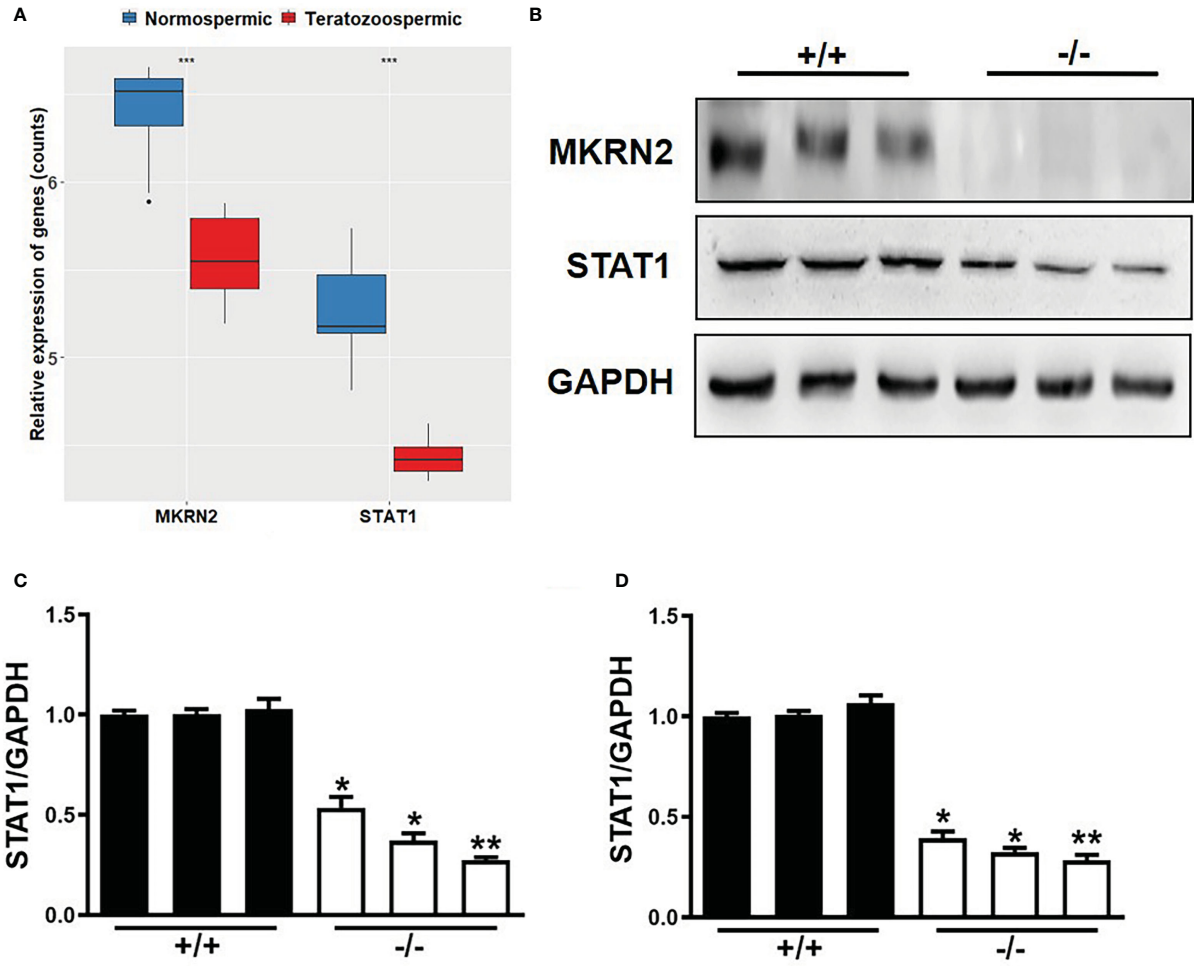

FIGURE 4
MKRN2 mediates male teratozoospermia by affecting the expression level of STAT1. **(A)** The expression levels of MKRN2 and STAT1 in the sperms of 13 normally fertile male patients and eight infertile individuals with severe and consistent heterogeneous teratozoospermia. Data are from the GEO dataset GSE6872. **(B–D)** The protein and mRNA expression levels of MKRN2 and STAT1 in the testis of *Mkrn2*-WT and *Mkrn2*-KO mice. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0001$—significant difference.

MKRN2 regulates the expression of SIX4 and TNC

To further investigate the molecular mechanism of the above reproductive-related genes in male teratozoospermia, we verified expression levels of SIX4 and TNC in *Mkrn2*-WT MEFs and *Mkrn2*-KO MEFs using the proteomics data. The results showed that the expression levels of SIX4 and TNC were significantly downregulated in the *Mkrn2*-KO MEFs (Figure 6A). We then overlapped the putative upstream transcription factors of SIX4 or TNC according to the Jaspar website and differentially expressed proteins from proteomics and found only one transcription factor, EBF2 (Figure 6B). The mRNA expression levels of SIX4, TNC, and EBF2 were detected in *Mkrn2*-WT MEFs and *Mkrn2*-KO MEFs, and we found that the expression levels of SIX4, TNC, and transcription factor EBF2 were significantly downregulated in *Mkrn2*-KO MEFs (Figure 6C). We also used Western blot to examine the testis tissues of *Mkrn2*-WT and *Mkrn2*-KO mice, and the results showed that the protein expression levels of EBF2, SIX4, and TNC were decreased in *Mkrn2*-KO mice (Figure 6D). These findings indicated that MKRN2 regulated the expression level of SIX4 and TNC in *Mkrn2* knockout mice *via* the transcription factor EBF2.

Discussion

The *MKRN2* gene is a member of the makorin gene family, which also includes MKRN1, MKRN2, and MKRN3. MKRN2 has been found to be highly conserved throughout evolution, and its ancestral origin can be traced back to 450 million years ago, possibly as a result of *MKRN1* gene duplication (1). We have illustrated the functional role of Mkrn2 in *Xenopus* embryos to negatively regulate neurogenesis through PI3K/Akt signaling. However, the potential functions and molecular mechanisms of MKRN2 in mammals remain to be studied. This *Mkrn2* knockout mouse model and an independent GEO dataset of human sperm samples were used in

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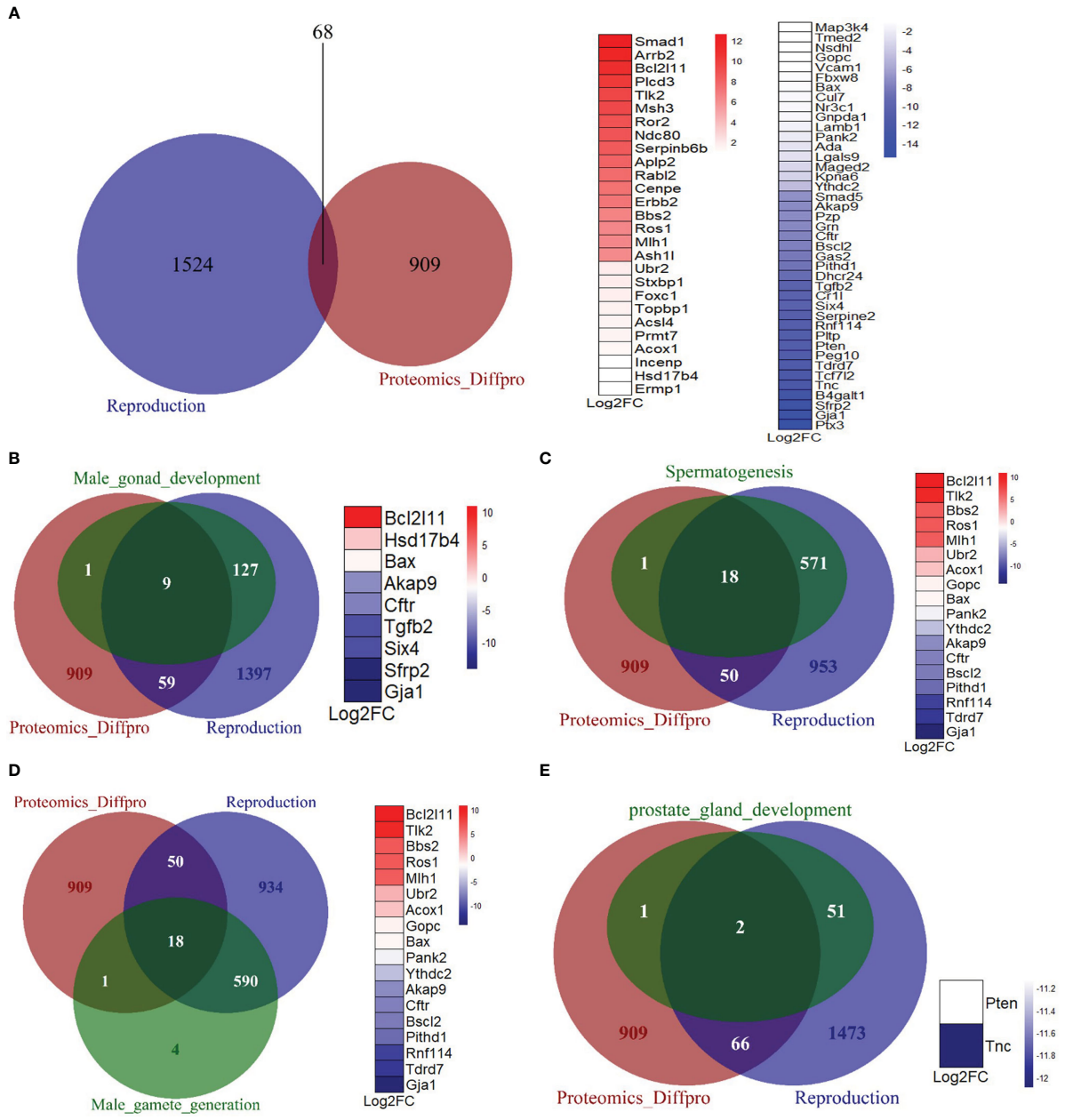

FIGURE 5
MKRN2 results in a disorder of the reproductive pathway. **(A)** The overlapping between differentially expressed proteins from proteomics (Proteomics_Diffpro) and genes from the reproduction pathway (reproduction). Upregulated proteins (blue) and downregulated proteins (red) from proteomics analysis were ranked by log2FC. **(B–E)** The overlapping of differentially expressed proteins from proteomics (Proteomics_Diffpro), genes from reproduction pathway (reproduction), and genes from related molecular function sets of male infertility, including male gonad development, spermatogenesis, male gamete generation, and prostate gland development. The overlapped genes are ranked by log2 fold change (Log2FC).

this study to demonstrate the important role of MKRN2 in male fertility.

Previous studies have reported that MKRN2 inhibits the cell migration and invasion of non-small cell lung cancer by downregulating the PI3K/Akt pathway and is associated with lymph node metastasis, TNM stage, and cell differentiation (7). MKRN2 also acts as an E3 ligase for the NF-κB p65 subunit and has a negative regulatory effect on the inflammatory response (25).

Few reports have focused on the regulation of MKRN2 in male infertility. Our previous results demonstrated that *Mkrn2* deletion in somatic Sertoli cells disrupted ectoplasmic specialization (ES), resulting in abnormalities in sperm heads and spermiation failure, both of which are essential to spermiogenesis. Moreover, *Mkrn2* is crucial for protecting germ cells from apoptosis in spermatogenesis and male fertility *via* the p53/PERP signaling pathway. In this study, we focus on another phenotype of *Mkrn2* knockout mice: no sperm generated, which may indicate the essential role of MKRN2 in male fertility. In this study, we found a new regulatory mechanism between MKRN2 and STAT1/SIX4/TNC, which is a novel mechanism of MKRN2 in regulating male infertility.

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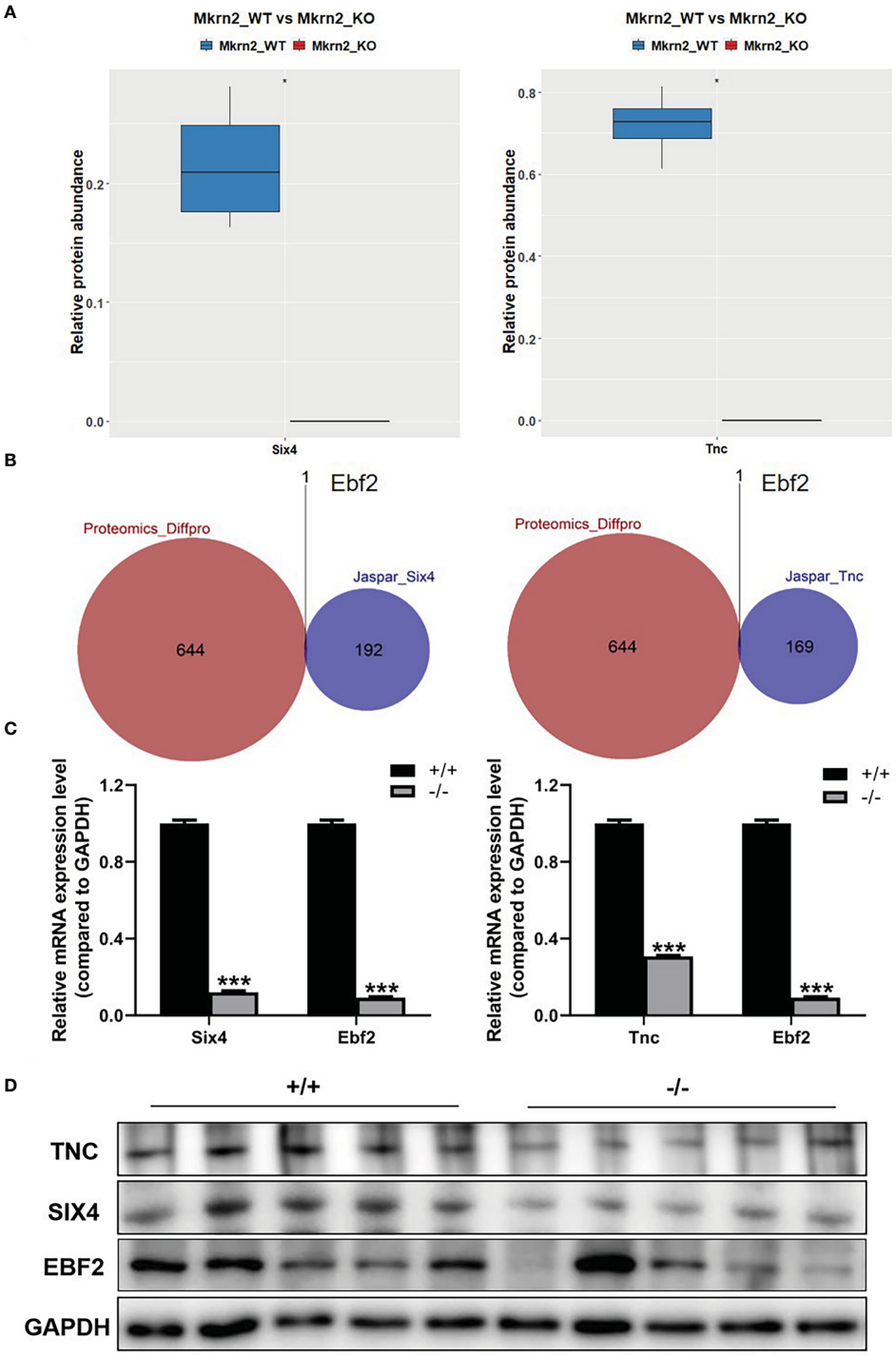

FIGURE 6
MKRN2 regulates the expression of SIX4 and TNC. **(A)** Proteomics data show SIX4 and TNC expression levels in *Mkrn2*-WT and *Mkrn2*-KO MEFs. **(B)** The overlapping between the putative upstream transcription factors of Six4 or Tnc according to the Jaspar website and differentially expressed proteins from proteomics. Ebf2 is the only differentially expressed transcription factor. **(C)** The mRNA expression levels of Six4 and Ebf2 were detected *via* Q-PCR, and the mRNA expression levels of Tnc and Ebf2 in *Mkrn2*-WT MEFs and *Mkrn2*-KO MEFs were also detected *via* Q-PCR. **(D)** The protein expression levels of EBF2, SIX4, and TNC were detected *via* Western blot in the testis tissues of *Mkrn2*-WT and *Mkrn2*-KO mice. $^{*}p < 0.05$ and $^{***}p < 0.0001$—significant difference.

Recent studies have shown that the testis shares many similarities with cancerous tissues, including cell division, immigration, and immortalization. Cancer–testis (CT) antigens are usually expressed only in testis tissues, with the exception of early-developing embryos. In addition, CT antigens are expressed in various tumor types (26). The CT genes, OIP5, TAF7L, and AURKC have been identified as biomarkers for breast cancer and may be promising and potent candidates for therapeutic cancer

vaccines (27, 28). MKRN2 may function as a cancer–testis antigen, with dual roles in tumorigenesis and spermiogenesis that need to be further investigated.

In this study, MKRN2 could interact with STAT1 and regulate its expression level, as well as the expression levels of SIX4 and TNC *via* the transcription factor EBF2. These results suggested that MKRN2 played a key role in testicular development and male 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 authors.

Ethics statement

The animal study was reviewed and approved by Committee of Laboratory Animal Experimentation of Zhengzhou University.

Author contributions

J-GQ and B-HJ designed experiments and prepared the manuscript. LW, Y-LY, and K-KW performed experiments and analyzed the data. Y-XX, Y-CQ, and F-MZ analyzed clinical samples and provided technical support. B-HJ provided advice. 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 of BMI with erectile dysfunction: A cross-sectional study of men from an andrology clinic

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Abnormal body mass index (BMI) is associated with an increased risk of erectile dysfunction (ED). However, the relationship between different BMI categories and the levels of ED severity remains unclear. In the current study, 878 men from the andrology clinic in Central China were recruited. Erectile function was assessed by the International Index of Erectile Function (IIEF) scores. Questionnaires included questions about demographic characteristics (age, height, weight, educational status), lifestyle habits (drinking, smoking, sleep time), and medical history. Logistic regression was used to examine the association between ED risk and BMI. The incidence of ED was 53.1%. BMI was significantly higher in men from the ED group than in those from the non-ED group (P = 0.01). Compared with the normal weight group, obese men had a higher risk of ED (OR = 1.97, 95% CI = 1.25-3.14, P = 0.004), even after adjustment for potential confounders (OR = 1.78, 95% CI = 1.10-2.90, P = 0.02). Moreover, the positive correlation between obesity and moderate/severe ED severity was confirmed by logistic regression analysis (moderate/severe ED, OR = 2.71, 95% CI = 1.44-5.04, P = 0.002), even after adjusting for potential confounders (OR = 2.51 95% CI = 1.24-5.09, P = 0.01). Collectively, our findings indicate a positive correlation between obesity and the risk of moderate/severe ED. Clinicians could pay more attention to moderate/severe ED patients to maintain a healthy body weight to improve erectile function.

KEYWORDS

BMI, erectile dysfunction, obesity, IIEF-5, China

Introduction

Erectile dysfunction (ED), also known as impotence, is defined as the persistent inability to attain and maintain a penile erection sufficient for satisfactory sexual intercourse (1). A previous study by the Massachusetts Male Aging Study (MMAS) reported that ED is the most common sexual health issue that affected over 150 million men worldwide in 1995 and will reach over 300 million in 2025 (2). ED is associated with substantial adverse effects on physical and psychological health (3). Moreover, ED is also an independent predictive factor for cardiovascular disease and male infertility (4, 5).

Overweight/obesity, defined as abnormal or excessive fat accumulation, causes impaired quality of life. With its rapidly increasing prevalence, obesity has become a global public health concern. Sexual dysfunction, especially ED, is common among obese males. Bacon et al. studied the risk of ED in overweight/obese males and reported that obesity increases the risk of ED by approximately 1.3 times (6). Further study by Fillo et al. showed that the incidence rate of ED was directly attributed to the growing prevalence of obesity (7). Recently, a regional study found that the prevalence of ED was 42.1% and presented increasing BMI and waist circumference in young nondiabetic obese men (8). Among obese middle-aged men, 15% weight loss increased the mean International Index of Erectile Function (IIEF) score (9). In addition to weight loss by lifestyle changes, bariatric surgery has been reported to decrease ED among patients with obesity (10).

To date, several studies have shown that an elevated body mass index (BMI) is associated with an increased risk of ED among men from Europe and North America (11, 12). However, in China, whether overweight/obesity is associated with ED remains unclear. The aim of this study was to investigate the association of BMI with erectile dysfunction in young men from Central China.

Methods

Patients

From May 2021 to July 2022, a total of 878 men from the andrology clinic at the First Affiliated Hospital of University of Science and Technology of China (USTC) were recruited for this cross-sectional study. The exclusion criteria were as follows: (1) incomplete data for individual information; (2) incomplete data for the IIEF-5 score; and (3) unmarried. This study was approved by The First Affiliated Hospital of USTC Ethical Committee (2021-RE-064).

Questionnaires

Questionnaires included questions about demographic characteristics (age, height, weight, educational status), lifestyle habits (drinking, smoking, sleep time), and medical history.

Erectile function

For all participants, erectile function was assessed by the International Index of Erectile Function-5 (IIEF-5) questionnaire, which is a sensitive and specific measure for ED. The IIEF-5 questionnaire contains 5 questions on a 5-point scale. A score below 21 is considered ED, and the degree of ED is divided into three classes: severe (5–11), moderate (12–16), and mild (17–21).

Statistical analysis

BMI was calculated as weight in kilograms divided by height in meters squared. Qualitative variables are reported as frequencies, and quantitative variables are reported as the mean ± standard deviation (SD) or as the median (interquartile range, IQR). For comparisons between the two groups, Student's t test was used for parametric data, and Pearson's chi-squared test (for trend) was used when needed. For comparison of more than two groups, Pearson's chi-squared test and chi-squared test for trend were used for normal and ordinal categorical variables, respectively. Logistic regression was used to examine the association between ED risk and BMI. Covariates initially included factors possibly associated with ED risk, including age, smoking, drinking, education, chronic diseases, sex frequency, urogenital infections, varicocele and prepuce length. All tests were 2-sided using GraphPad Prism 9.0 (GraphPad Software, CA, USA), and $P < 0.05$ was considered to indicate statistical significance.

Results

The clinical characteristics of the recruited men are shown in Table 1. Among 878 participants, the mean age and BMI were 31.5 years and 24.7 kg/m^2, respectively. According to the IIEF-5 score, all subjects were divided into two groups: the ED group (IIEF-5 score > 21, 466 subjects) and the non-ED group (IIEF-5 score ≤ 21, 412 subjects). The age did not differ between the two groups ($P = 0.35$), but BMI was significantly higher in men from the ED group than in those from the non-ED group ($P = 0.01$). Analysis of four BMI categories (underweight, normal weight, overweight and obese), we found that obese men were more likely to be in the ED group than in the non-ED group (15.0% vs 8.2%, $P = 0.02$). Educational state and sex frequency were more likely to be lower in the ED group ($P = 0.003$ and $P = 0.001$, respectively). No significant differences were found in lifestyle factors (smoking and drinking) or medical history (chronic diseases, urogenital infections, varicocele and the length of prepuce).

The prevalence of ED according to sex frequency and BMI category is shown in Table 2. Among all participants, the prevalence of men with sex inactivity (less than one time per week) was 65.5%, which was higher than that of men with a sex frequency of more than one time per week (Table 2). Notably, among men with

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TABLE 1 Characteristics and descriptive statistics of the whole cohort.

Clinical characteristics	Total (n=878)	ED (n=466)	Non-ED (n=412)	*P*
Age (years), mean ± SD	31.5 ± 4.9	31.6 ± 4.9	31.3 ± 4.8	0.35
BMI (kg/m^2), mean ± SD	24.7 ± 3.5	24.9 ± 3.7	24.4 ± 3.1	0.01
< 18.5 (underweight)	30 (3.4)	17 (3.6)	13 (3.2)	0.02
18.5-23.9 (normal weight)	360 (41.0)	184 (39.5)	176 (42.7)	
24.0-28.9 (overweight)	384 (43.7)	195 (41.8)	189 (45.9)	
≥ 29 (obese)	104 (11.9)	70 (15.0)	34 (8.2)	
Smoking, n (%)				0.95
Nonsmoker	504 (57.4)	267 (57.3)	237 (57.5)	
Smoker	374 (42.6)	199 (42.7)	175 (42.5)	
Drinking, n (%)				0.93
Nondrinker	412 (46.9)	218 (46.8)	194 (47.1)	
Drinker	466 (53.1)	248 (53.2)	218 (52.9)	
Education, n (%)				0.003
Primary school	12 (1.3)	5 (1.1)	7 (1.7)	
Junior high school	148 (16.9)	99 (21.3)	49 (11.9)	
High school	148 (16.9)	78 (16.7)	70 (17.0)	
College/University	570 (64.9)	284 (60.9)	286 (69.4)	
Chronic diseases				0.11
No	788 (89.7)	411 (88.2)	377 (91.5)	
Yes	90 (10.3)	55 (11.8)	35 (8.5)	
Sex frequency (weekly), n (%)				0.001
< 1	226 (25.7)	148 (31.7)	78 (18.9)	
1-2	536 (61.1)	258 (55.4)	278 (67.5)	
> 2	116 (13.2)	60 (12.9)	56 (13.6)	
Urogenital infections, n (%)				0.31
No	774 (88.2)	406 (87.1)	368 (89.3)	
Yes	104 (11.8)	60 (12.9)	44 (10.7)	
Varicocele, n (%)				0.70
No	813 (92.6)	430 (92.3)	383 (93.0)	
Yes	65 (7.4)	36 (7.7)	29 (7.0)	
Prepuce length, n (%)				0.32
I	446 (50.8)	241 (51.7)	205 (49.8)	
II	174 (19.8)	93 (20.0)	81 (19.7)	
III	179 (20.4)	96 (20.6)	83 (20.1)	
IV	79 (9.0)	36 (7.7)	43 (10.4)	
IIEF−5 score (Median, IQR)	21 (19-23)	19 (16-20)	23 (22-24)	< 0.001

ED, erectile dysfunction; BMI, body mass index. P values were derived from Pearson's chi-square test and Student's t test. Prepuce length was classified into four levels according to Zhao et al (13).

TABLE 2 Prevalence of ED according to sex frequency and BMI category.

	Participants (n=878)		
	N	Prevalence (%)	95% CI (%)
Sex frequency (weekly)			
< 1	226	65.5	59.1-71.4
1-2	536	48.1	43.9-52.4
> 2	116	51.7	42.7-60.6
BMI (kg/m^2)			
< 18.5 (underweight)	30	56.7	39.2-72.6
18.5-23.9 (normal weight)	360	51.1	46.0-56.2
24.0-28.9 (overweight)	384	50.8	45.8-55.7
≥ 29 (obese)	104	67.3	57.8-75.6
Overall	878	53.1	49.8-56.4

BMI, body mass index; CI, confidence interval; ED, erectile dysfunction.

different BMI categories, the fraction of ED patients with sexual inactivity was higher than that of sexually active participants, although the significant difference was only in men with normal weight and overweight (Table S1). In addition, the prevalence of ED in our study was 53.1%, with obese men having a higher prevalence than those with a normal BMI (67.3% vs 51.1%) (Table 2).

To analyze the association between ED and clinical characteristics, we performed a logistic regression analysis model, and the results are shown in Table 3. Participants with a low education level (junior high school) had a higher risk of ED (OR = 2.04, 95% CI = 1.40-2.99, P = 0.002). In addition, the frequency of sex less than once a week was significantly associated with the risk of ED (OR = 1.77, 95% CI = 1.12-2.80, P = 0.01). Compared with the normal weight group, obese men had a higher risk of ED (OR = 1.97, 95% CI = 1.25-3.14, P = 0.004), even after adjustment for potential confounders (OR = 1.78, 95% CI = 1.10-2.90, P = 0.02).

In terms of all participants, a significantly higher frequency of obesity was found in mild (3.8%), moderate (18.3%) and severe ED (45.2%) in comparison with men with normal weight (P = 0.001) (Table 4).

The positive correlation between obesity and ED severity (mild and moderate) was confirmed by logistic regression analysis (moderate/severe ED, OR = 2.71, 95% CI = 1.44-5.04, P = 0.002; mild ED, OR = 1.74, 95% CI = 1.06-2.86, P = 0.03) (Table 5).

TABLE 3 Logistic regression analyses for ED and BMI, education and sex frequency.

	Crude		Adjusted	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age	1.01 (0.98-1.04)	0.35		
Smoking	1.01 (0.77-1.32)	0.95		
Drinking	1.01 (0.78-1.32)	0.93		
Education				
Primary school	0.72 (0.21-2.28)	0.58		
Junior high school	2.04 (1.40-2.99)	0.0002		
High school	1.12 (0.78-1.61)	0.53		
College/University	Ref			
Chronic diseases	1.44 (0.93-2.27)	0.11		
Sex frequency				
< 1	1.77 (1.12-2.80)	0.01		
1-2	0.87 (0.58-1.29)	0.48		
> 2	Ref			

(Continued)

TABLE 3 Continued

	Crude		Adjusted	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Urogenital infections	1.24 (0.82-1.88)	0.32		
Varicocele	1.11 (0.67-1.85)	0.70		
Prepuce length				
I	Ref			
II	0.98 (0.69-1.39)	0.90		
III	0.98 (0.70-1.39)	0.93		
IV	0.71 (0.44-1.15)	0.17		
BMI	1.05 (1.01-1.10)	0.01		
< 18.5 (underweight)	1.25 (0.59-2.70)	0.56	1.18 (0.54-2.62)	0.68
18.5-23.9 (normal weight)	Ref		Ref	
24.0-28.9 (overweight)	0.99 (0.74-1.32)	0.93	1.00 (0.74-1.34)	0.98
≥ 29 (obese)	1.97 (1.25-3.14)	0.004	1.76 (1.09-2.86)	0.02

BMI, body mass index; ED, erectile dysfunction. ED-adjusted model, adjusted for age, smoking, drinking, education, chronic diseases, sex frequency, urogenital infections, varicocele and prepuce length.

Furthermore, after adjusting for potential confounders, a high risk of moderate/severe ED was still observed in obese men (OR = 2.51 95% CI = 1.24-5.09, *P* = 0.01).

Discussion

ED is a common sexual disorder and is a concern as a factor of metabolic syndrome in men (14, 15). This observational study investigated the relationship between BMI and ED risk and found that participants with obesity had a 1.3-fold greater risk of ED than participants with normal weight. After adjusting for potential confounders, obese men had an increased risk of moderate-severe ED.

The guidelines of the European Association of Urology (EAU) show a high incidence and prevalence of ED worldwide (16). In China, the prevalence of ED is approximately 20%-40% in men below 50 years of age (17). In the present study, the prevalence of ED was 53.1%, with a mean age of 31.5 years, and more than 99% were younger than 50 years. The frequency of sexual activity in approximately one-third of the ED patients was less than one time a week, which was significantly higher than the frequency in non-ED patients (19%), indicating that sexual inactivity was more common in ED patients. Notably, a subset of subjects were men who underwent fertility evaluation. Since sexual dysfunction, including ED, leads to impaired fertility through natural conception in men of reproductive age, the ED prevalence in our study population is relatively high, although sexual dysfunction accounted for only 0.4-4.6% of male infertility (5). These results suggest that clinical screening for the risk of ED should include the assessment of male reproductive health.

We observed a U-shaped relationship between BMI and ED prevalence, with a higher prevalence in underweight and obese participants than in normal and overweight participants. The results were similar to those of a population-based study by Cheng et al. but inconsistent with those of the Health Professionals Follow-Up Study (HPFS, a prospective cohort study) (18, 19). Analysis of BMI categories used in these studies

TABLE 4 Frequency distribution of BMI according to IIEF-5 score.

	Total (n=878)	< 18.5 (Q1, n=30)	18.5-23.9 (Q2, n=360)	24.0-28.9 (Q3, n=384)	≥ 29 (Q4, n=104)	^{a}P	^{b}P	^{c}P
IIEF–5 score, n (%)						0.57	0.94	0.001
5-11	20 (2.3)	1 (3.4)	7 (1.9)	8 (2.1)	4 (3.8)			
12-16	97 (11.1)	3 (10.0)	37 (10.3)	38 (9.9)	19 (18.3)			
17-21	349 (39.7)	13 (43.3)	140 (38.9)	149 (38.8)	47 (45.2)			
22-25	412 (46.9)	13 (43.3)	176 (48.9)	189 (49.2)	34 (32.7)			

BMI, body mass index; IIEF-5, International Index of Erectile Function questionnaire. Chi-square test for trend.
[a]P, Q2 vs Q1;
[b]P, Q2 vs Q3;
[c]P, Q2 vs Q4.

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TABLE 5 Logistic regression for the association between BMI and ED severity.

	IIEF-5 score				
	5-16		17-21		22-25
	OR (95% CI)	*P*	OR (95% CI)	*P*	
Crude					
< 18.5	1.23 (0.33-3.68)	0.73	1.26 (0.56-2.82)	0.58	Ref
18.5-23.9	Ref		Ref		Ref
24.0-28.9	0.97 (0.61-1.55)	0.91	0.99 (0.73-1.35)	0.95	Ref
≥ 29	2.71 (1.44-5.04)	0.002	1.74 (1.06-2.86)	0.03	Ref
Adjusted					
< 18.5	1.01 (0.26-3.30)	0.98	1.15 (0.50-2.68)	0.74	Ref
18.5-23.9	Ref		Ref		Ref
24.0-28.9	0.95 (0.58-1.56)	0.85	0.99 (0.72-1.36)	0.94	Ref
≥ 29	2.51 (1.24-5.09)	0.01	1.48 (0.89-2.50)	0.13	Ref

BMI, body mass index; ED, erectile dysfunction. ED-adjusted model, adjusted for age, smoking, drinking, education, chronic diseases, sex frequency, urogenital infections, varicocele and prepuce length.

revealed that both Cheng and our studies divided the underweight (BMI < 18.5 kg/m^2) subgroup, while the HPFS study used a BMI less than 23 kg/m^2 as the reference group. It is possible that different BMI cutoffs in European and Asian populations classified by the WHO result in different BMI categories being used (20). As a result, the decreasing range of the U-shaped relationship between BMI and ED was not present in the HPFS study. Nevertheless, the present study extended the high prevalence of ED in underweight and obese men.

Evidence from animals to humans has reported that obesity plays a key role in male sexual dysfunction, including premature ejaculation and ED, although ED is mainly considered an age-related disease (21, 22). It has been demonstrated not only that obesity is a strong and independent risk factor for ED but also that ED risk is positively correlated with the level of obesity (7). A recent study showed that the prevalence of ED was 42% in nondiabetic young obese men from a primary care-based cohort (8). Moreover, a meta-analysis including three studies demonstrated that the prevalence of ED in obese men with a BMI > 30 kg/m^2 was approximately 71-73% (21), which was similar to the value (67%) obtained from men with BMI > 29 kg/m^2 in the current study. The prevalence of ED is approximately 1.3 times higher for obese men than for normal weight men, confirming the relationship between obesity and the incidence of ED.

Although obesity is a well-documented risk factor for ED, the association between obesity and the levels of ED severity remains unclear. Our results show that the prevalence of severe ED in underweight and obese men was higher than that in those who were normal weight. Notably, overweight (BMI = 24.0-28.9 kg/m^2) and normal weight men had similar incidence rates of mild, moderate and severe ED, indicating that the prevalence of ED was not increased even though they were overweight. Considering that the mean BMI (24.7 kg/m^2) reached the overweight range, we assumed that the observations of our study could be partly explained by the enrolled populations.

Our data also revealed a positive correlation of obesity with moderate/severe ED risk after adjusting for confounders. According to the role of obesity in metabolic syndrome, the high risk of moderate/severe ED may be due to pathophysiologic processes, including oxidative stress, inflammation, and insulin and leptin resistance. This might partly explain the elevated prevalence of chronic diseases (hypertension, hyperlipidemia, and diabetes) in the ED groups (ED: 11.8%, non-ED: 8.5%) in the current study. Moreover, the efficacy of phosphodiesterase type 5 inhibitors (PDE5-i) on erectile function is reduced in obese men (23). Obesity evaluation can be a useful biomarker in those with severe ED and PDE5-i nonresponders. However, unlike obesity, both crude and adjusted analyses showed that underweight was not associated with ED risk. The relatively small number of underweight men enrolled may limit the statistical power.

It is important to note that a randomized controlled trial was performed to evaluate weight reduction on ED in obese men (9). The results showed that lifestyle changes are associated with improvements in erectile function. In addition, weight loss by bariatric surgery has been reported to improve erectile function in obese men (10). Accordingly, it seems to be reversible that weight loss from exercise, diet, or surgery could reverse ED and provide a view of the cost-effectiveness for the treatment of ED.

The current study has several strengths. First, the mean age of the recruited men was 31.5 years, reducing the effect of age on ED risk. Second, several potential confounders, including age, BMI, lifestyle styles (smoking and drinking), educational status and medical history (chronic diseases, urogenital infections, varicocele and the length of prepuce), were included in our study, which gives more statistical power. Third, classification of the BMI categories was based on the recommendations of WHO Asian BMI cutoffs. Fourth, the relationship between BMI categories and the severity of ED according to the IIEF-5 was assessed.

We also acknowledge that this study has several limitations. First, our study was limited to an association based on the cross-sectional study design. Second, only healthy or subfertile men who underwent infertility investigations were selected, and further studies across other populations will be needed. Third, metabolic parameters and penile duplex ultrasound were not measured in the present study. Finally, recall bias may have occurred in this study when data were obtained from questionnaires.

In conclusion, the findings in this study indicate that obesity is associated with an increased risk of moderate/severe ED. Given the high prevalence of ED in obese men, clinicians could pay more attention to moderate/severe ED patients to maintain a healthy body weight to improve erectile function.

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

This study was approved by The First Affiliated Hospital of USTC Ethical Committee (2021-RE-064). The patients/participants provided their written informed consent to participate in this study.

Author contributions

YL, SB, and XZ designed the research study. XH, MX, JL, XJ, YW, and SB contributed to the data acquisition. YL, XH, SB, and XZ analyzed the data. YL and SB 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1135024/full#supplementary-material

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Dietary flaxseed oil and vitamin E improve semen quality *via* propionic acid metabolism

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Introduction: Flaxseed oil (FO) and vitamin E (VE) both have antioxidant effects on sperm. The present study investigated the effects of dietary supplementation with FO and/or VE on semen quality.

Methods: 16 fertile Simmental bulls were selected and randomly divided into 4 groups (n = 4): the control group (control diet), FO group (control diet containing 24 g/kg FO), VE group (control diet containing 150 mg/kg VE) and FOVE group (control diet containing 150 mg/kg VE and 24 g/kg FO), and the trial lasted 10 weeks.

Results: The results showed that the addition of FO independently can increase sperm motion parameters, the levels of catalase (CAT), glutathione peroxidase (GSH-Px), testosterone (T) and estradiol (E2), while reduce oxidative stress in seminal plasma ($P < 0.05$). Supplement of VE independently can increased the motility, motility parameters, CAT and superoxide dismutase (SOD) levels, and reduce oxidative stress in seminal plasma ($P < 0.05$). There was an interaction effect of FO × VE on motility and reactive oxygen species (ROS), while GSH-Px and ROS were affected by week × VE 2-way interaction, levels of T and E2 were also affected by the dietary FO × week interaction ($P < 0.05$). The triple interaction effects of FO, VE and week were significant for malondialdehyde (MDA) ($P < 0.05$). Compared with the control group, sperm from the FOVE group had a significantly higher in vitro fertilization (IVF) rate, and subsequent embryos had increased developmental ability with reduced ROS levels at the eight-cell stage, then increased adenosine triphosphate (ATP) content and gene expression levels of CAT, CDX2, Nanog, and SOD at the blastocyst stage ($P < 0.05$). Metabolomic and transcriptomic results indicated that dietary supplementation of FO and VE increased the expression of the metabolite aconitic acid, as well as the expression of ABAT and AHDHA genes.

Conclusion: With in-silico analysis, it can be concluded that the effects of dietary FO and VE on improving semen quality and embryo development may be related to increased aconitic acid via the ABAT and AHDHA genes involved in the propionic acid metabolism pathway.

KEYWORDS

cryopreservation, bull semen, flaxseed oil, vitamin E, metabolomics, transcriptomics

1 Introduction

Cryopreservation is the most practical approach for long-term storage of bull sperm. However, most sperm are damaged by oxidative stress during cryopreservation. This damage causes a loss and decline in motility, viability and fertilization ability. Therefore, improving the antioxidant capacity of frozen-thawed sperm and reducing the level of oxidative stress is particularly important for maintaining semen quality (1).

ω-3 Polyunsaturated fatty acids (ω-3 PUFAs) play important roles in the regulation of reproductive processes such as testosterone synthesis, sperm acrosome integrity and membrane fluidity (2). Ruminants lack the relevant fatty acid desaturase enzymes and are not able to synthesize ω-3 PUFAs. Thus, these animals must obtain ω-3 PUFAs or their pre-cursors from dietary sources (3). Flaxseed oil (FO) contains up to 50% ω-3 PUFAs, of which α-linolenic acid is converted to eicosapentaenoic and docosahexaenoic acids in mammals (4). In addition, ω-3 PUFAs contain stearic, oleic, linoleic and palmitic acids, and phenolic compounds, all of which have important roles in spermatogenesis and antioxidant enzyme activity (5).

Vitamin E (VE), one of the major antioxidants against reactive oxygen species (ROS) and lipid peroxidation, has been shown to be a major component of the antioxidant system of sperm (6, 7). VE deficiency may lead to developmental arrest of reproductive organs, including testicular tissue damage, and reduction of spermatogonia and spermatocytes (8). Previous studies demonstrated that supplementation of diets with VE could improve semen quality by reducing mitochondrial protection from oxidative stress damage (9).

In the past decade, *in vitro* fertilization (IVF) technology has made significant progress in animal reproduction (10). Compared with artificial insemination, IVF is more cost-effective and less time-consuming (11). Seminal plasma metabolomics has been used to screen for potential biomarkers of fertility in bulls (12). To investigate male fertility, transcriptome sequencing technology has been used to assess mRNA expression in sperm (13, 14). Therefore, metabolomic and transcriptomic analyses are important tools for identifying semen fertility.

At present, there is no relevant report on the effect of the combination of FO and VE in the diet on the frozen-thawed semen of bulls. Therefore, we speculate that the combined addition of FO and VE in the diets could further reduce oxidative stress and improve motility and motion parameters of frozen-thawed sperm, compared with FO or VE alone. At the same time, the combined addition of FO and VE could further improve the ability of IVF and early embryonic development by enhancing the antioxidant capacity. The aim of this study was to evaluate the effects of combined FO and VE supplementation on bull semen quality and subsequent embryonic development *in vitro*, and to explore the mechanism of the effects of FO and VE on semen quality using transcriptome and metabolome analyses.

2 Materials and methods

2.1 Experimental design

The trial lasted for 10 weeks, and 16 fertile Simmental bulls (approximately 2–3 years of age and 550–650 kg each) were selected from Changchun Xinmu Sciences & Technologies Co., Ltd, and divided randomly into four groups (n = 4). They were all kept under similar management. Ingredients and chemical composition of the diets are shown in Table 1. The dietary treatments were as follows: 1) control group: control diet, 2) FO group: control diet containing 24 g/kg FO (15), 3) VE group: control diet containing 150 mg/kg VE (16), and 4) FOVE group: control diet containing 150 mg/kg VE and 24 g/kg FO (17). In the current study, FO was purchased from Inner Mongolia Jiuding Food Co., Ltd., China. The molar percentages of linolenic acid, linoleic acid, oleic acid and saturated acyl groups in FO was 55.7 ± 0.0%, 14.2 ± 0.3%, 20.5 ± 1.2% and 9.5 ± 0.9% respectively (18). VE was purchased from Zhejiang NHU Co., Ltd., China. This study showed that the combination of FO and VE improved the motion parameters of sperm and the antioxidant capacity of seminal plasma after frozen-thawed at week 10. Therefore, the control and FOVE groups of week 10 samples were selected for subsequent IVF, seminal plasma metabolome and sperm transcriptome studies.

TABLE 1 Ingredients and chemical composition of diets.

Ingredient	% of dry matter	Chemical composition	% of dry matter
corn	45	Crude protein	20
Soybean	32	Crude ash	7
Wheat bran	5	Ca	0.7
Rice bran	5	P	0.7
Soy Germ Powder	6	NaCl	1
Molasses	2		
Premix	5		

The premix contains vitamin A (5×10^5IU/kg), vitamin D (2×10^5IU/kg), Cu (250mg/kg), Fe (1250mg/kg), Zn (1500mg/kg) and Mn (1000mg/kg).

2.2 Semen collection and assessment

Semen was collected by experienced technicians, twice a week per bull, into a heated (38°C) artificial vagina connected to a disposable tube, and quickly transferred to an adjacent laboratory where the physiological parameters were assessed after semen collection. Semen samples were expanded in a BioXcell® (IMV Technologies, L'agile, France) extender to a concentration of 1.28×10^8, then loaded into thin tubes (IMV Technologies), and the semen was equilibrated at 4°C for 2 h, and subsequently cooled at approximately 8 min from 4°C to −140°C by a turbofreezer (Minitube, Germany) as previously described by Memon et al. (19). After that, samples were stored in a liquid nitrogen tank (−196°C). The sperm motility and motion parameters were assessed using the Hamilton Thorne IVOS II automatic sperm analyzer (Hamilton-Thorne, Beverly, MA, USA). For each bull, 2 straws were thawed by immersion in a water bath at 37°C for 30 s. 10 µL were collected from each straw, and eight fields were randomly examined.

2.3 Oxidative stress index and hormones

The oxidative stress index and hormones of embryo culture medium and seminal plasma included superoxide dismutase (SOD, MM-34758O1, 1.2U/mL-42 U/mL), catalase (CAT, MM-50463O1, 0.5 U/mL-18U/mL), glutathione peroxidase (GSH-Px, MM-2465O1, 20 IU/L-480 IU/L), ROS (MM-50460O1, 5 IU/mL-160 IU/mL), malondialdehyde (MDA, MM-34745O1, 0.5 nmol/mL-16 nmol/mL), testosterone (T, MM-2382O1, 0.3nmol/L-12nmol/L) and estradiol (E_2, MM-0023O1, 4 pmol/L-120 pmol/L) were determined using specific enzyme-linked immunoassay kits (Jiangsu Meimian Industrial Co., Ltd, Yancheng, China), according to the manufacturer's instructions. Briefly, semen was centrifuged at 3000 rpm for 15 min, and the seminal plasma was extracted before analysis. Results were measured at 450 nm on a spectrophotometer (Shanghai Spectrophotometer Co., Ltd, Shanghai, China).

2.4 *In vitro* maturation

Oocyte collection and IVM were performed as described previously (20). Briefly, a 10 mL syringe was used to aspirate cumulus–oocyte complexes from antral follicles with a diameter between 2 and 8 mm. Oocytes surrounded by at least three cumulus cell layers were selected for subsequent experiments, then washed in collection medium M199 supplemented with 2% fetal bovine serum (FBS, Hyclone) and transferred to maturation medium modified M199 medium supplemented with alanyl-glutamine (10 µL/mL), sodium pyruvate (50 µL/mL), epidermal growth factor (10 µL/mL), follicle-stimulating hormone (4 µL/mL), luteinizing hormone (4 µL/mL), E_2 (10 µL/mL), cysteamine (10 µL/mL), and 10% FBS, incubated for 22 h at 38.5°C, 5% CO_2 in air and high humidity.

2.5 *In vitro* fertilization

After 22 h, cumulus–oocyte complexes were added to SOF-IVF medium (6.29 mg/mL NaCl, 0.53 mg/mL KCl, 0.16 mg/mL NaH_2PO_4, 0.1 mg/mL $MgCl_2{\cdot}6H_2O$, 0.25 mg/mL $CaCl_2{\cdot}6H_2O$, 0.758 mg/mL sodium lactate, 2.1 mg/mL $NaHCO_3$, 6.25 mg/mL BSA, 10.417 µL/mL MEM non-essential amino acids, 10 µL/mL fructose, 10 µL/mL heparin and 10 µL/mL sodium pyruvate). Frozen-thawed pooled semen was washed through 45% and 90% Percoll gradients (21). Semen, layered on the top, was centrifuged (700 ×g, 15 min). 5 mL of sperm TALP medium (5.84 mg/mL NaCl, 0.23 mg/mL KCl, 35 µg/mL NaH_2PO_4, 2.1 mg/mL $NaHCO_3$, 2.38 mg/mL HEPES, 80 µg/mL $MgCl_2{\cdot}6H_2O$, 310 µg/mL $CaCl_2{\cdot}6H_2O$, 3.07 µL/mL sodium lactate, 6 mg/mL BSA and 0.11 mg/mL sodium pyruvate) were used to wash the pellet (300 ×g, 5 min). Sperm concentration was calculated with a hemocytometer and adjusted to 10^6/mL with SOF-IVF medium.

2.6 *In vitro* culture

After 18 h of co-incubating cumulus–oocyte complexes and sperm at 38.5°C, 5% CO_2 in air and high humidity, Zygotes were mechanically separated *via* pipetting and washed three times through IVC medium SOF-aa with 62.94 mg/mL NaCl, 5.33 mg/mL KCl, 1.62 mg/mL NaH_2PO_4, 0.46 mg/mL $MgCl_2$, 4.7 mg/mL sodium lactate, 4.32 mg/mL glucose, 26.07 mg/mL $NaHCO_3$, 2.52 mg/mL $CaCl_2{\cdot}2H_2O$, 0.36 mg/mL sodium pyruvate, 20 µL/mL essential amino acids, 10 µL/mL non-essential amino acids, 4 mg/mL BSA and 0.15 mg/mL glutamine. The zygotes were cultured at 38.5°C, 5% CO_2 in air and high humidity. The number of two-, four- and eight-cell embryos were recorded at 32, 42 and 72 h, respectively, and blastocyst yield was assessed at Day 7.

2.7 Measurement of intracellular ROS levels

Intracellular ROS levels in embryos were measured with a ROS detection kit (S0033S; Beyotime, China). Briefly, embryos were incubated with 10 mM DCFH-DA for 20 min at 37°C. After washing three times with 1% phosphate-buffered saline/PVP360 (PBS-PVP), the embryos were examined using a fluorescence microscope (Olympus, Tokyo, Japan) with a 460 nm excitation filter. Fluorescence images of all embryos were recorded using a digital

camera (Nikon 990, Tokyo, Japan), and analyzed using the NIH ImageJ by Wayne Rasband from the National Institute of Health (Bethesda, MD, USA). Fluorescence intensities of the embryos were compared with that of the control after deducting the background value.

2.8 Measurement of intracellular ATP levels

Adenosine triphosphate (ATP) of embryos was detected using an ATP assay kit (S0063, Invitrogen). Briefly, embryos were collected, washed twice in PBS-PVP and then fixed in formaldehyde-PVP for 0.5 h at room temperature. Afterwards, the embryos were washed three times with PBS-PVP, 5 min each time, and then incubated with PBS-PVA containing 0.1% Triton X-100 for 1 h for membrane permeabilization. After thoroughly washing with PBS-PVP, the fluorescence signal was captured using an epifluorescence microscope, and the fluorescence intensity of embryos was analyzed using NIH ImageJ software.

2.9 RNA isolation, cDNA synthesis and q-PCR

Total RNA from bovine blastocysts was extracted using the RNeasy Mini kit (KIT0204, Qiagen, Hilden, Germany). A total of 18 blastocysts were used to extract RNA. The One-Step gDNA Removal and cDNA Synthesis SuperMix (AT311-03, TransGen Biotech, Beijing, China) was used to synthesize cDNA. The real-time PCR mix (25 µL) consisted of 2 µL of cDNA, 12.5 µL of SYBR green master mix, 9.5 µL of RNase-free water, and 0.5 µL each of primers (10 pmol). The primers used for the octamer-binding transcription factor 4 (*OCT-4*), caudal type homeobox 2 (*CDX-2*), *Nanog*, *SOD*, *CAT*, B-cell lymphoma 2 (*Bcl2*), *BCL2* Associated X (*Bax*) genes and 18s rRNA are listed in Table 2. The program used for the amplification consisted of a denaturing cycle of 3 min at 95°C, 40 cycles of PCR (95°C for 10 s, 55°C for 45 s, and 95°C for 1 min), a melting curve analysis consisting of 95°C for 1 min followed by 55°C for 1 min, and a step cycle starting at 55°C for 10 s with a 0.5°C/s transition rate, and cooling at 4°C. Relative gene expression data were analyzed using q-PCR and the $2^{-\triangle\triangle CT}$ method.

2.10 Metabolite extraction and analysis

Approximately 100 µL of each seminal plasma sample was transferred to a new 1.5 mL Eppendorf tube. Then an equal volume of each sample was sampled and mixed into 100 µL of a quality control (QC) sample. Then, 300 µL of cold methanol was added, shaken and mixed, and placed at −20°C for 2 h. After centrifugation at 25,000 ×g for 10 min at 4°C, 350 µL supernatant was placed in a new Eppendorf tube, and the centrifugation step was repeated. Then 25 µL supernatant was added to 225 µL 50% methanol, and 50 µL of supernatant from each sample was taken and mixed with a QC sample, and the remaining transferred to a new 1.5 mL Eppendorf tube for testing. An Acquity UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 µm, Waters, UK) was used for reversed-phase separations. The column temperature was maintained at 40°C and the metabolites were eluted at a flow rate of 0.5 mL/min. The injection volume for each sample was 5 µL. A high-resolution

TABLE 2 Primer sequences for real-time q-PCR.

Gene	Primers	Length (bp)
18s rRNA-F	TTGATCTTCATTGTGCTGGGTG	189
18s rRNA-R	CTTCCTGGGCATGGAATCCT	
Oct4-F	CCACCCTGCAGCAAATTAGC	184
Oct4-R	CCACACTCGGACCACGTCTT	
Nanog-F	ATAATGGTTTTGGTGAGATTGGTAG	161
Nanog-R	ATAAAACTCAACCATACTTAACCCC	
CDX2-F	AAGACAAATACCGGGTCGTG	154
CDX2-R	CTGCGGTTCTGAAACCAAAT	
CAT-F	TGGGACCCAACTATCTCCAG	178
CAT-R	AAGTGGGTCCTGTGTTCCAG	
SOD-F	AGAGGCATGTTGGAGACCTG	189
SOD-R	CAGCGTTGCCAGTCTTTGTA	
Bax-F	CGAGTTGATCAGGACCATCAT	168
Bax-R	ATGTGGGTGTCCCAAAGTAG	
Bcl-2-F	TGGATGACCGAGTACCTGAA	124
Bcl-2-R	GAGACAGCCAGGAGAAATCAAA	

tandem mass spectrometer Xevo G2 XS QTOF (Waters, UK) was used to detect small molecules eluted from the column. A quality control sample was acquired after every 10 samples to evaluate the stability of the LC-MS.

2.11 Data processing of metabolites

Peak extraction was performed primarily through the commercial software Progenesis QI (version 2.2). The resulting LC-MS data were inputted to the R software package for principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to evaluate differential ions, identify differentially expressed genes, and analyze metabolite and metabolic pathways.

2.12 Total RNA extraction of transcriptomics

Total RNA was extracted from sperm using the RNeasy Micro kit (KIT0204, Qiagen, Hilden, Germany) according to manufacturer's instruction. Briefly, samples were transferred to tubes containing Buffer RL and one volume of 70% ethanol and then centrifuged. Buffer RW1, DNase I, Buffer RPE and 80% ethanol were added then samples centrifuged. High quality RNA was used for mRNA library construction.

2.13 mRNA library construction of transcriptomics

High-quality RNA was amplified (more than 200 pg) with oligo-dT and dNTPs to generate full-length cDNA by PCR. The Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA) was used to determine the average molecular length of PCR products. The DNA products were purified by an Agencourt AMPure XP-Medium kit (Thermo Fisher Scientific, USA). Single-stranded circular DNA was formatted as the final library, which used phi29 (Thermo Fisher Scientific, MA, USA) to prepare a DNA nanosphere. The DNA nanosphere was loaded into a patterned nanoarray, with single end 50 base reads generated on a BGISEQ500 platform (BGI-Shenzhen, China).

2.14 Statistical analysis

All data are presented as least squares means ± SD. Each individual bull was considered an experimental unit in all statistical analysis. Data on oxidative stress index, T, E_2 and the quality of semen were analyzed for the main effects of FO and VE. The FO, VE and time interactions used the General Linear Model procedure of the SPSS Institute (19.0) with a repeated measurement analysis. Differences among least square means were determined by the Tukey test and $P < 0.05$ was considered a significant difference. Differences in embryo development, CAT, SOD and ROS levels in embryo culture medium, and relative levels of gene expression, ROS and ATP in embryos, between the control and FOVE groups were compared by the T-test (SPSS 19.0), and $P < 0.05$ was considered a significant difference.

3 Results

3.1 Effect of dietary FO and VE on frozen-thawed sperm motility and motion parameters

The diet supplemented with VE significantly enhanced motility, straight distance (DSL), straight line velocity (VSL), curvilinear velocity (VCL) at week 8 and increased average path (DAP), DSL, curvilinear distance (DCL), velocity of the average path (VAP), VSL and VCL at week 10 compared with groups with no VE supplementation ($P < 0.05$) (Table 3). Sperm from bulls fed a diet containing FO had significantly higher values of DAP, DSL, DCL, VAP, VSL, VCL and amplitude of lateral head displacement (ALH) at week 10 compared with other groups ($P < 0.05$). There was an interaction effect of FO × VE on motility ($P < 0.05$).

3.2 Effect of dietary FO and VE on frozen-thawed semen plasma antioxidant status and hormones

As shown in Table 4, dietary FO significantly increased GSH-Px and decreased MDA at week 10. Supplementation with VE significantly increased SOD and decreased MDA at week 8 compared with groups with no VE supplementation ($P < 0.05$). The diet supplemented with FO or VE alone significantly increased CAT and decreased ROS at weeks 8 and week 10 compared with no FO or VE supplementation ($P < 0.05$). There was an effect of week × VE interaction on GSH-Px and ROS, and ROS was also affected by a dietary FO × VE 2-way interaction ($P < 0.05$). The triple interaction effects of FO, VE and week were significant for MDA ($P < 0.05$). Levels of T and E_2 in frozen-thawed semen plasma are also shown in Table 4. Samples from bulls fed the diet with FO had increased levels of T and E_2 at weeks 4, 8 and 10 compared with those from bulls fed the diet without FO. At week 8, elevated T content was observed with supplementation of VE in the diet ($P < 0.05$). Levels of T and E_2 were also affected by the dietary FO × week interaction ($P < 0.05$).

3.3 Effect of dietary FO and VE on thawed sperm and subsequent embryo development outcomes

The above results show that adding FO and VE to the diet for 10 weeks can improve the motility and motion parameters of frozen-thawed sperm, and also improve the antioxidant capacity, and the

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TABLE 3 Frozen-thawed sperm motility and motion parameters.

	Control	FO	VE	FOVE	Overall
Motility, %					
0 week	41.43 ± 3.52	46.35 ± 7.38	44.95 ± 11.66	43.5 ± 6.92	44.06 ± 7.33[c]
4 week	43.05 ± 1.85	40.33 ± 14.48	40.85 ± 18.49	56.87 ± 5.93	45.27 ± 12.92[c]
8 week $	53.63 ± 5.1	48.27 ± 4.12	51.03 ± 7.53	68.27 ± 3.4	55.3 ± 9.27[b]
10 week	61 ± 2.45	59.93 ± 5.46	58.05 ± 7.49	74.28 ± 2.78	63.31 ± 7.99[a]
overall	49.78 ± 8.82	48.72 ± 10.77	48.72 ± 12.77	60.73 ± 12.95	–
DAP (μm)					
0 week	11.93 ± 2.57	13.04 ± 2.59	13.16 ± 3.87	12.47 ± 2.25	12.65 ± 2.63[c]
4 week	11.1 ± 0.96	10.52 ± 4.76	10.52 ± 4.76	12.14 ± 1.04	11.07 ± 3.15[c]
8 week	16.38 ± 4.23	12.48 ± 1.38	14.36 ± 2.64	19.82 ± 1.34	15.76 ± 3.69[b]
10 week #$	16.08 ± 1.52	17.2 ± 3.3	17.54 ± 2.91	26.15 ± 4.55	19.24 ± 5.07[a]
overall	13.87 ± 3.4	13.31 ± 3.84	13.9 ± 4.17	17.65 ± 6.44	–
DSL (μm)					
0 week	9.45 ± 2.27	9.19 ± 1.38	10.43 ± 2.89	9.53 ± 1.44	9.65 ± 1.93[c]
4 week	8.46 ± 1.07	8.45 ± 3.77	8.45 ± 3.77	9.41 ± 1.03	8.69 ± 2.51[c]
8 week $	12.86 ± 4.34	9.18 ± 1.09	11.88 ± 2.28	15.51 ± 1.43	12.36 ± 3.31[b]
10 week #$	12 ± 1.65	12.61 ± 1.73	13.85 ± 2.37	19.72 ± 3.51	14.54 ± 3.84[a]
overall	10.69 ± 3	9.86 ± 2.62	11.15 ± 3.29	13.54 ± 4.86	–
DCL (μm)					
0 week	22.6 ± 4.72	23.1 ± 8.2	24.41 ± 7.86	24.33 ± 5.22	23.61 ± 6.03[c]
4 week	21.57 ± 2.15	19.5 ± 8.97	19.5 ± 8.97	23.03 ± 1.63	20.9 ± 6[c]
8 week	31.2 ± 6.75	24.13 ± 2.68	26.25 ± 4.81	37.86 ± 1.39	29.86 ± 6.73[b]
10 week #$	31.01 ± 2.28	33.23 ± 7.58	32.48 ± 5.88	51.19 ± 9.87	36.98 ± 10.56[a]
overall	26.6 ± 6.11	24.99 ± 8.35	25.66 ± 7.94	34.1 ± 12.87	–
VAP (μm/sec)					
0 week	33.04 ± 6.15	36.22 ± 6.52	35.55 ± 12.3	35.52 ± 5.64	35.08 ± 7.37[c]
4 week	32.67 ± 2.01	29.72 ± 15.73	29.72 ± 15.73	37.88 ± 3.98	32.5 ± 10.71[c]
8 week	43.8 ± 9.11	36.97 ± 3.73	37.56 ± 6.38	56.34 ± 4.99	43.67 ± 9.86[b]
10 week #$	49.64 ± 3.55	52.39 ± 9.56	48.38 ± 8.81	74.09 ± 9.55	56.13 ± 13.1[a]
overall	39.79 ± 9.13	38.83 ± 12.37	37.8 ± 12.33	50.96 ± 17.1	–
VSL (μm/sec)					
0 week	26.25 ± 4.69	26.07 ± 3.3	28.28 ± 9.19	27.47 ± 3.57	27.02 ± 5.19[c]
4 week	25.23 ± 2.77	24.03 ± 12.7	24.03 ± 12.7	30.04 ± 3.94	25.83 ± 8.7[c]
8 week $	34.44 ± 9.11	27.73 ± 2.72	31.28 ± 5.71	44.76 ± 4.98	34.55 ± 8.52[b]
10 week #$	37.85 ± 3.41	39.49 ± 4.83	38.65 ± 6.94	56.94 ± 7.39	43.23 ± 9.73[a]
overall	30.94 ± 7.44	29.33 ± 8.9	30.56 ± 9.78	39.8 ± 13.13	–
VCL (μm/sec)					
0 week	61.23 ± 12.05	69.09 ± 14.7	64.59 ± 24.03	67.41 ± 13.04	65.58 ± 15.21[c]
4 week	61.7 ± 4.69	53.77 ± 28.76	53.77 ± 28.76	69.88 ± 7.06	59.78 ± 19.82[c]

(Continued)

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TABLE 3 Continued

	Control	FO	VE	FOVE	Overall
8 week $	82.05 ± 15.17	69.25 ± 6.99	67.41 ± 11.66	104.88 ± 6.23	80.9 ± 18.14[b]
10 week #$	93 ± 7.07	98.21 ± 21.53	87.78 ± 17.44	140.84 ± 21.6	104.96 ± 27[a]
overall	74.5 ± 16.93	72.58 ± 24.23	68.39 ± 23.03	95.75 ± 33.2	–
ALH (μm)					
0 week	3.45 ± 0.56	4.06 ± 0.97	3.57 ± 1.32	3.89 ± 0.76	3.74 ± 0.88[c]
4 week	3.78 ± 0.32	3.02 ± 1.56	3.02 ± 1.56	4.22 ± 0.28	3.51 ± 1.14[c]
8 week	4.82 ± 0.62	4.26 ± 0.5	3.78 ± 0.49	6.06 ± 0.28	4.73 ± 0.98[b]
10 week #	5.52 ± 0.42	5.55 ± 1.26	4.88 ± 1	7.54 ± 0.96	5.87 ± 1.34[a]
overall	4.39 ± 0.96	4.22 ± 1.38	3.81 ± 1.25	5.43 ± 1.63	–

Data shown are the mean ± SD (n = 4 replicates per treatment, one ejaculate per replicate). For each parameter, values within columns with different superscript letters differ significantly ($P < 0.05$). #Significant ($P < 0.05$) main effect of flaxseed oil (FO). $Significant ($P < 0.05$) main effect of vitamin E (VE). CASA, computer-aided sperm analysis; Means (± SD) of the distance of the average path (DAP, μm), straight distance (DSL, μm), curvilinear distance (DCL, μm), velocity of the average path (VAP, μm/sec), straight line velocity (VSL, μm/sec), curvilinear velocity (VCL, μm/sec), amplitude of lateral head displacement (ALH, μm) were assessed after thawing. "-" This symbol has no meaning.

TABLE 4 Frozen-thawed semen plasma antioxidant status and hormones.

	Control	FO	VE	FOVE	overall
CAT					
0 week	187.67 ± 31.81	194.32 ± 20.57	192.65 ± 30.21	182.69 ± 25.16	189.33 ± 24.86[b]
4 week	181.03 ± 46.79	174.38 ± 23.26	177.7 ± 23.26	192.65 ± 28.71	181.44 ± 29.49[b]
8 week #$	187.67 ± 9.97	240.83 ± 19.84	249.13 ± 27.4	252.46 ± 12.13	232.52 ± 31.82[a]
10 week #$	139.5 ± 19.56	177.7 ± 23.88	182.69 ± 8.58	200.96 ± 9.97	175.21 ± 27.54[b]
overall	173.97 ± 34.16	196.81 ± 33.7	200.55 ± 36.41	207.19 ± 33.38	–
GSH-Px					
0 week	431.54 ± 32.9	448.73 ± 36.51	402.24 ± 44.73	451.6 ± 16.13	433.53 ± 36.69[b]
4 week	420.39 ± 27.17	461.15 ± 41.98	466.88 ± 68.15	479.62 ± 30.69	457.01 ± 46.28[b]
8 week	577.39 ± 43.67	611.47 ± 27.84	543.95 ± 51.65	589.18 ± 51.59	580.5 ± 47.3[a]
10 week #	339.82 ± 15.05	377.08 ± 64.77	430.58 ± 17.85	467.2 ± 24.38	403.67 ± 60.1[b]
overall	442.28 ± 92.83	474.61 ± 96.81	460.91 ± 70.27	496.9 ± 63.44	–
SOD					
0 week	42.78 ± 5.13	43.15 ± 1.61	42.44 ± 2.1	40.88 ± 8.73	42.31 ± 4.76[b]
4 week	44.04 ± 2.68	44.86 ± 4.5	45.08 ± 3.52	49.35 ± 2.27	45.83 ± 3.68[b]
8 week $	49.98 ± 7.22	57.74 ± 6.43	58.74 ± 8.98	58.89 ± 3.04	56.34 ± 7.16[a]
10 week	38.99 ± 4.91	44.37 ± 3.03	41.7 ± 2.29	44.56 ± 1.7	42.41 ± 3.72[b]
overall	43.95 ± 6.21	47.53 ± 7.22	46.99 ± 8.44	48.42 ± 8.2	–
MDA					
0 week	12.71 ± 3.92	13.95 ± 3.87	14.15 ± 2.8	13.99 ± 2.18	13.7 ± 2.99b
4 week	14.41 ± 1.52	13.36 ± 1.56	13.78 ± 0.68	10.05 ± 3.12	12.9 ± 2.45b
8 week $	17.51 ± 3.83	17.21 ± 2.44	16.71 ± 1.9	13.04 ± 1.11	16.12 ± 2.92a
10 week #	13.45 ± 2.62	7.55 ± 0.79	8.79 ± 0.89	8.92 ± 2.21	9.68 ± 2.83c

(Continued)

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TABLE 4 Continued

	Control	FO	VE	FOVE	overall
overall	14.52 ± 3.38	13.02 ± 4.21	13.36 ± 3.36	11.5 ± 2.96	–
ROS					
0 week	126.86 ± 25.94	130.56 ± 13.73	129.82 ± 27.3	131.02 ± 12.53	129.56 ± 18.86^{b}
4 week	123.08 ± 16.84	127.23 ± 9.42	139.05 ± 11.17	146.81 ± 12.85	134.04 ± 15.07^{b}
8 week #$	204.72 ± 21.8	176.92 ± 19.98	159.83 ± 11.32	156.97 ± 8.05	174.61 ± 24.45^{a}
10 week #$	131.39 ± 4.4	93.34 ± 6.34	78.65 ± 11.04	85.58 ± 4.21	97.24 ± 21.98^{c}
overall	146.51 ± 38.78	132.01 ± 32.96	126.84 ± 34.29	130.1 ± 29.61	–
T (nmol/L)					
0 week	15.28 ± 1.46	13.99 ± 1.22	14.54 ± 0.55	15.28 ± 1.14	14.77 ± 1.16^{b}
4 week #	15.92 ± 0.98	17.68 ± 0.97	16.66 ± 2.01	19.61 ± 1.43	17.47 ± 1.91^{b}
8 week #$	19.7 ± 0.46	22.1 ± 2.73	21.36 ± 2.12	25.33 ± 1.09	22.13 ± 2.67^{a}
10 week #	9.37 ± 1.42	14.26 ± 0.6	9.93 ± 0.18	13.99 ± 0.55	11.89 ± 2.44^{c}
overall	15.07 ± 3.95	17.01 ± 3.68	15.62 ± 4.45	18.55 ± 4.68	–
E_2 (pmol/L)					
0 week	232.06 ± 28.15	212.07 ± 30.18	238.91 ± 36.85	222.62 ± 19.8	226.41 ± 28.28^{c}
4 week #	226.59 ± 10.17	269.16 ± 12.26	217.82 ± 7.76	278.88 ± 18.09	248.11 ± 29.45^{b}
8 week #	264.78 ± 27.96	299.42 ± 54.78	292.71 ± 19.57	317.77 ± 44.5	293.67 ± 40.19^{a}
10 week #	113.64 ± 12.68	129.24 ± 22.32	116.79 ± 14.21	135.82 ± 4.78	123.87 ± 16.24^{d}
overall	209.27 ± 62.02	227.48 ± 73.43	216.56 ± 68.81	238.77 ± 74.39	–

Data shown are the mean ± SD (n = 4 replicates per treatment, one ejaculate per replicate). For each parameter, values within columns with different superscript letters differ significantly ($P < 0.05$). #Significant ($P < 0.05$) main effect of flaxseed oil (FO). $Significant ($P < 0.05$) main effect of vitamin E (VE).

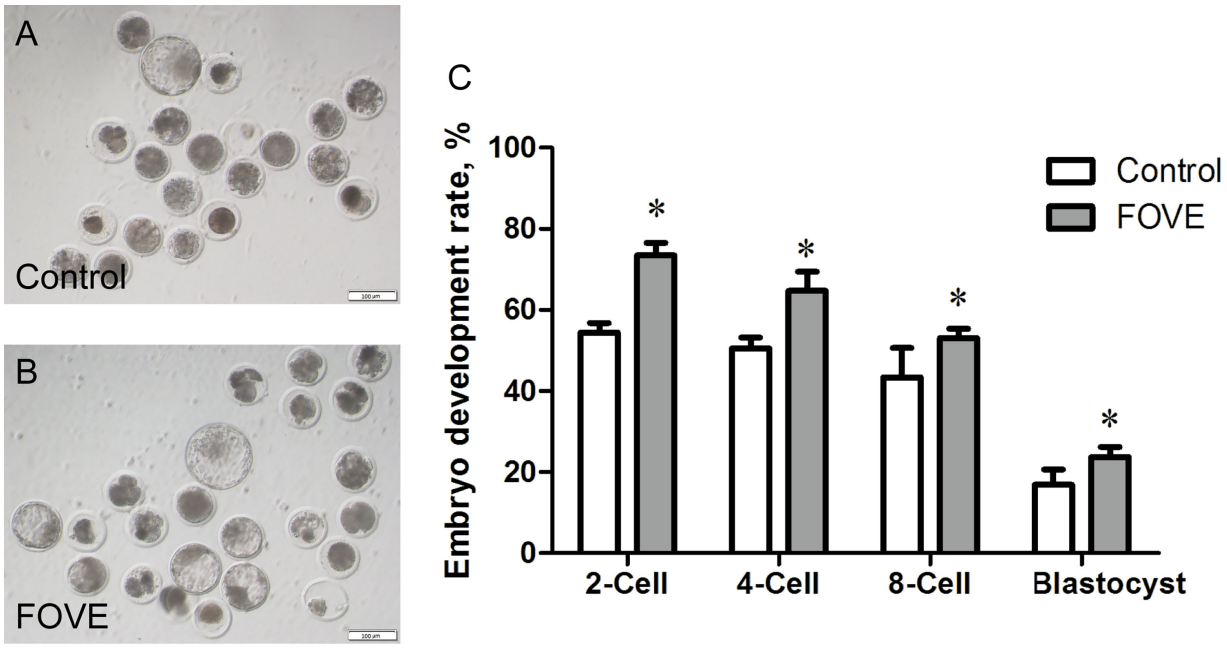

FIGURE 1
The above results show that adding FO and VE to the diet for 10 weeks can improve the motility and motion parameters of frozenthawed sperm, and also improve the antioxidant capacity, and the combined addition of FO and VE has a better effect. The control group and FOVE group samples of week 10 were used for further experiments. IVF results **(A–C)** showed that the diet supplemented with FO and VE significantly increased the twocell, four-cell, eight-cell and blastocyst rate compared with the control group ($P < 0.05$).

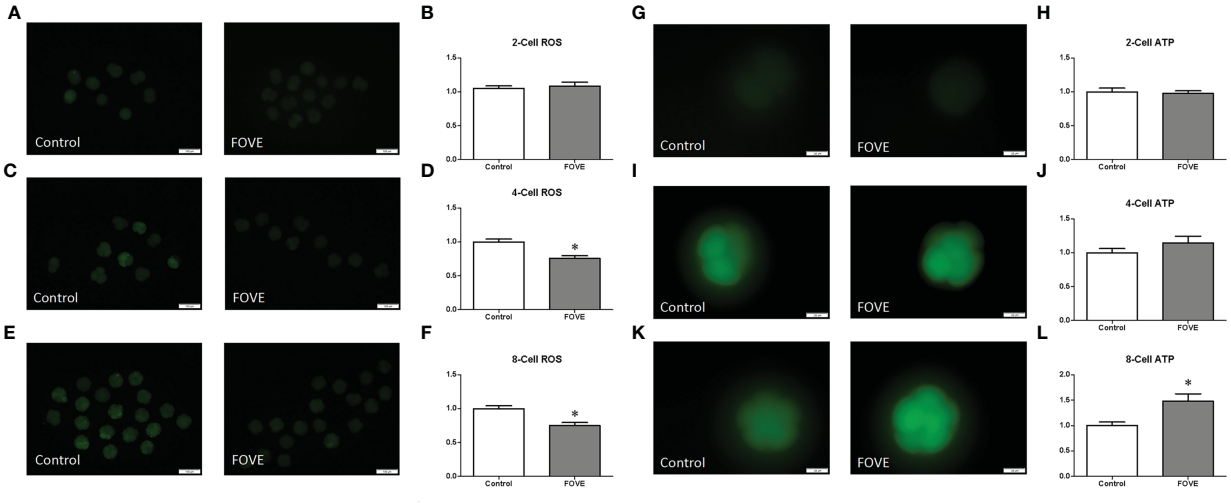

FIGURE 2
Intracellular ROS levels were measured by assessing DCFH fluorescence **(A–F)**. Quantitative analysis showed that the ROS levels of four- and eight-cell embryos were significantly decreased (P < 0.05) in the FOVE group compared with the control group **(C–F)**. There were no significant difference in ATP content between two- and four-cell shown in **(G–J)** (P > 0.05). As shown in Figures 2K, L, the ATP levels of eight-cell embryos were significantly higher in the FOVE group than in the control group (P < 0.05).

combined addition of FO and VE has a better effect. The control group and FOVE group samples of week 10 were used for further experiments. IVF results (Figure 1) showed that the diet supplemented with FO and VE significantly increased the two-cell, four-cell, eight-cell and blastocyst rate compared with the control group ($P < 0.05$).

3.4 Effects of dietary FO and VE on the ROS and ATP of bovine embryos

Intracellular ROS levels were measured by assessing DCFH fluorescence (Figures 2A–F). Quantitative analysis showed that the ROS levels of four- and eight-cell embryos were significantly decreased ($P < 0.05$) in the FOVE group compared with the control group (Figures 2C–F). As shown in Figures 2K, L, the ATP levels of eight-cell embryos were significantly higher in the FOVE group than in the control group ($P < 0.05$).

3.5 The effect of dietary FO and VE on the antioxidant status of embryo culture medium

Figure 3 shows the effect of dietary FO and VE on the antioxidant status of embryo culture medium. The activity of CAT was increased when the diet was supplemented with FO and VE ($P < 0.05$). In contrast, FO and VE supplementation resulted in decreased MDA levels in embryo culture medium, but the activity of SOD did not differ among the groups ($P > 0.05$).

3.6 The effect of dietary FO and VE on the relative levels of gene expression in blastocysts

The effects of dietary FO and VE combined on blastocyst gene expression were examined by q-PCR. The relative abundance of

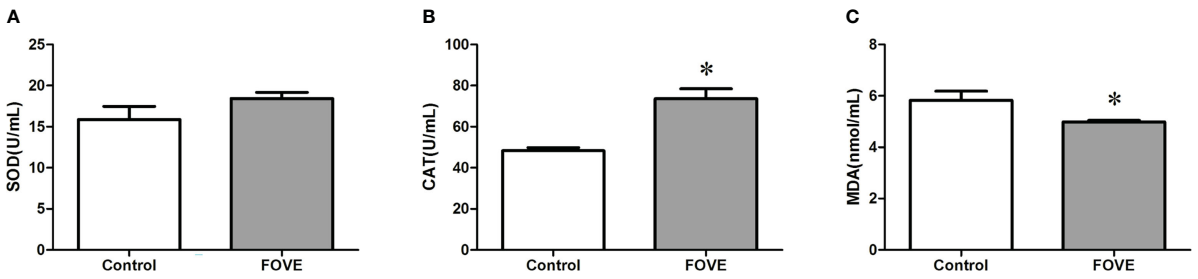

FIGURE 3
Antioxidant status of embryo culture medium. Values are shown as mean ± SD. *Significant difference compared with the control group ($P < 0.05$). **(A–C)** shows the effect of dietary FO and VE on the antioxidant status of embryo culture medium. The activity of CAT was increased when the diet was supplemented with FO and VE (P < 0.05). In contrast, FO and VE supplementation resulted in decreased MDA levels in embryo culture medium, but the activity of SOD did not differ among the groups (P > 0.05)

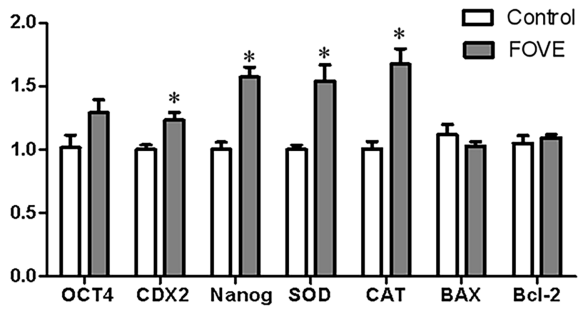

FIGURE 4
Relative levels of gene expression in blastocysts. Values are shown as mean ± SD. *Significant difference compared with the control group ($P < 0.05$).

gene expression in blastocysts is presented in Figure 4. The results showed that blastocysts from the sperm of bulls consuming a diet supplemented with FO and VE had significantly increased expression of *CDX2*, *Nanog*, *SOD* and *CAT* compared with control group ($P < 0.05$).

3.7 Metabolomic changes and pathway enrichment analysis

The above experimental results suggest that the combined addition of FO and VE in the diet may improve the IVF rate of sperm and the developmental ability of early embryos by improving antioxidant capacity. Therefore, we used metabolomics and transcriptomics to deeply explore the mechanism of improving the motion parameters of frozen-thawed sperm motility and IVF ability. Significantly differences in metabolites were identified according to the PLS-DA VIP (variable importance in the projection) > 1, fold change ≥ 1.2 and $P < 0.05$ (Figures 5A, B). We identified a total of 12,252 positive ions (pos) and 10,101 negative ions (neg). As shown in Figures 5C, D, compared with the control group, 198 and 167 differentially expressed ions were identified in the pos and neg mode, respectively. Among them, 90 and 108 were up-regulated and down-regulated, respectively, in the pos mode, and 86 and 81 were up-regulated and down-regulated, respectively, in neg mode (Figures 5E, F). The main differences in metabolites are shown in Table 5. Pathway enrichment analysis was performed to study the effect of dietary FO and VE on sperm, as shown in Figures 5G, H. The results revealed that the metabolites with altered levels of expression were mainly associated with androgen and estrogen metabolism, riboflavin metabolism, fatty acid metabolism, steroid biosynthesis and bile acid biosynthesis in positive ion mode (Figure 5G), and mitochondrial electron transport chain, glycerolipid metabolism, citric acid cycle and tyrosine metabolism in negative ion mode (Figure 5H).

3.8 KEGG and Gene ontology analysis of differentially expressed genes

The overall distribution trend of all samples was investigated by PCA, and the PCA score plots of the two groups are shown in Figure 6A. Variations were identified in the PC1 and PC2 score plots. Differentially expressed genes were chosen with the DESeq package using the following criteria: |log2| (fold change) > 3 and adjusted *P*-value < 0.01. The results showed that 1336 genes and 987 genes were up-regulated and down-regulated, respectively, in the FOVE group compared with the control group (Figures 6B, C), and the top 5 significantly up-regulated genes in the two groups are listed in Table 6. Gene ontology (GO) analysis of DEGs was categorized functionally based on GO annotation terms. Figure 6D shows the top 20 enriched GO terms. To further characterize DEGs, we performed pathway analysis using the KEGG pathway database. As shown in Figure 6E, the major enrichment pathways included cell motility, lipid metabolism, amino acid metabolism, nucleotide metabolism, and energy metabolism.

3.9 Integrated analysis of the metabolomic and transcriptomic data

To further explore the potential relationship between DEGs and metabolites, we integrated metabolomic and transcriptomic data at the pathway level. As shown in Figure 7, there were a total of 77 enriched pathways, including purine metabolism, nicotinate and nicotinamide metabolism, glycerolipid metabolism, glycerophospholipid metabolism, valine, leucine and isoleucine degradation, propanoate metabolism, fructose and mannose metabolism, tricarboxylic acid cycle (TCA cycle), phenylalanine, tyrosine and tryptophan biosynthesis and steroid biosynthesis.

4 Discussion

In this study, metabolomics and transcriptomics were used to clarify the mechanism of dietary supplementation of FO and VE on semen quality and embryonic development. FO contains up to 50% ω-3 PUFAs, especially α-linolenic acid (22), which plays important roles in the regulation of membrane properties (23). Previous studies have shown that ω-3 PUFAs in diets improved sperm morphology in the boar (15). Dietary supplementation of VE could reduce the rate of sperm deformity in goats (9). Results of the present study indicated that dietary supplementation with 24 g/kg FO and 150 mg/kg VE had positive effects on bull sperm. The positive effects may be due to an increased level of aconitic acid *via* upregulated expression of 4-aminobutyrate aminotransferase (*ABAT*) and Hydroxyacyl-CoA dehydrogenase subunit alpha

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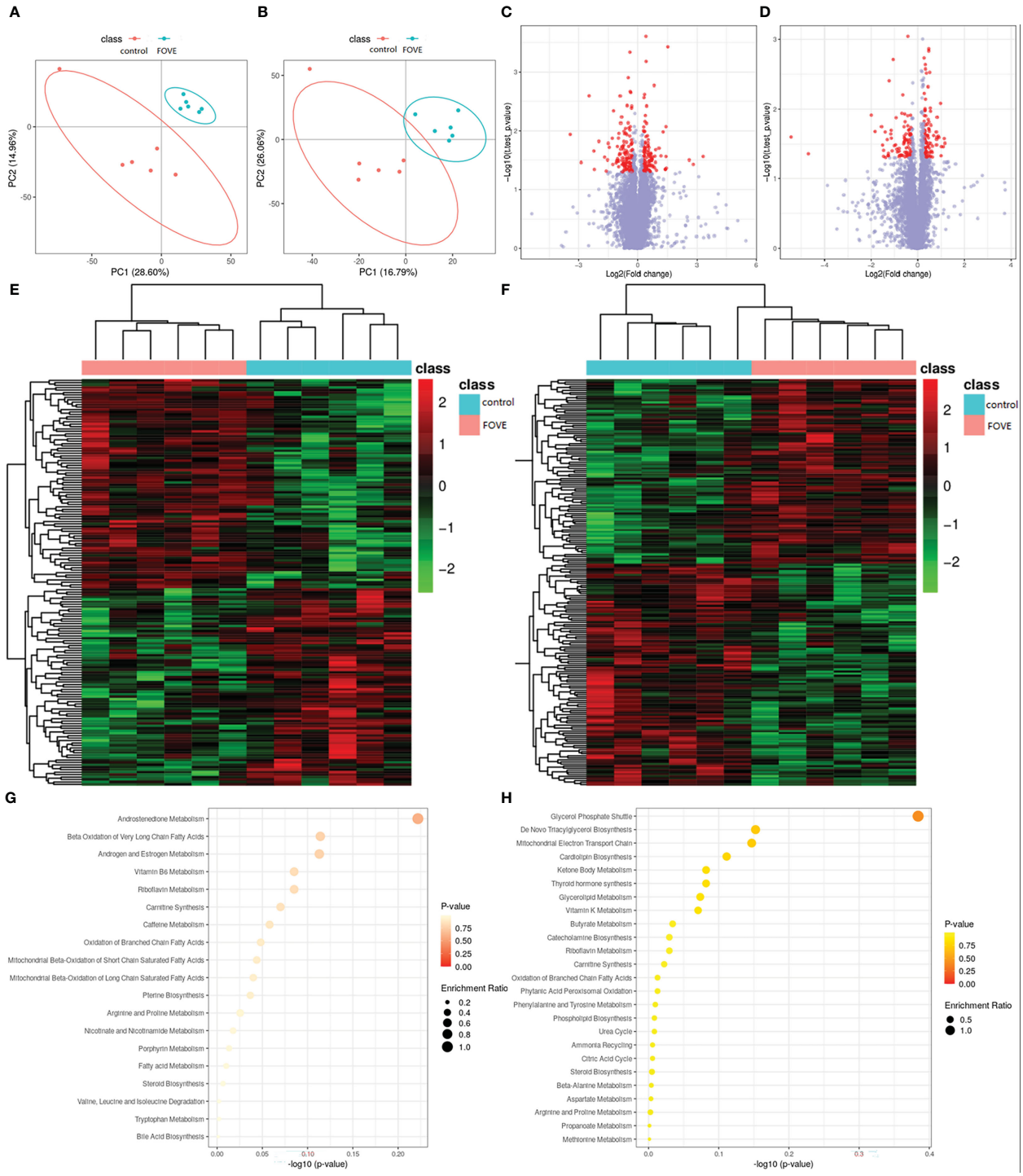

FIGURE 5
Seminal plasma metabolomic changes and pathway enrichment. Positive ion mode **(A)** and negative ion mode **(B)** for the partial least square-discriminate analysis (PLS-DA) score charts of metabolite profiling data. Analysis of the flaxseed oil + vitamin E (FOVE) group and control group based on metabolomics. Volcano figure under positive ion mode **(C)** and negative ion mode **(D)**, with the red points representing different levels of metabolites. Hierarchical clustering analysis of positive ion mode **(E)** and negative ion mode **(F)**, with red denoting increased expression and blue denoting decreased expression. The pathway analysis for positive ion mode **(G)** and negative ion mode **(H)**. Colors (varying from yellow to red) show metabolites in the data with different levels of significance. The x-axis represents the pathway impact value computed from pathway topological analysis, and the y-axis is the-log of the *P*-value obtained from pathway enrichment analysis. The pathways that were most significantly changed are characterized by both a high-log (*P*) value and high impact value (top right region).

TABLE 5 Changes in the main metabolites between the control and FOVE groups.

Compared sample	Metabolite name	KEGG ID	ratio	*P*-value	VIP
Steroid biosynthesis					
FOVE: control	Lanosterol	C01724	1.454291	0.009641	2.252628
Vitamin digestion and absorption					
FOVE: control	Riboflavin	C00255	1.341744	0.0474189	1.28584909
Tryptophan metabolism;					
FOVE: control	lactate	C22006	1.243668	0.0291399	1.34673036
Cutin, suberine and wax biosynthesis					
FOVE: control	palmitic acid	C08285	1.958944	0.008367	3.23004716
Phenylalanine, tyrosine and tryptophan biosynthesis					
FOVE: control	Protocatechuic acid	C00230	1.253955	0.00267	1.884392
Propanoate metabolism					
FOVE: control	aconitate	C21250	1.214776	0.019896	1.399021

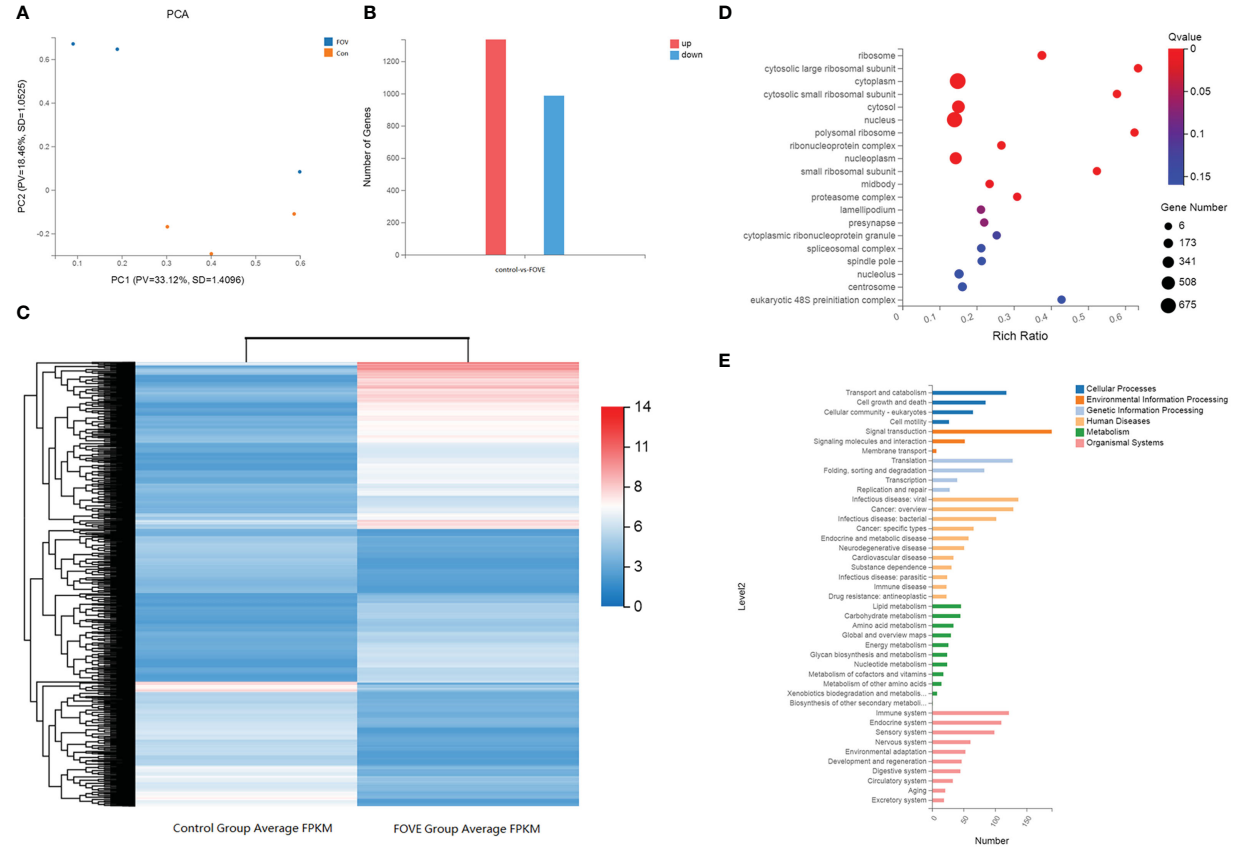

FIGURE 6
KEGG and GO analysis of differentially expressed genes. Principal component analysis (PCA) score plots of the two groups **(A)**. The number of differentially expressed genes (DEGs), red column = increased expression (n=1336) and blue column = decreased expression (n=987) **(B)**. Hierarchical clustering analysis of DEGs. Red denotes increased expression and blue denotes decreased expression **(C)**. Function analysis of differentially expressed genes (DEGs) between two treatment groups based on Gene Ontology **(D)** and KEGG pathway **(E)** analysis. D, The size of the dot designates the number of DEGs in the pathway, and the color of the dot corresponds to a different Q value (for the most enriched top 20 pathway terms). E, The ordinate is the enriched KEGG pathway term, and the abscissa is the number of DEGs for the term. Different colors are used to differentiate cellular processes, environmental information processing, genetic information processing, human diseases, metabolism and organismal systems.

TABLE 6 Information of up-regulated expresse differentially genes.

Gene. ID	Gene Name	log2 Fold Change	*P*-value	KEGG pathway
100125267	*LIPA*	4.14909	2.80E-05	Steroid biosynthesis Cholesterol metabolism
280969	*ABAT*	3.820472	2.78E-10	Propanoate metabolism
281810	*HADHA*	7.471023	5.09E-41	Propanoate metabolism Fatty acid metabolism
338074	*AOX1*	8.803598209	1.27E-260	Vitamin B6 metabolism Nicotinate and nicotinamide metabolism
518852	*EHHADH*	7.202206	1.2E-167	Propanoate metabolism Fatty acid metabolism

(*HADHA*) genes of the propionate metabolic pathway, providing energy for bull sperm and promoting the IVF rate and subsequent embryonic development.

Our results demonstrated that the diet supplemented with FO and VE increased frozen-thawed sperm motion parameters. These results are consistent with previous studies that showed dietary supplementation with FO alone increased the motility of sperm, progressive motility, vitality, membrane integrity and quality of frozen-thawed sperm (16, 24). Similarly, it has been shown that 8% FO in goat diets increased the antioxidant capacity of testicular tissue and promoted testicular development and spermatogenesis (17). ω-3 PUFAs can affect the hypothalamic-pituitary-gonadal axis, thereby promoting the secretion of important hormones related to reproduction, such as T and E_2 (25, 26). Similarly, FO promoted the secretion of T and E_2 in our study. It can be speculated that FO may promote the production of steroid hormones and then stimulate the production of sperm.

Numerous studies have shown that high concentrations of VE in the diet improved semen quantity (27, 28). As the main antioxidant of sperm, vitamin E can scavenge oxygen free radicals in sperm membrane and avoid oxidative damage to sperm (29). It has been reported that diets supplemented with VE at 200 IU/sheep/d significantly reduced MDA levels and increased the activities of SOD and GSH-Px in semen (9). Consistent with

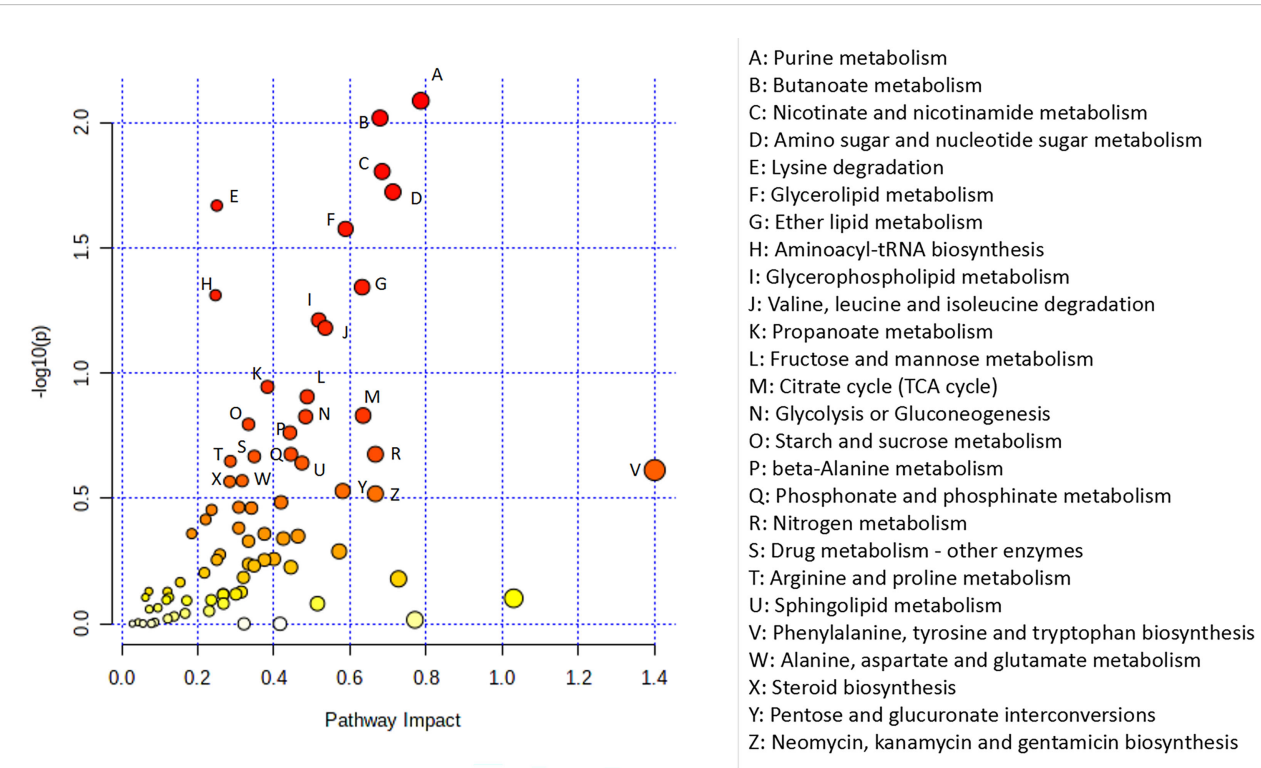

FIGURE 7
Integrated analysis of the metabolomic and transcriptomic data. Integrated analysis of the metabolomic and transcriptomic data performed with Metaboanalyst (https://www.metaboanalyst.ca/). Each point represents one metabolic pathway. The size of the dot represents a positive correlation with the metabolic pathway.

these findings, our results showed that adding VE increased frozen-thawed sperm motility and increased SOD and CAT levels and reduced MDA and ROS levels in bull seminal plasma. Taken together, it was observed in this study that VE may enhance sperm fertility through antioxidant effects (30).

It was reported that the combined addition of ω-3 PUFAs and VE may have reduced oxidation of sperm plasma membranes and maintained semen quality during cryopreservation (31). In the current study, the addition of FO and VE in the diet may have prevented sperm lipid peroxidation caused by MDA and ROS. In addition, FO and VE in the diet improved sperm motility and motion parameters, which is consistent with the results of Kargar et al., suggesting that dietary supplementation with FO and VE improved sperm motility (17). Supporting these findings, supplementation of FO and VE in the diet improved rooster semen quality and fertility potential (32), and increased the motility and membrane integrity of stallion semen (31).

Accumulating evidence has revealed that sperm motility and motion parameters are positively correlated with early embryonic development (33). Low motility sperm may affect the acrosome reaction, and the rate of fertilization and blastocyst formation (34). Sperm with low motion parameters and motility can decrease embryo cleavage rates (35). The present results suggested that dietary FO and VE improves embryo development by improving sperm motility.

During embryonic development, mitochondria provide ATP to cleavage-stage embryos by uptake and oxidation of various substrates (36). ROS is a by-product of ATP generation through oxidative phosphorylation, and cause oxidative stress and embryo damage (37). The synthesis of ROS and ATP in the cytoplasm of embryonic cells may be affected by sperm quality (38). In this study, embryos from the FOVE group showed increased ATP levels at the eight-cell stage, and decreased ROS levels at the four- and eight-cell stage, suggesting that sperm from bulls fed a diet with FO and VE may be beneficial for subsequent embryonic development.

It is reported that concentrations of ROS in spent culture media may play a critical role in the success of IVF (39). Our research showed that sperm from bulls fed a diet containing FO and VE reduced the oxidative stress of embryo medium and up-regulated the expression *CAT* and *SOD* genes in blastocysts. In addition, this sperm led to significantly increased blastocyst expression of *CDX2* and *Nanog* genes, which are important for normal embryonic development (40).

It is reported that lanosterol could affect oocyte maturation and subsequent embryonic development (41). Our results showed that a diet supplemented with FO and VE may promote the sperm fertilization rate through increased lanosterol levels, supporting previous findings that supplementation with 50 μM lanosterol during *in vitro* maturation improved preimplantation development of embryos by elevating lipid levels (42). A lack of riboflavin in the diet was reported to reduce murine sperm motility, morphology, and bioenergetic metabolism (43). Riboflavin has antioxidant properties while protecting cells from oxidative stress (44, 45). Similarly, protocatechuic acid can positively affect the reproductive function of male rats through anti-inflammatory and antioxidant effects (46–48). In the current study, we found that dietary FO and VE may enhance bull semen quality by increasing the levels of riboflavin and protocatechuic acid.

Lactate and palmitic acid provide an important energy source for sperm (49, 50). Thus, when high energy is required, sperm efficiently metabolize glycolysable substrates to yield ATP (51). The present study has shown that dietary FO and VE increased the content of lactate and palmitic acid, suggesting the sperm may utilize anaerobic glycolysis more efficiently (52). The current study also found that dietary FO and VE increased aconitase levels in sperm. Studies have shown that mitochondrial aconitase is an

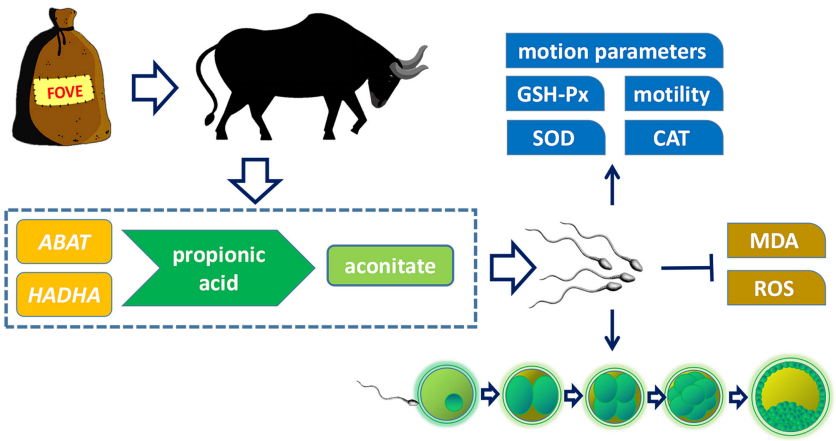

FIGURE 8
Effects of dietary supplementation of FO and VE on semen quality of bulls. The combination of FO and VE in diet can improve frozen-thawed sperm motility, motion parameters, antioxidant capacity and *in vitro* fertilization ability, which may be caused by the regulation of *ABAT* and *HADHA* genes and aconitate content in propionic acid metabolism pathway.

important regulator of the TCA cycle in asthenozoospermia, and aconitase was reduced in asthenozoospermia (53). Therefore, these results indicate that high expression of aconitase may promote the TCA cycle and improve sperm motility.

Our results showed that dietary FO and VE increased the expression of lipase A, lysosomal acid type (*LIPA*) and *ABAT* genes in sperm. It has been reported that *LIPA* and *ABAT* are involved in the TCA cycle and maintain mitochondrial membrane function (54, 55). As a key lipid metabolism enzyme, *HADHA* is associated with increased hepatic lipid accumulation when *HADHA* is inhibited, whereas lipid droplet formation is reduced when *HADHA* is overexpressed (56). Enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase (*EHHADH*) is a part of the fatty acid β-oxidation pathway (57). Our results showed that a diet supplemented with FOVE increased *EHHADH* and *HADHA* gene expression, indicating that sperm lipid metabolism may be regulated through the fatty acid pathway. Aldehyde oxidase 1 (*AOX1*) induces antioxidant defense pathways and increases H_2O_2 levels, and subsequently promoting the production of antioxidant enzymes (58). The elevated expression of *AOX1* genes in this study shows that the antioxidant capacity of sperm was increased.

Finally, we performed a combined analysis of metabolomic and transcriptomic data to identify the pathways by which dietary FO and VE affect sperm. We found that propanoate metabolism played an important role in improving sperm motility. Propionate metabolism is downstream of lipid metabolism and contributes to the tricarboxylic acid cycle and provides energy (59). A previous study showed that propanoate metabolic pathways play an important role in sperm cryopreservation (60). In addition, sperm must reserve sufficient energy to sustain motility, capacitation, and fertilization (61). Therefore, dietary FO and VE may affect sperm motility and subsequent embryonic developmental through the propionate pathway.

Based on transcriptome and metabolome analysis, as shown in Figure 8, we found that sperm from bulls fed a diet with FO and VE may regulate expression of the *ABAT* and *HADHA* genes in the propionic acid metabolism pathway to increase aconitate, thereby improving semen quality and subsequent embryo development. However, Due to the small number of bulls available for study under actual conditions of the experiment, as a large livestock animal, we cannot obtain a large number of experimental animals to carry out the experiment. In addition, an inherent limitation of this analysis was the in silico platform with results only based on bioinformation instead of direct investigation of biological mechanisms. Therefore, more method validation should be used to validate our hypothesis.

5 Conclusions

In conclusion, combined supplementation of FO and VE in bull diets could improve frozen-thawed sperm motion parameters, IVF rate and subsequent embryonic development by enhancing antioxidant capacity, which may be related to increased aconitic acid *via* the *ABAT* and *AHDHA* genes involved in the propionic acid metabolism pathway.

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 authors.

Ethics statement

The animal study was reviewed and approved by Jilin Agricultural University in P.R. China and approved by the Experimental Animal Welfare and Ethics Committee of Jilin Agricultural University. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

CY wrote the paper. KZ and XM performed the experiments. ZW and JZ did the statistical analysis. HL collected the samples and data. WL and JW edited and reviewed 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.

Publisher's note

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Association between neutrophil to lymphocyte ratio and erectile dysfunction among US males: a population-based cross-sectional study

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Objective: The purpose of the study was to investigate the relationship between neutrophil-to-lymphocyte ratio (NLR) and erectile dysfunction (ED) in adult American males using a large database.

Methods: We adopted a series of statistical analyses of the relationship between NLR indices and ED prevalence among participants in the 2001-2004 National Health and Nutrition Examination Survey (NHANES) database using the R software.

Results: The study included a total of 3012 participants, of whom 570 (18.9%) presented with ED. NLR levels were 2.13 (95% CI: 2.08,2.17) in those without ED and 2.36 (95% CI: 2.27,2.45) in those with ED. After adjusting for confounding variables, NLR levels were higher in patients with ED, (β, 1.21, 95% CI, 1.09-1.34, $P < 0.001$). In addition, a U-shaped relationship between NLR and ED was observed after controlling for all confounders. A more significant correlation (β, 1.35, 95% CI, 1.19 to 1.53, $P < 0.001$) existed to the right of the inflection point (1.52).

Conclusion: The results of the large cross-sectional study showed a statistically significant association between the occurrence of ED and NLR, a simple, inexpensive, and readily available parameter of inflammation, in US adults. Further studies are still needed in the future to validate and replicate our findings and to investigate the specific mechanisms involved.

KEYWORDS

neutrophil to lymphocyte ratio, NHANES, erectile dysfunction, cross-sectional study, inflammation

1 Introduction

Erectile dysfunction (ED) was defined as the inability to achieve or maintain an erection sufficient for satisfactory sexual intercourse, according to the International Medical Association (1). And ED can have negative impacts on both the physical and mental health of men, as well as their partners' quality of life, even if it's not life-threatening (2). A large follow-up study in Massachusetts reported a crude prevalence of ED of 26/1000 person-years (3), and it's expected that the worldwide rate of ED prevalence will increase rapidly with the rise of comorbidities associated with ED, potentially affecting about 320 million men worldwide by 2025 (4). The etiology of ED is complex, and the current research suggests that it may result from a multifactorial process involving vascular, hormonal, neurological, and anatomical factors (5). It has been reported that the presence and severe degree of ED are related to markers of inflammation and endothelial dysfunction (6). The neutrophil-to-lymphocyte ratio (NLR) has recently been recognized as a prospective biological marker of a generalized inflammatory state with the advantage of convenience and inexpensive and has been reported to have prognostic value in several diseases (7–9). However, only a limited number of studies have investigated the relationship between NLR and ED in the population, and most of them are from Asia, with inconsistent results. A relevant meta-analysis revealed that the NLR was higher in ED patients than in the healthy subjects, but only seven studies were included, and there was significant heterogeneity (10). Additional research is needed due to the slightly smaller sample size of previous studies and the limited adjustment for confounding variables. We hypothesize that there is a potential association between NLR and ED. In the study, we aimed to determine the relations between NLR and ED by using a large database, incorporating more comprehensive population data, and adjusting for confounders as much as possible. Our findings will contribute to a better understanding of the mechanisms of inflammation and exploration of valuable biomarkers.

2 Materials and methods

2.1 Study population

We obtained relevant data for this study from the National Health and Nutrition Examination Survey (NHANES) database, which is conducted by the National Center for Health Statistics (NCHS), a division of the Centers for Disease Control and Prevention (CDC). The NHANES database uses a complex, probability-based sampling design to assess the health and nutritional status of noninstitutionalized civilians in the United States through standardized interviews, physical examinations, and laboratory tests, providing information from diverse populations (11). The data have been available for research since 1999 and have been issued every two years. For this study, we collected data from the two NHANES cycles (2001-2002, 2003-2004), with more information on the data available on the NHANES website (www.cdc.gov/nchs/nhanes/).

The data sets from two NHANES research cycles (2001-2002 and 2003-2004) were selected for cross-sectional analysis, as ED and NLR index values were only available for these two cycles. From 2001 to 2004, a total of 21161 individuals participated in NHANES. Exclusion criteria were as follows: female (n=10860); missing information on ED (n=6185); age >70 years (n=747); missing information on education level (n=1); missing information on marriage (n=2); missing information on NLR index (n=109); missing information on household income (n=186); missing information on smoking (n=3); missing Alcohol information (n=5); missing BMI information (n=50); missing coronary artery disease (n=1). Finally, a total of 3012 cases were included in this study, including 570 ED patients and 2442 controls.

2.2 Data collection and definition

For the assessment of ED, participants were asked to evaluate their competence to achieve and maintain an erection sufficient to enable sexual intercourse in the 2001-2004 information collection, and the response options were "never", "sometimes", "usually", and "almost often or almost always", and we classified subjects who answered "never" or "sometimes" as individuals with ED. In the sensitivity analysis, only men who selected "never" were considered to suffer from ED (12). Based on these, we started a correlation analysis to uncover the factors associated with ED. The target variable for our primary study was NLR, which was analyzed for neutrophil and lymphocyte counting by whole blood count from a Beckman Coulter automated analyzer. Detailed analysis procedures are described in Chapter 7 of the NHANES Laboratory/Medical Technician Procedures Manual (https://wwwn.cdc.gov/nchs/nhanes/).

Covariates including age, BMI, race, marital status, education, alcohol consumption status, poverty income ratio, smoking status, history of diabetes, and history of hypertension were selected for analysis. The results of BMI were divided into three groups: BMI ≤ 25, 25 < BMI ≤ 30, and BMI > 30. Race was classified as Mexican American, Non-Hispanic White, Non-Hispanic Black, Other Hispanic, and other races. Educational attainment was divided into less than high school, high school, and high school or higher. The marital status was divided into: married/cohabiting with partner and living alone. The poverty income ratio (PIR) is an index of the household income to poverty ratio, which reflects social economic status. These guidelines are published annually by the Department of Health and Human Services (HHS) and are categorized as PIR ≤ 1.3, 1.3 < PIR ≤ 3.5, and PIR > 3.5. These covariates were considered potential confounders that may affect the relationship between NLR and ED and were included in the multivariate model.

2.3 Statistical analysis

Since NHANES performs a complex multistage sampling design for the American population, to avoid obtaining unrealistic statistical results, we applied information on the sample weights, subgroups, and substrata to all analyses of the statistics thus enabling the accurate assessment of the included population as much as possible. Weights

for the combined survey periods were obtained by dividing the weight of each 2-year period by 2 based on the analysis rules of NHANES (13). Using the survey design R package in R programming, we provided weights to characterize the demographic and clinical parameters of all participants according to the presence or absence of ED in the subject population. Means and standard errors (SE) were used for continuous variables, and frequencies and percentages were used for categorical variables. To analyze the differences between the two groups, we performed linear regression (continuous variables) and chi-square tests (categorical variables). We conducted a cointegration test to eliminate any issues and filtered out valid covariates according to guidelines (14). Further, multiple models were run to adjust for potential confounders and to compare coefficients across adjusted models. In Model 1, no variables were adjusted. Model 2 adjusted for age, marital status, race, and educational attainment. The third model adjusted for alcohol consumption status, smoking, diabetes, hypertension, cardiovascular disease (CVD), PIR, and BMI based on model 2. We further assessed the association between the NLR index and ED with smooth curve fitting (penalized spline method) and generalized additive model regression (GAM). If a non-linear relationship was observed, a dichotomous linear regression method model was used to calculate the threshold effect of NLR. In addition, we assessed multicollinearity with the Variance Inflation Factor (VIF) for all variables, and covariates were excluded if VIF was > 5, indicating cointegration problems. When a nonlinear association was found, we performed a likelihood ratio test to find the inflection point value. Most past studies on the relationship between NLR and ED have not been adjusted for clinical conditions, such as hypertension, diabetes, and cardiovascular disease. Some researchers have suggested adjusting for clinical conditions or health markers to avoid methodological inconsistencies that could affect the reproducibility of the work (15). Therefore, in sensitivity analyses, we did not exclude patients with a history of hypertension, diabetes, or CVD and were stratified by age, race, education level, BMI, diabetes, hypertension, and CVD in the final model. All analyses were performed with the R version 4.2.0 package. All significance tests were two-tailed, and the significance level was set at $P < 0.05$.

3 Results

3.1 Characteristics of participants

A total of 21,161 individuals were included in the two NHANES cycles conducted between 2001 and 2004. Following the exclusion criteria described in the Methods, 3012 participants were identified for the study, out of which 570 (18.9%) had ED. The process flow diagram for the specific selection of study participants is shown in Figure 1. The baseline characteristics of the included population and the weighted analysis of the study population characteristics for the total sample are detailed in Table 1. The levels of NLR were 2.13 (95% CI: 2.08,2.17) in those without ED and 2.36 (95% CI: 2.27,2.45) in those with ED, and the levels of NLR were higher in those with ED, p<0.001. Moreover, the ED group exhibited higher rates of age, BMI, smoking, diabetes, CVD, and hypertension, while education levels and PIR levels were significantly lower. Additionally, rates of being married or cohabiting with a partner were higher in those with ED.

3.2 The relationship between NLR and ED

The detailed relationship between NLR as a continuous variable or quartile of a categorical variable and ED is presented in Table 2. In the crude model, NLR was positively associated with severity (β,

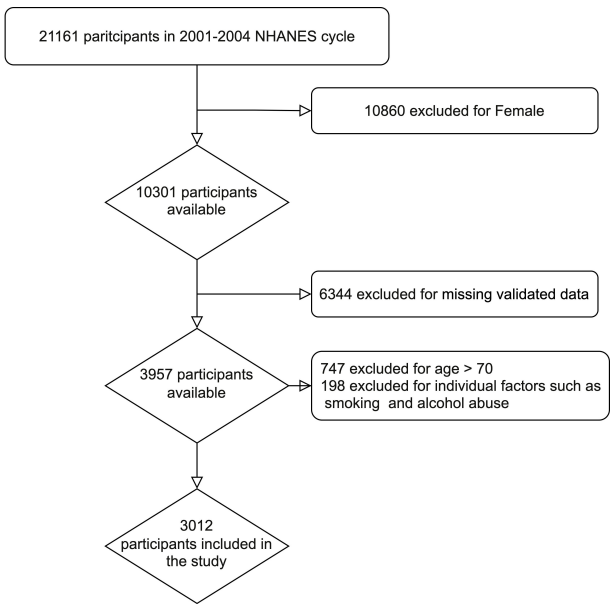

FIGURE 1
Flow chart of the study.

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TABLE 1 Baseline characteristics of study participants in NHANES 2001–2004, weighted.

Characteristics	History of erectile dysfunction (ED)		P-value
	No	Yes	
Number (n)	2442	570	
Age, year	40.26 (39.67,40.85)	53.55 (52.41,54.68)	<0.0001
BMI (kg/m^2), n (%)			<0.0001
BMI≤25	30.67 (28.67,32.74)	22.07 (17.71,27.16)	
25<BMI≤30	41.17 (38.93,43,47)	37.92 (33.43,42.64)	
BMI>30	28.15 (25.85,30.58)	40.00 (34.86,45.37)	
Race, n (%)			0.2544
Mexican American	8.28 (6.40,10.66)	8.99 (5.62,14.07)	
Other Hispanic	4.03 (2.56,6.29)	6.57 (2.82,14.58)	
Non-Hispanic White	73.56 (69.26,77.46)	70.95 (62.52,78.15)	
Non-Hispanic Black	9.75 (7.75,12.19)	9.58 (6.86,13.23)	
Other races	4.38 (3.20,5.96)	3.91 (2.21,6.82)	
Educational level, n (%)			<0.0001
Below high school	13.58 (12.25,15.03)	27.87 (22.44,34.03)	
High school	27.92 (25.49,30.50)	23.30 (19.35,27.79)	
Above high school	58.49 (55.80,61.14)	48.83 (43.41,54.27)	
Marital status, n (%)			<0.0001
Married or living with a partner	68.86 (65.79,71.77)	79.46 (75.38,83.03)	
Living alone	31.14 (28.23,34.21)	20.54 (16.97,24.64)	
PIR, n (%)			0.0053
PIR≤1.3	16.01 (13.97,18.28)	20.62 (16.11,26.00)	
1.3<PIR≤3.5	33.52 (30.89,36.25)	36.40 (31.79,41.27)	
PIR>3.5	50.47 (47.04,53.90)	42.99 (37.69,48.46)	
Alcohol intake, n (%)			0.8229
No	6.96 (4.13,11.49)	6.66 (4.11,10.61)	
Yes	93.05 (88.51,95.88)	93.34 (89.39,95.89)	
Smoking, n (%)			<0.0001
No	45.54 (42.44,48.67)	30.53 (26.33,35.07)	
Yes	54.46 (51.33,57.57)	69.47 (64.93,73.67)	
History of diabetes, n (%)			<0.0001
No	94.61 (93.45,95.57)	71.06 (66.87,74.92)	
Yes	5.39 (4.43,6.55)	28.94 (25.08,33.13)	
History of CVD, n (%)			
No	95.71 (94.62,96.49)	81.43 (76.10,85.79)	<0.0001
Yes	4.29 (3.41,5.39)	18.57 (14.21,23.90)	
History of hypertension, n (%)			

(Continued)

TABLE 1 Continued

Characteristics	History of erectile dysfunction (ED)		P-value
	No	Yes	
No	71.28 (68.80,73.63)	44.18 (38.86,49.63)	<0.0001
Yes	28.72 (26.37,31.20)	55.83 (50.37,61.14)	
NLR	2.13 (2.08,2.17)	2.36 (2.27,2.45)	0.0003

BMI, body mass index; PIR, poverty income ratio; CVD, cardiovascular disease; NLR, neutrophil to lymphocyte ratio.
For continuous variables: survey-weighted mean (95% CI), P-value was by survey-weighted linear regression.
For categorical variables: survey-weighted percentage (95% CI), P-value was by survey-weighted Chi-square test.

1.26, 95% CI, 1.16-1.38, P<0.001). After adjusting for age, race, education, and marital status (Model I), the results did not change significantly (β, 1.214, 95% CI, 1.10,1.35, P < 0.001). Even after adjusting for all covariates (Model III), a significant association between NLR and ED was still observed (β, 1.21, 95% CI, 1.09-1.34, P < 0.001). When NLR was considered as a categorical variable (quartiles), in the crude model, only the population in the Q4 (>2.57) interval was statistically significant (β, 1.50, 95% CI, 1.17-1.93, p=0.001) compared to Q1 (<1.47), while Q2 (1.47-1.94) and Q3 (1.95-2.56) were not statistically significant. In Model II and in the fully adjusted model, Q2, Q3, and Q4 were not statistically significant compared to Q1. In addition, a U-shaped relationship between NLR and ED was observed after adjusting for all covariates (Figure 2). With the two-piecewise linear regression model, we found an inflection point of 1.52 (Table 3). Although on both sides of the inflection point, there is a positive correlation, the correlation is obviously higher on the right side (β, 1.35, 95% CI, 1.19 to 1.53, P < 0.001) than on the left side (β, 0.46, 95% CI, 0.26 to 0.81, P < 0.01).

3.3 Subgroup analysis

Further analyses of subgroups were performed according to various confounding factors, as detailed in Table 4, showing that age >50 years (OR = 1.34, 95% CI: 1.17, 1.54), Mexican American (OR = 1.34, 95% CI: 1.07, 1.69) and Non-Hispanic White (OR = 1.27, 95% CI: 1.09, 1.47), Below high school (OR = 1.26, 95% CI: 1.05, 1.50) and High school (OR = 1.32, 95% CI: 1.05, 1.66), and BMI between 25 and 30 (OR = 1.27, 95% CI: 1.08, 1.50) subgroups were at higher risk of ED. Furthermore, all subgroups analyzed were examined for interaction, and no statistically significant association was found (P > 0.05 for the interaction).

4 Discussion

In this large cross-sectional study, we investigated the relationship between ED and NLR levels in American adult males using the NHANES database. After adjusting for appropriate skewed variables, we found a significant association between high levels of NLR and a higher prevalence of ED. Furthermore, we can still observe a clearly existing association after analysis by subgroups. So far as we know, this would be the first research to be performed examining the relationship between the NLR index and ED in a large population through the NHANES database.

With the increasing prevalence of ED, the erectile function of men has received increasing concerns. According to large-scale surveys, older men with ED show a higher frequency of comorbidities with diseases or conditions such as CVD, diabetes, obesity, lower urinary symptoms, and which have been considered risk factors for ED (16, 17). Our study revealed a significantly higher prevalence of CVD in the ED group compared to the control group. And endothelial damage plays a substantial contribution in the development of ED and CVD (18–20). Several studies have

TABLE 2 Multivariable logistic regression analyses for NLR and ED, weighted.

Exposure	Crude Model		Adjusted Model 1		Adjusted Model 2	
	OR (95%CI)	P value	OR (95%CI)	P value	OR (95%CI)	P value
NLR (continuous)	1.26 (1.16,1.38)	<0.0001	1.214 (1.10,1.35)	0.0002	1.21 (1.09,1.34)	0.0005
NLR (quartile)						
Q1 (<1.47)	1.0		1.0		1.0	
Q2 (1.47-1.94)	0.86 (0.65.1.13)	0.27	0.77 (0.57,1.04)	0.09	0.74 (0.54,1.02)	0.06
Q3 (1.95-2.56)	1.02 (0.78,1.32)	0.91	0.89 (0.66,1.21)	0.46	0.84 (0.62.1.15)	0.28
Q4 (≥2.57)	1.50 (1.17,1.93)	0.001	1.29 (0.96,1.72)	0.09	1.26 (0.94,1.70)	0.12
P for trend	<0.001		0.03		0.05	

NLR, neutrophil to lymphocyte ratio; ED, erectile dysfunction.
Crude Model: no covariates were adjusted.
Model 2: age, race, education and marital status were adjusted.
Model 3: Model 2+alcohol use, smoking, diabetes, hypertension, CVD, PIR and BMI were adjusted.

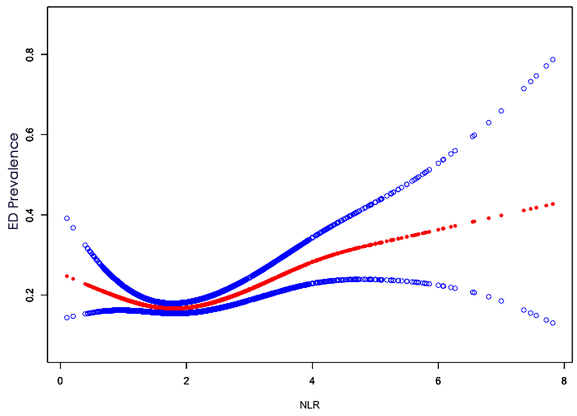

FIGURE 2
Non-linear relationship plot between NLR and ED.

suggested that the pathogenesis and severe degree of ED is related to an increase in inflammatory markers, and that lower-grade subclinical inflammation may affect endothelial function and lead to thrombosis. It was reported that there was an increased formation of inflammatory mediators (interleukin (IL)-1β, TNF-α, IL-6, CRP, IL-10), markers, and endokines in patients with ED (21, 22). NLR is a novel inflammatory marker that has drawn the attention of many scholars. Demirkol et al. demonstrated that NLR levels were elevated significantly in patients suffering from cardiac syndrome and CAD. Moreover, they proved a statistically significant association between carotid intima-media thickness and NLR (23). Sambel et al. suggested that the NLR is associated with the diagnosis of ED and that the index is available easily without additional charges (24). Based on these foundations, we aimed to study the relationship between ED and NLR in a sizeable group of American adult men. Our findings revealed that the NLR levels in the subjects group were significantly higher than those in the control group, and there was a significant positive correlation between NLR at more than 1.52 (inflection point) and ED.

Previous studies have demonstrated a range of associations between erectile dysfunction (ED) and various indicators, such as age, BMI, smoking frequency, hypertension, diabetes, CVD, and some inflammatory indices like leukocytes and CRP (25, 26). Recently, the NLR index, derived from routine blood neutrophils and lymphocytes, has emerged as an intuitive and reliable predictor of inflammation levels that contributes to clinical decision-making (12). In our study, age, BMI, smoking prevalence, diabetes, CVD, and hypertension factors were observed to be correlated with an increased prevalence of ED by subgroup analysis. Besides, Mexican-American, non-Hispanic white, low-economic level patients and low-education (below high school and high school) subgroups were found to be at higher risk for ED. It has been previously found that Mexican-American men have a higher prevalence of ED (27), and a correlation between low-economic status and the risk of ED occurrence has also been proposed (28). Similar results have been reported from the NHSLS data, where they first observed an association between income in and ED in the NHSLS sample. The lower the education level, the higher the probability of ED, although the association was not statistically significant. However, such an association was not adjusted for the comorbidities and lifestyle risk factors (29). A more comprehensive study reported an association between ED and education level and occupation, and after adjusting for all risk factors, only occupation had a statistically significant association with ED, which was a higher risk of ED in blue-collar men compared to white-collar men (30), unfortunately, they did not include income as a variable. Results from the MARSH research similarly indicated that higher levels of education were associated with lower odds of ED (31).

In addition to the well-established association between ED and traditional cardiovascular risk factors such as obesity, hypertension, smoking, and diabetes, many authors have found a close link between ED and factors related to CVD (25, 32). This suggests that ED may serve as an early warning sign for CVD. And these risk factors can lead to endothelial dysfunction and eventually to atherosclerosis. The degree of impact from atherosclerosis is similar for all vessels, but the appearance of symptoms varies

TABLE 3 Threshold effect analysis for NLR and ED.

Outcome	β (95%CI)	P value
Fitting model by standard linear regression	1.21 (1.09,1.34)	0.0005
Fitting model by two-piecewise linear regression Inflection point		
<1.52	0.46 (0.26,0.81)	0.0069
≥1.52	1.35 (1.19,1.53)	<0.0001
P for log likelihood ratio test		<0.0001

TABLE 4 Subgroup analysis for NLR and ED, weighted.

Characteristics	Crude model	Adjusted Model 1	Adjusted Model 2	P for interaction
	OR (95%CI)	OR (95%CI)	OR (95%CI)	
Age				0.049
Age≤50y	1.07 (0.89,1.28)	1.07 (0.89,1.29)	1.05 (0.87,1.27)	
Age>50y	1.29 (1.14,1.46)	1.36 (1.19,1.54)	1.34 (1.17,1.53)	
Race				0.11
Mexican American	1.52 (1.25,1.85)	1.37 (1.11,1.69)	1.34 (1.07,1.69)	
Other Hispanic	0.90 (0.56,1.44)	0.87 (0.52-1.44)	0.69 (0.40,1.20)	
Non-Hispanic White	1.35 (1.19,1.53)	1.27 (1.10,1.47)	1.27 (1.09,1.47)	
Non-Hispanic Black	1.09 (0.87,1.36)	1.09 (0.84,1.42)	1.08 (0.83,1.42)	
Other races	0.80 (0.45,1.42)	0.78 (0.42,1.45)	0.82 (0.43,1.56)	
Educational level, n (%)				0.43
Below high school	1.28 (1.09,1.50)	1.21 (1.02,1.44)	1.26 (1.05,1.50)	
High school	1.35 (1.12,1.64)	1.33 (1.07,1.66)	1.32 (1.05,1.66)	
Above high school	1.25 (1.09,1.43)	1.16 (0.99,1.37)	1.11 (0.94,1.32)	
BMI				0.68
BMI≤25	1.19 (1.00,1.41)	1.14 (0.94,1.40)	1.16 (0.96,1.41)	
25<BMI≤30	1.33 (1.15,1.54)	1.24 (1.05,1.46)	1.27 (1.08,1.50)	
BMI>30	1.29 (1.10,1.51)	1.21 (1.01,1.47)	1.15 (0.95,1.40)	
History of diabetes				0.49
No	1.28 (1.15,1.42)	1.23 (1.09,1.38)	1.22 (1.08,1.38)	
Yes	1.19 (0.97,1.47)	1.11 (0.88,1.40)	1.12 (0.89,1.40)	
History of Hypertension				0.53
No	1.22 (1.07,1.40)	1.17 (1.01,1.35)	1.17 (1.01,1.36)	
Yes	1.27 (1.12,1.45)	1.25 (1.08,1.46)	1.25 (1.07,1.46)	
History of CVD				0.15
No	1.26 (1.14,1.39)	1.19 (1.07,1.33)	1.19 (1.06,1.33)	
Yes	1.27 (0.96,1.67)	1.52 (1.08,2.13)	1.55 (1.09,2.20)	

BMI, body mass index; PIR, poverty income ratio; CVD, cardiovascular disease; NLR, neutrophil to lymphocyte ratio.
The subgroup analysis was stratified by age, race, educational level, BMI, diabetes, Hypertension, and CVD, not adjusted for the stratification variable itself.
Crude Model: no covariates were adjusted.
Model 2: age, race, education and marital status were adjusted.
Model 3: Model 2+alcohol use, smoking, diabetes, hypertension, CVD, PIR and BMI were adjusted.

depending on the diameter of the affected artery (33–35). Since the penile arteries have a smaller diameter (1-2 mm) compared to coronary arteries (3-4 mm), the same extent of endothelial dysfunction and atherosclerosis is more likely to result in a significant decrease in blood flow to the penile tissue at an early stage (33, 35). It has been suggested that the development of atherosclerosis is an active process of inflammation instead of passive damage to blood vessels resulting from lipid infiltration (36, 37). Several clinical studies have shown that inflammation plays a key role in the development and progression of atherosclerosis and can even transform stable atherosclerotic lesions into unstable plaques (37). Besides, subclinical inflammation at a lower level may impair endothelial function and trigger thrombotic events. Therefore, inflammation likely contributes significantly to the progression of ED. There are many researches have investigated the role of NLR in the development of CVD. Considering NLR as a novel marker of inflammation levels, Kalay et al. (38) suggested that NLR levels are elevated markedly in patients with atherosclerosis, and can serve as a biomarker for the development of atherosclerosis.

The normal vascular endothelium is typically resistant to inflammatory properties; however, under conditions of inflammation and increased oxidative stress, the endothelial function can be impaired (39). Moreover, it has been demonstrated that inflammatory stimulation may cause acute or

chronic damage to arterial function to some extent (40–43). Notably, plasma levels of C-reactive protein (CRP) were found to be statistically higher in patients with ED who were matched with age and coronary risk scores compared to subjects without ED (21). Besides, in men with ED without clinically significant CVD, CRP levels correlated significantly with the severity of disease of the penile arteries as assessed by penile Doppler ultrasonography (44). In a primary research, increasing levels of fibrinogen were found in ED patients when compared to men with a normal erectile function (45). Some studies have also found that ED is associated with an increased state of inflammation in males presenting with obesity syndrome or metabolic disorders (46, 47). However, it should be emphasized that while the findings of the aforementioned observational and cross-sectional studies are important, they do not necessarily prove a causal relationship. While the penile vessels can be targets of extensive inflammation originating elsewhere, the organ itself may contribute to the general development of inflammation. The male's corpus cavernosum acts as a paracrine system for the production of angiotensin II (48), and studies have shown that deletion polymorphisms in the gene encoding angiotensin-converting enzyme are more common in men with organic ED (49). Angiotensin II contributes to inflammation in blood vessels by causing oxidative stress and modulating the distribution of inflammatory mediators such as IL-6 (50). It also enhances the expression of adhesion molecules and increases the infiltration of monocytes/macrophages into the vascular wall (51). Although many studies have indicated a relationship between ED and inflammation, the relationship between the two is complex, and the specific causal and pathological mechanisms remain to be further explored.

NLR is a simple, inexpensive, and accessible inflammatory parameter with high sensitivity and low specificity. It can detect dynamic changes in NLR levels before clinical manifestations occur, providing clinicians with early warning signs of an ongoing pathological process. NLR is a novel marker of cellular immune activation and a validated indicator of systemic inflammation, which can open up a new dimension in clinical medicine (52). Based on the relationship between ED and inflammation, we found an association between NLR levels and ED in our study as well, which means that NLR may also be applied in the initial evaluation of ED patients.

However, there are several limitations to our study. First, it is not permissible to draw causal inferences due to the design of the cross-section. Besides, the NLR data came from only an individual blood test and it would be more accurate to evaluate the chronic inflammatory status of the subjects by repeating the test multiple times. Additionally, the findings of the study were acquired in an American population and cannot be generalized to other races. Larger studies in multiracial populations may be more helpful in the future. In the meantime, there are several strengths of the study. First, it is based on a large-scale sample size with a complex survey design that provides a good overview of the US population. Next, we included other confounding factors such as age, cardiovascular history, and economic status that have not been adjusted for concurrently in earlier studies. Finally, the large sample size allowed us to conduct subgroup analyses without significantly reducing statistical power.

In conclusion, the results of this large cross-sectional study suggest a significant association between high levels of NLR and ED in US adults. We were able to observe a clear correlation between the two after subgroup analysis. The positive association between NLR and ED was more apparent when NLR was higher than 1.52. In the future, more research is still needed to verify and replicate our findings and examine the specific mechanisms.

Data availability statement

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

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. 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

Data analysis and manuscript writing: XF, YM, and LC; Study design and statistical advice: XF, YM, XW, LC, and RX; Manuscript editing: XF, YM, XW, LC, and RX; Validation and review: LC, and RX; Quality control: XF. All authors contributed to the article and approved the submitted version.

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Conflict of interest

YM was employed by Department of Urology, Jiangyin People's Hospital of Jiangsu Province, Jiangyin, China.

The remaining 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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Influence of body mass index and waist–hip ratio on male semen parameters in infertile men in the real world: a retrospective study

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Background: It is suggested that body mass index (BMI) can affect male semen quality; however, the results remain controversial. In addition, most studies have focused on the effect of obesity on semen quality. Evidence on the relationship of underweight or waist-hip ratio (WHR) with semen quality is rare. This study aimed to assess the association of BMI and WHR with semen quality.

Methods: Data, including BMI and WHR, was collected from 715.00 men who underwent a fertility evaluation. BMI (kg/m^2) was categorized as <18.50 (underweight), 18.50–24.90 (normal), 25.00–27.90 (overweight), and ≥28.00 (obese) kg/m^2 for analysis. WHR was categorized as <0.81 (normal) and ≥0.81 (high). Semen volume, sperm concentration, progressive motility, and total motile sperm count were detected by experienced clinical technicians.

Results: Spearman's correlation showed that BMI was weakly associated with sperm progressive motility (r = 0.076, $P < 0.05$), while WHR showed no relationship with semen parameters. The azoospermia rate was significantly higher (33.33% vs. 2.10%, $P < 0.001$) and the sperm concentration was lower ($P < 0.05$) in the underweight group. The nonlinear correlation analysis showed that BMI was negatively associated with sperm concentration while BMI was more than 22.40 kg/m^2 ($P < 0.05$), while WHR was negatively related to sperm progressive motility within 0.82 to 0.89 ($P < 0.05$). Furthermore, the multivariate logistic analysis showed that follicular stimulating hormone (FSH) was an independent risk factor for normal sperm concentration (odds ratio [OR]: 0.791, $P = 0.001$) and morphology (OR: 0.821, $P = 0.002$), BMI was an independent risk factor for normal sperm progressive motility, and testosterone was an independent risk factor for sperm morphology (OR: 0.908, $P = 0.023$).

Conclusion: BMI and WHR were significantly associated with semen parameters, while BMI was an independent risk factor for normal sperm progressive motility. Reproductive hormones, including FSH and testosterone, had a significant influence on sperm concentration and sperm morphology.

KEYWORDS

body mass index, waist-hip ratio, semen parameters, testosterone, follicular stimulating hormone

1 Introduction

Infertility is a global clinical concern, affecting 10.00–15.00% of reproductive-age couples. It is believed that 40% of cases are due to male factors (1–3). Poor semen quality is the major condition leading to male infertility. Although studies have reported several risk factors—including environmental pollutants, Mumps virus infection, and alcohol intake—that may be related to decreased semen quality, the underlying causes are still uncertain (4–6).

Recently, an increasing number of studies have explored the relationship between abnormal body mass index (BMI) and semen quality; however, the results have remained controversial. For example, Michael et al. (7) suggested that an increased BMI and waist circumference (WC) were associated with a reduction in ejaculate volume and the total sperm count, but no relationship was found between BMI and sperm concentration, sperm motility, sperm vitality, sperm morphology, or the DNA fragmentation index in the United States. Wang et al. (8) reported that an increased BMI was linked with a lower total sperm number and sperm concentration in northern China. Meanwhile, another large single-center clinical study by Ma et al. found that being underweight or overweight were both factors associated with a decreased total motile sperm count (9). On the right hand, the researchers found that being overweight was related with a reduction in semen volume and total sperm number, while no correlation with sperm concentration was observed, which was consistent with the study by Michael et al. (7, 9). In addition, Lu et al. (10) found that BMI, the waist–hip ratio (WHR), and WC cannot predict male semen quality; however, semen quality was significantly related to levels of follicular stimulating hormone (FSH) and luteinizing hormone (LH).

Taken together, the evidence on the relationship between body size (BMI or WHR) and semen quality is limited and inconclusive. In the present study, we performed a retrospective study of 715 healthy sperm donors to assess the association of BMI and WHR with semen quality.

2 Methods

2.1 Study design

A total of 715, male partners of infertile couples who could not achieve pregnancy after 12, consecutive months without contraception at the Reproductive and Genetic Hospital, Division of Life Sciences and Medicine, The First Affiliated Hospital of USTC, University of Science and Technology of China, were recruited for our study. The experimental study protocol was administrated by the Research Ethics Committee of The First Affiliated Hospital of USTC, University of Science and Technology of China, and informed consent was obtained from all participants. Semen samples from the patients with azoospermia were analyzed at least twice at an interval of 3 weeks according to the World Health Organization (WHO) Laboratory Manual for the Examination and Processing of Human Semen (5th edition). Participants were divided into four groups by different BMI values: <18.50 (underweight), 18.50–24.90 (normal), 25.00–27.90 (overweight), and ≥28.00 (obese) kg/m2 (11).WHR was categorized into two groups: <0.81 (normal) and ≥0.81 (high) (12). The patients with a history of cryptorchidism, varicocoele or testicular trauma, administration of hormones, genital infections, or other diseases during the previous 3.00 months were excluded.

2.2 Semen and hormone analysis

The semen volume was detected by the weighing method according to the WHO guidelines (2010). Computer-assisted sperm analysis was used to measure sperm concentration, progressive motility, and total motility (SAS, Beijing, China). Sperm morphology was determined through Diff-Quick staining (Anke Biotechnology, Hefei, China). The result of semen analysis including oligospermia, asthenospermia and teratospermia were undertaken according to the WHO Semen Manual, 5th edition. In detail, oligospermia was defined as the sperm concentration <15.00 * 10^6/ml, asthenospermia as P R< 32.00%, teratospermia as normal morphology of spermatozoa < 4.00% while azoospermia was defined as the absence of spermatozoa in the semen. Blood samples were obtained at 8–11 a.m. and were centrifuged for 10 min at 1800 g. The levels of testosterone, LH, and FSH were determined by radioimmunoassay (Beckman Coulter, Brea, USA).

2.3 Statistical analysis

All data were evaluated for the normal distribution by the Kolmogorov–Smirnov test. The variables departing from the normal distribution were summarized as medians and interquartile intervals. Correlations between BMI, WHR, and semen parameters were analyzed by Spearman's correlation coefficient, as appropriate. A one-way ANOVA or Kolmogorov–Smirnov test was used to evaluate the differences among the groups. Univariate and multivariate logistic analysis was performed to seek the independent risk factors for semen parameters. Statistical analyses were performed using IBM SPSS 26 for windows and P value < 0.05 was considered statistically significant. The non-linear relationship between BMI, WHR, and semen quality were analyzed by the restricted cubic spline.

3 Results

3.1 Characteristics of studied men and correlation analysis

The basic data of the participants are shown in Table 1. BMI, WHR, and semen parameters were non-normally distributed,

TABLE 1 Characteristics of men in the study population.

Variables	Mean ± SD or Median (25%-75% quartiles)	Total N
Age (years)	29 (26-31)	715
BMI (kg/m^2)	23.63 (21.88-25.61)	715
WHR	0.85 (0.81-0.89)	715
Time of abstinence (days)	4 (3-6)	715
Semen volume (ml)	3.20 (2.50-4.00)	715
Sperm concentration (×10^6/ml)	54.08 (29.47-93.68)	715
Total sperm count (×10^6/ml)	176.51 (89.50-296.67)	715
Progressive motility (a+b, %)	31.33 (21.70-39.35)	715
Normal morphology rate (%)	5.33 (4.07-6.33)	715
TMSC (×10^6)	2.56 (0.99-5.32)	715
Acrosome integrity (%)	56.62 (50.46-60.62)	715
Anti-sperm antibody	92 (12.87%)	715
FSH (IU/l)	4.82 (3.66-5.94)	170
T (nmol/l)	14.12 (11.61-17.83)	151
LH (IU/l)	4.64 (3.28-6.36)	170
Alcohol	444 (62.10%)	715
Smoking	316 (44.20%)	715

BMI, Body Mass Index; WHR, Waist-Hip Ratio; TMSC, Total Motile Sperm Count; FSH: Follicular Stimulating Hormone; LH, Luteinizing Hormone; T, Testosterone.

which were presented as medians and interquartile intervals. The correlations of BMI and WHR with semen parameters are shown in Table 2; a weak positive correlation was found between BMI and sperm progressive motility while both BMI and WHR were negatively correlated with serum testosterone. Other semen parameters, such as total motile sperm count (TMSC) and acrosome integrity, were not related to BMI or WHR.

3.2 Comparison of semen parameters in different groups divided by BMI and WHR

Firstly, the prevalence of oligospermia, asthenospermia, and teratospermia in each group was compared by the chi-square test. There was a reduction of the normal sperm concentration rate in the underweight group compared with other groups (Figure 1A). Interestingly, the prevalence of asthenospermia was higher in the normal BMI group and a higher rate of teratospermia was found in the normal WHR group (Figures 1B, F). However, no difference was found in the rate of teratospermia in different BMI groups while both the rate of oligospermia and asthenospermia showed no statistically significant differences in the different WHR groups (Figures 1C–E). Furthermore, the Kolmogorov–Smirnov test was used to evaluate the differences among each group. The sperm concentration in the underweight group was significantly lower than the normal BMI group and overweight group. No difference was found in any other group (Figure 2A). Furthermore, the level of serum testosterone did not show a consistent change; serum testosterone in the overweight group and obese group were significantly higher than in the normal BMI group (Figure 2B). In addition, we found that the BMI level in the azoospermia group was lower compared with the oligospermia group and normal sperm concentration group (Figure 2C). The BMI was lower in the asthenospermia group than the normal sperm motility group (Figure 2D).

3.3 Restricted cubic spline analysis of the nonlinear correlation of BMI and WHR with sperm concentration, sperm morphology, and sperm progressive motility

The nonlinear correlation between BMI and semen quality parameters was expressed in Figures 3A–C, while Figures 3D–F shows the nonlinear correlation between WHR and semen quality. The result indicated that when the BMI was lower

TABLE 2 Correlations of BMI and WHR with semen parameters, serum hormones.

Variables	BMI (kg/m2)	*P*	WHR	*P*
Sperm concentration (×106/ml)	-0.035	0.347	-0.021	0.576
Total sperm count (×106/ml)	-0.025	0.497	-0.028	0.449
Semen volume (ml)	0.019	0.605	-0.025	0.511
Progressive motility (a+b, %)	0.076	**0.042**	0.036	0.340
Normal forms (%)	0.044	0.241	0.030	0.428
TMSC (*10^6)	0.016	0.670	0.010	0.794
Acrosome integrity (%)	0.042	0.257	0.059	0.116
Anti-sperm antibody	-0.062	0.095	-0.027	0.477
FSH (IU/l)	-0.073	0.346	-0.149	0.052
T (nmol/l)	-0.469	**<0.001**	-0.484	**<0.001**
LH (IU/l)	-0.046	0.555	-0.170	0.027

TMSC, Total Motile Sperm Count; FSH, Follicular Stimulating Hormone; LH, Luteinizing Hormone; T, Testosterone. Statistically significant data were represented in bold.

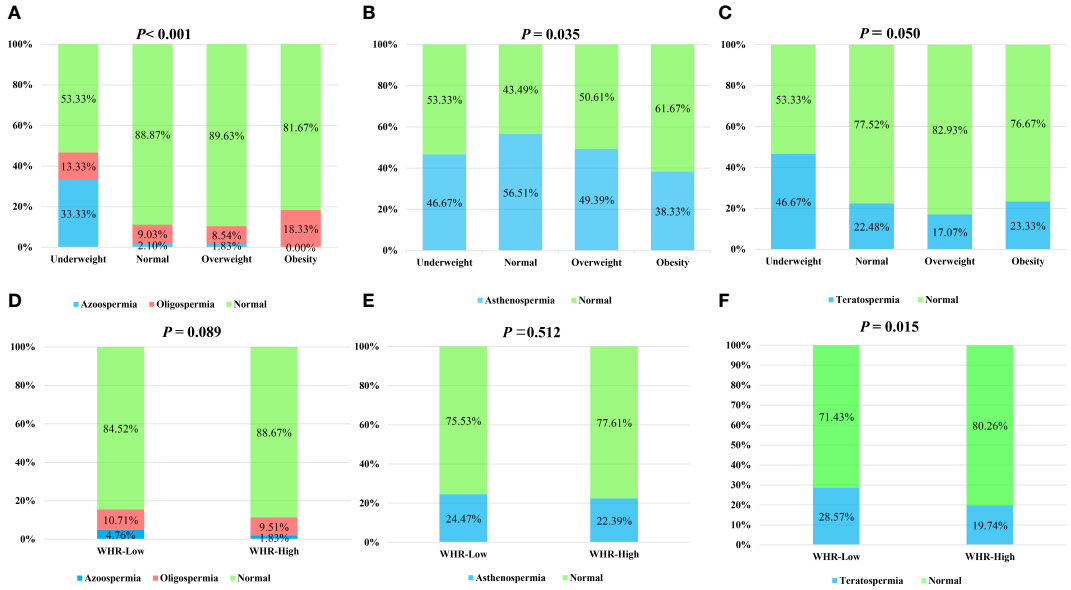

FIGURE 1
Chi-square test to analyze the influence of body mass index (BMI) and waist–hip ratio (WHR) on the sperm concentration, sperm progressive motility, and sperm morphology. BMI was categorized as: underweight (<18.0 kg/m^2), normal weight (18.0–24.9kg/m^2), overweight (25–27.9 kg/m^2), and obese (≥28 kg/m^2). WHR was categorized into two groups: <0.81 (normal) and ≥0.81 (high). **(A)** The rate of azoospermia, oligospermia, and normal sperm concentration in the different BMI groups. **(B)** The rate of asthenospermia and normal sperm progressive motility in the different BMI groups. **(C)** The rate of teratospermia and normal sperm morphology in the different BMI groups. **(D)** The rate of azoospermia, oligospermia, and normal sperm concentration in the different WHR groups. **(E)** The rate of asthenospermia and normal sperm progressive motility in the different WHR groups. **(F)** The rate of teratospermia and normal sperm morphology in the different WHR groups.

than the median BMI in normal weight subjects (22.40 kg/m^2), the sperm concentration appeared to increase monotonically with increasing BMI. The sperm concentration was inversely associated with increasing BMI While BMI was more than 22.4 kg/m^2. No significant relationships were observed between BMI and sperm progressive motility or sperm morphology. Interestingly, the sperm progressive motility was positively associated with the WHR when <0.82 or >0.89 and negatively associated with the WHR within 0.82 to 0.89. No relationship was observed between the WHR and sperm concentration or sperm morphology.

3.4 Multivariate logistic analysis of the independent risk factors for sperm concentration, sperm progressive motility, and sperm morphology

The odds ratios (ORs), 95% confidence interval (CIs), and *P* values of the sperm concentration, sperm morphology, and sperm progressive motility are summarized in Tables 3–5. The results of the ordinal multivariate logistic analysis revealed that age (OR: 1.184, 95% CI: 1.015–1.381, $P = 0.031$) and FSH (OR: 0.791, 95% CI: 0.692–0.904, $P = 0.001$) were independent risk factors of male sperm concentration; while BMI, WHR, serum testosterone, LH, alcohol and smoke intake were not. Interestingly, referring to sperm motility, the results of the multivariate logistic analysis showed that BMI (OR: 1.072, 95% CI: 1.019–1.128, $P = 0.007$) and abstinence time (OR: 0.913, 95% CI: 0.853–0.978, $P = 0.009$) were the only independent risk factors among the clinical parameters counted. Finally, the multivariate logistic analysis suggested that FSH (OR: 0.821, 95% CI: 0.727–0.927, $P = 0.002$) and testosterone (OR: 0.908, 95% CI: 0.835–0.987, $P = 0.023$) were the independent risk factors for sperm morphology, while BMI or WHR were not found to be associated with it.

4 Discussion

In this observational study, the association between BMI, WHR, and semen quality was investigated in 715, sperm donors who underwent semen examinations between 2019 and 2021 in Hefei, China. We found that BMI was weakly associated with sperm progressive motility, while WHR showed no relationship with semen parameters. The RCS showed that BMI was negatively associated with sperm concentration while BMI was more than 22.40 kg/m^2 and the WHR was negatively related to sperm progressive motility within 0.82 to 0.89. Furthermore, the multivariate logistic analysis showed that BMI was an independent risk factor for normal sperm progressive motility, FSH was an independent risk factor for normal sperm concentration and morphology, and testosterone was an independent risk factor for sperm morphology. This study highlights that BMI is a more important factor in the assessment of semen quality than WHR. BMI showed a weak influence on sperm progressive motility; however, serum FSH and testosterone were the main factors affecting sperm concentration and morphology.

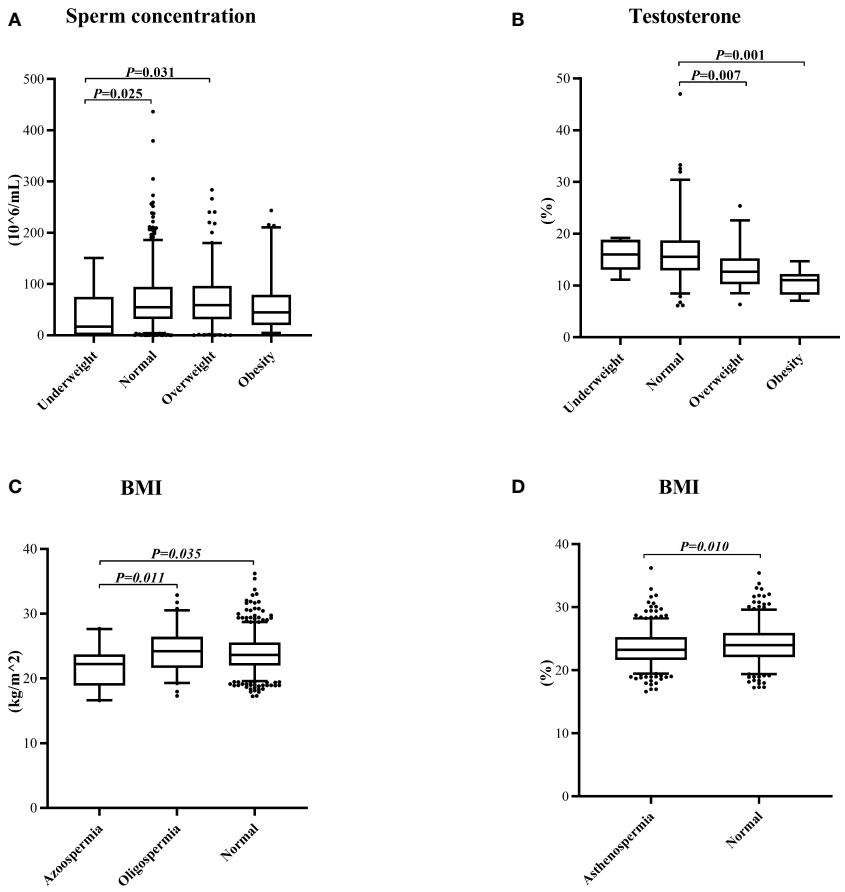

FIGURE 2
Kolmogorov–Smirnov test to evaluate the differences among the groups. **(A)** The sperm concentration in the different body mass index (BMI) groups. **(B)** The testosterone concentration in the different BMI groups. **(C)** The BMI in the different sperm concentration groups. **(D)** The BMI in different sperm progressive motility groups.

The relationship between BMI and semen quality remains controversial. Some studies have demonstrated that an increased BMI is significantly linked with sperm concentration, sperm motility, and sperm morphology (8, 9, 13). However, other studies reported different conclusions (7, 9, 14). In 2013, Sermondade et al. performed a meta-analysis on a large sample of data (15). They found that being overweight and obesity were significantly associated with an increased risk of azoospermia and oligozoospermia. However, our study showed that BMI was positively associated with sperm progressive motility, but not with sperm concentration or morphology. The results of the multivariate logistic analysis showing that BMI was an independent risk factor confirmed this. Therefore, an elevated BMI may be beneficial for sperm progressive motility.

Few studies have focused on the relationship between being underweight and semen quality. In 2004, Jensen et al. assessed military readiness in 1558.00 young Danish men young men between 1996 and 1998; a significant reduction in sperm concentration was observed in underweight men, which was defined as BMI <20.00 kg/m^2 (16). A later study by Qin et al. (17) reported that being underweight was significantly associated with a lower sperm concentration in fertile men from the general population. Consistent with these two studies, being underweight was inversely related to sperm concentration and total sperm number, but not to sperm motility or semen volume, in Jixuan Ma's study, which recruited 3966.00 healthy sperm donors (9). In the present study, an increased prevalence of azoospermia was observed in the underweight group and the sperm concentration was lower compared with the normal bodyweight or overweight group. This was consistent with previous research. Malnutrition may be the key factor of the association between being underweight and worse semen quality, which is known to have negative effects on male reproductive hormones (18).

It is well known that reproductive hormones, including FSH and testosterone, are closely related to male semen quality (19–21). FSH plays an important role in spermatogenesis. Clinical studies reported that FSH levels were higher in infertile patients compared with fertile controls, and that FSH was negatively correlated to sperm concentration in the population surveyed (22). However, the relationship between FSH and sperm morphology has been rarely reported. In 2020, Wei Zhao et al. (23) reported that FSH was inversely associated with sperm morphology after mutual adjustment, which was consistent with our finding. This indicated that FSH is important for sperm to maintain normal morphology. However, the mechanism remains to be studied in the future. In addition,

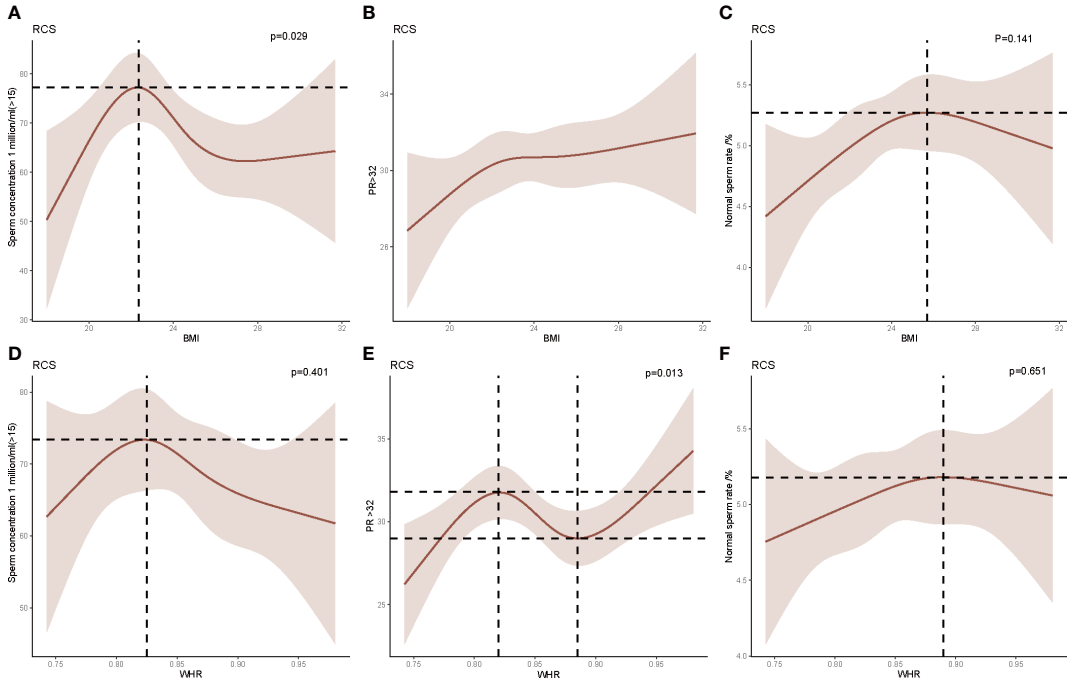

FIGURE 3
Restricted cubic spline (RCS) analysis of the relationship between body mass index (BMI), waist–hip ratio (WHR), and sperm concentration, sperm progressive motility, and sperm morphology. **(A)** The relationship between BMI and sperm concentration. **(B)** The relationship between BMI and sperm progressive motility. **(C)** The relationship between BMI and sperm morphology. **(D)** The relationship between WHR and sperm concentration. **(E)** The relationship between WHR and sperm progressive motility. **(F)** The relationship between WHR and sperm morphology.

testosterone is known to be the key factor to sustain sperm production. Therefore, testosterone may be strongly associated with sperm concentration, sperm morphology, or sperm motility. As reported, using human chorionic gonadotropin or tamoxifen citrate in infertile men with testosterone deficiency led to an improvement of the sperm concentration, sperm motility, and sperm morphology (24). Nevertheless, the results of the present study showed that testosterone was an independent risk factor for sperm morphology but was not related with sperm concentration or sperm morphology. A possible explanation is that the levels of testosterone in the serum in the present study did not reflect the true intratesticular testosterone level, which is believed to directly act in the spermatogenic process.

TABLE 3 Univariate and multivariate ordinal logistic analysis for screening the independent factors of sperm concentration.

Variables	OR	Univariate analysis	*P*	B	OR	Multivariate analysis	*P*
		95%CI				95%CI	
Age (years)	1.051	0.674-1.105	**0.05**	0.169	1.18	1.015-1.381	**0.031**
BMI (kg/m^2)	1.008	0.934-1.087	0.84				
WHR	1.489	0.028-80.238	0.84				
Time of	1.040	0.941-1.149	0.44				
Anti-sperm	1.242	1.234-9.718	**0.01**	1.095	2.98	0.337-	0.325
FSH (IU/l)	0.757	0.668-0.858	**<0.0**	–	0.79	0.692-0.904	**0.001**
T (nmol/l)	0.953	0.8841-1.028	0.21				
LH(IU/l)	0.800	0.712-0.899	**<0.0**	–	0.88	0.783-1.001	0.053
Alcohol	1.150	0.846-1.564	0.37				
Smoke	0.707	0.452-1.105	0.12				

BMI, Body Mass Index; WHR, Waist-Hip Ratio; FSH, Follicular Stimulating Hormone; LH, Luteinizing Hormone; T, Testosterone. Statistically significant data were represented in bold.

TABLE 4 Univariate and multivariate logistic analysis for screening the independent Influencing factors of sperm progressive motility.

Variables	OR	Univariate analysis	*P*	B	OR	Multivariate analysis	*P*
		95%CI				95%CI	
Age (years)	0.975	0.946-1.006	0.112				
BMI (kg/m²)	1.071	1.018-1.127	**0.00**	0.070	1.07	1.019-1.128	**0.007**
WHR	14.19	1.010-199.315	**0.04**	0.783	2.18	0.080-	0.643
Time of	0.914	0.854-0.978	**0.01**	–	0.91	0.853-0.978	**0.009**
Anti-sperm	0.905	0.583-1.406	0.65				
FSH (IU/l)	0.975	0.899-1.057	0.53				
T (nmol/l)	0.995	0.938-1.056	0.87				
LH(IU/l)	0.940	0.845-1.046	0.25				
Alcohol	1.117	0.915-1.364	0.27				
Smoke	1.131	0.841-1.520	0.41				

BMI, Body Mass Index; WHR, Waist-Hip Ratio; FSH, Follicular Stimulating Hormone; LH, Luteinizing Hormone; T, Testosterone. Statistically significant data were represented in bold.

TABLE 5 Univariate and multivariate logistic analysis for screening the independent factors of sperm morphology.

Variables	OR	Univariate analysis	*P*	B	OR	Multivariate analysis	*P*
		95%CI				95%CI	
Age (years)	0.987	0.952-1.024	0.48				
BMI (kg/m²)	1.053	0.990-1.121	0.10				
WCR	2.958	0.122-	0.50				
Time of	1.041	0.963-1.125	0.31				
Anti-sperm	1.005	0.591-1.705	0.98				
FSH (IU/I)	0.820	0.723-0.931	**0.00**	–	0.82	0.727-0.927	**0.002**
T (nmol/l)	0.924	0.854-1.000	**0.04**	–	0.90	0.835-0.987	**0.023**
LH(IU/1)	0.884	0.794-0.985	**0.02**	–	0.97	0.844-1.115	0.670
Alcohol	1.233	0.965-1.576	0.095				
Smoke	0.792	0.555-1.130	0.199				

BMI, Body Mass Index; WHR, Waist-Hip Ratio; FSH, Follicular Stimulating Hormone; LH, Luteinizing Hormone; T, Testosterone. Statistically significant data were represented in bold.Statistically significant data were represented in bold.

There are some limitations in the present research. Firstly, the sample size collected was small, especially for people who had completed sex hormone tests. Secondly, the data was acquired in the infertile population, whose age were concentrated in the reproductive age. Therefore, the result of this study cannot represent the result of all populations. Thirdly, this is a single-center retrospective research, the conclusion needs to be verified by a multi-center prospective study in the future.

In conclusion, BMI is an independent risk factor for sperm motility while the WHR did not contribute to semen parameters. Our findings highlight the important role of serum FSH and testosterone in male semen quality.

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 authors.

Author contributions

XH and SW designed the research study. ZK, CW and PX contributed to the data acquisition. XH, BW and JX analyzed the data. SW wrote the paper. All authors approved the final manuscript.

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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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UBR4 deficiency causes male sterility and testis abnormal in *Drosophila*

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Introduction: It has been established that *UBR4* encodes E3 ubiquitin ligase, which determines the specificity of substrate binding during protein ubiquitination and has been associated with various functions of the nervous system but not the reproductive system. Herein, we explored the role of *UBR4* on fertility with a *Drosophila model*.

Methods: Different *Ubr4* knockdown flies were established using the UAS/GAL4 activating sequence system. Fertility, hatchability, and testis morphology were studied, and bioinformatics analyses were conducted. Our results indicated that *UBR4* deficiency could induce male sterility and influent egg hatchability in *Drosophila*.

Results: We found that *Ubr4* deficiency affected the testis during morphological analysis. Proteomics analysis indicated 188 upregulated proteins and 175 downregulated proteins in the testis of *Ubr4* knockdown flies. Gene Ontology analysis revealed significant upregulation of *CG11598* and *Sfp65A*, and downregulation of *Pelota* in *Ubr4* knockdown flies. These proteins were involved in the biometabolic or reproductive process in *Drosophila*. These regulated proteins are important in testis generation and sperm storage promotion. Bioinformatics analysis verified that *UBR4* was low expressed in cryptorchidism patients, which further supported the important role of *UBR4* in male fertility.

Discussion: Overall, our findings suggest that *UBR4* deficiency could promote male infertility and may be involved in the protein modification of *UBR4* by upregulating *Sfp65A* and *CG11598*, whereas downregulating Pelota protein expression.

KEYWORDS

male fertility, hatchability, drosophila, testes, UBR4

1 Introduction

Current evidence suggests that maintaining cellular protein homeostasis (proteostasis) largely relies on proteolysis (1). As a cellular mechanism that triggers proteasomal degradation, ubiquitin conjugation, based on ubiquitin and ubiquitin-like proteins (UBLs), plays an important role in controlling cell division, signal transduction, embryonic development, endocytic trafficking, and immune response (2).

UBR4 encodes the Ubiquitin Protein Ligase E3 Component N-Recognin 4; the homology of *Drosophila* is *poe*. It is an E3 ubiquitin ligase (E3 enzyme) that regulates protein degradation, removing damaged or misfolded proteins from the cellular environment, thereby regulating cellular homeostasis. The body utilizes the coordinated action of a series of enzymes in the ubiquitin–proteasome system (UPS), including ubiquitin-activating enzymes (E1 enzymes), ubiquitin-conjugating enzymes (E2 enzymes), and E3 ubiquitin ligases (E3 enzymes) (1–4). E3 enzymes are reportedly important in the ubiquitination process and determine substrate specificity (3). Protein error correction plays an extremely important role in physiological processes such as growth and development, reproductive inheritance, immunity, and endocrine of *Drosophila* and the human body.

Up to now, research on *UBR4* has mainly focused on the nervous system (5–7). Interestingly, the number of *Drosophila* pupae decreased after *Ubr4* gene knockdown in our preliminary experiments, suggesting that *Ubr4* may be related to reproductive capacity. This phenomenon may be attributed to erroneous protein synthesis in the genitals, which may cause physiological and behavioral changes, including reduced egg production and feeding, decreased willingness to mate and storage/utilization of sperm, and altered gene expression (8, 9). Spermatogenesis abnormality is also another important reason for reproductive problems. In *Drosophila*, spermatogenesis starts at the apical tip of the testis, which hosts two stem cell populations, including the germline stem cells (GSCs) and somatic cyst stem cells (CySCs) (10–12). Zygotes produced by GSCs undergo four rounds of mitosis and then differentiate into 16 spermatogonia. The spermatogonia then differentiates to spermatocytes, round spermatozoa, long spermatozoa, and mature spermatozoa and complete an entire spermatogenesis process. The cyst cells differentiated from CySCs encase germ cells and support germ cell growth (13). The hub cells mainly maintain the self-renewal and differentiation of GSCs and CySCs (14, 15).

Herein, loss-of-function *Drosophila* models were applied as research vectors to detect the effect of the *Ubr4* gene on *Drosophila* development and reproduction.

2 Materials and methods

2.1 Fly culture and qPCR

Drosophila was maintained on standard cornmeal and incubated under controlled conditions (temperature 25°C, humidity 60%–70%, and 12:12-h light/dark cycle). *UAS-Ubr4-RNAi* (THU1137, CG14472) was donated by Tsing Hua Fly Center (Tsinghua University, Beijing, China). The knockdown efficiency of RNAi was checked by qPCR (16). The primer sequencies were

Ubr4/Poe-F1: CCACCGTCACACACTTCAAT
Ubr4/Poe-R1: GGCAGCAGTCCATTACATCT
GAPDH-F1: CGTCAACGATCCCTTCATCGATGTC
GAPDH-R1: CAGCACTGGCCCAGTTGATGTTG.

2.2 Fertility and hatchability assays

The quantification of fertility and hatchability: Fertility refers to the ability of *Drosophila* to produce offspring, and our study mainly focused on the number of eggs it laid. Hatchability is the rate of eggs hatching into pupae and pupae hatching into adults. The details of analyses were shown below.

2.2.1 *Ubr4* knockdown male fertility and hatchability assay

UAS-Ubr4-RNAi males were mated with *tub-GAL4* females to generate *Ubr4* knockdown flies (*tub-GAL4>UAS-Ubr4-RNAi*). *UAS-Ubr4-RNAi* males were used as their genetically matched controls. Subsequently, 3-day-old virgin knockdown and control males, isolated within 24 h of their eclosion, were mated with 3–5-day-old wild-type (*Canton-S*) virgin female flies for different assays, as described below.

Three males were mated with *Canton-S* virgin females in pairs. Then, males were discarded after 48 h. Mated females were allowed to lay eggs for 10 days with the change to fresh food vials at 48-h intervals for the fertility assay. The number of eggs laid, egg-to-pupa hatchability rate, and pupa-to-adult hatchability rate was calculated. Mated females were discarded after 10 days, and the resultant progenies were counted to determine fertility. For each group, the experiments were repeated three times with five to six replicates each.

2.2.2 *Ubr4* knockdown female fertility and hatchability assay

Three-day-old *Ubr4* knockdown (*tub-GAL4>UAS-Ubr4-RNAi*) and control (*UAS-Ubr4-RNAi*) virgin females, isolated within 24 h of their eclosion, were mated with 3–5-day-old Canton-S males for fertility assays.

After three males were mated with *Canton-S* virgin females in pairs, the males were discarded. We allowed mated females to lay eggs for 10 days, with fresh food vials changed every 48 h for the fertility assay. A fertility test was carried out by counting the progeny of females discarded after 10 days of mating. Hatchability is defined the same as above.

2.3 Larva development experiment

Newly *Ubr4* knockdown (*tub-GAL4>UAS-Ubr4-RNAi*) and control (*UAS-Ubr4-RNAi>Canton-S*) instar larvae cultured in standard medium for 24, 48, 72, 96, and 120 h after egg laying

(AEL) were heated with 40°C–50°C water for 15 min to foster the larvae to come out from the medium. Afterward, the collected larvae were washed with phosphate-buffered saline (PBS), and their length was measured under the microscope. Next, the number of larvae developing into pupae was counted at 6-h intervals each day until all larvae had hatched. Subsequently, the number of adults hatched from pupae at 6-h intervals was counted. At the same time, the time of hatching from larvae to pupae and pupae to adults were counted independently.

2.4 Testes morphology

Drosophila testes from 2-day-old young adults were dissected in 1× PBS, fixed in 1× PBS/4% PFA for 30 min at room temperature. After being washed three times with PBS, the testes were transferred to glass slides. A mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Abcam, USA) was dripped and reacted with the testes for 10 min before being covered with a coverslip. Testes morphology was observed with an inverted phase-contrast microscope (SP8; Zeiss, Jena, Germany). The number of spermatogenic cells was counted by ImageJ software (National Institutes of Health, Bethesda, MD, USA), and data were analyzed by GraphPad Prism 7.0.

2.5 Mass spectrometry and bioinformatics analyses

2.5.1 Extraction of protein and enzymatic hydrolysis of peptides

Testes were collected and frozen in liquid nitrogen before protein extraction. Tissue protein was extracted by SDT (4% (w/v) SDS, 100 mm Tris/HCl pH 7.6, 0.1 m DTT), then protein was quantified by BCA. AFign appropriate amount of protein was obtained from each sample, and enzymatic hydrolysis of trypsin was performed by filter-aided proteome preparation (FASP). Next, the peptide was desalted by the C18 Cartridge. After freeze-drying, the peptide was lyophilized and redissolved in 40 µl of 0.1% formic acid solution (OD280).

2.5.2 Protein identification and quantitative analysis

The MaxQuant software (version number 1.6.14) (17) was used for database identification and quantitative analysis. Mass spectrometry was carried out by Shanghai Applied Protein Technology Co., Ltd.

2.5.3 Bioinformatics analyses

The CTD (Comparative Toxicogenomics Database), which can be used for the prediction of correlations between genes, diseases, and chemicals (18), indicates that changes in *UBR4* may be associated with cryptorchidism (http://ctdbase.org/). As one of the reasons for male infertility disorders, cryptorchidism, teratospermia, and azoospermia may be risk factors for the disease (19). To validate the findings, we downloaded the cryptorchidism dataset (GSE16191), teratospermia (GSE6872), and azoospermatism (GSE108886) from the GEO (Gene Expression Omnibus) database. With the screening criteria that (1) there were clear experimental and control groups, (2) the experimental group did not have any treatment other than cryptorchidism, (3) the samples were all from humans, and (4) the dataset had been borrowed from other articles, we finally chose the cryptorchidism dataset GSE16191 (20) and completed a bioinformatics analysis using R software (R.4.2.2), containing 16 diseased samples and 4 healthy control samples with tissue taken from whole testes (http://www.ncbi.nlm.nih.gov/geo/). The expression of three key genes, *UBR4*, *PELO*, and *LIPA*, was compared in groups to validate their up- and downregulation, and ROC curve analysis of key genes was performed to predict their diagnostic efficacy for cryptorchidism. A single-gene GSEA was performed to identify downstream genes in the functional pathway of the key genes. GO and KEGG analyses of downstream genes and key genes were also performed to search for related biological processes and enrichment pathways mediated by key genes.The quantitative information of the target protein set was normalized (normalized to (-1,1) interval). Then, the ComplexHeatmap R package (R Version 3.4) was used to classify the two dimensions (distance algorithm: Euclid, connection mode: average linkage) of the expression of samples and protein simultaneously. Finally, a hierarchical clustering heat map was generated.

GO annotation of the target protein set by Blast2GO can be summarized into four steps: sequence comparison (Blast), GO item mapping, GO annotation, and InterProScan annotation. The ProScan software package, which runs a scanning algorithm from the InterPro database in an integrated way to characterize the sequence, was used to obtain the domain annotation information of the target protein sequence in the Pfam database.

2.6 Statistical analysis

All quantitative data were expressed as mean ± SD. The Student's t-test was used to compare two independent or paired samples. Multiple samples were compared using one-way ANOVA, and differences between the two groups were evaluated using Tukey's *post-hoc* test. The data were analyzed using GraphPad Prism 7.00 and SPSS 20. A P-value < 0.05 was statistically significant.

3 Results

3.1 *Ubr4* deficiency influences larval development

Flies crossing strategy in larval development assay was showed in Figure 1A. The Knockdown efficiency of RNAi is about 20% (Figure 1B). Compared with the control group, the length of *tub-GAL4>UAS-Ubr4-RNAi* larvae was lower than that of *UAS-Ubr4-RNAi> Canton-S*, especially for 48-h AEL and 72-h AEL. The length of 48h AEL *tub-GAL4>UAS-Ubr4-RNAi* larvae was significantly lower than that of *UAS-Ubr4-RNAi> Canton-S* (1.694 ± 0.544 mm [n = 28]

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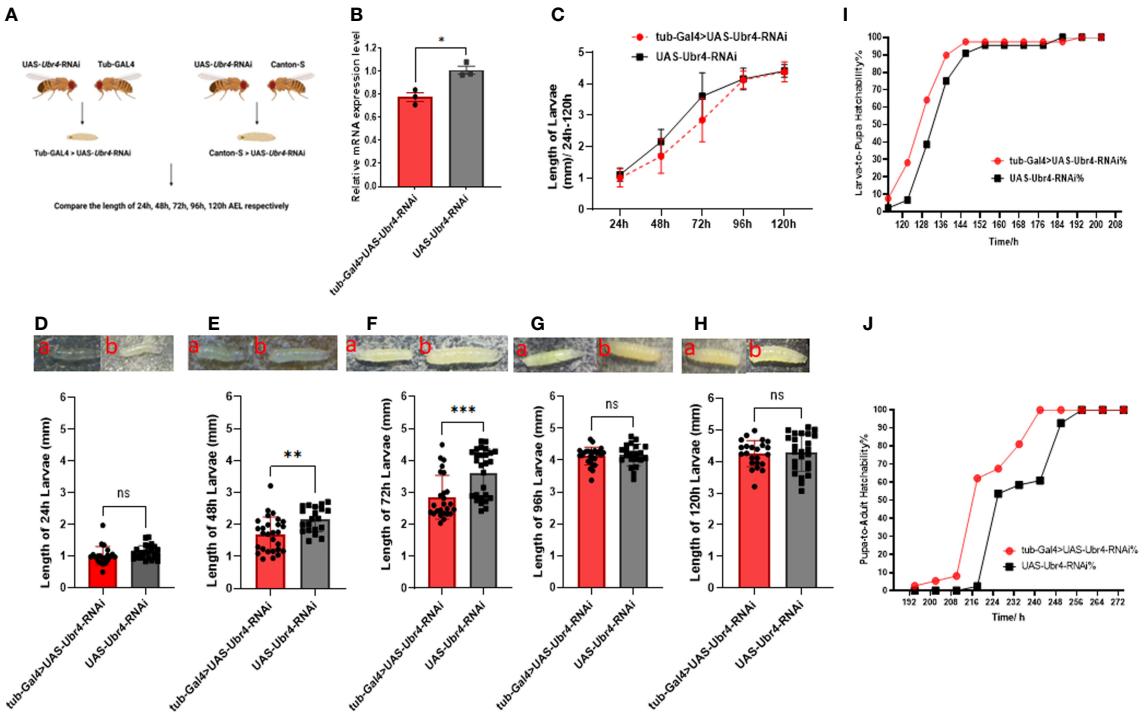

FIGURE 1
Comparison of larval development between *Ubr4* knockdown and wild type. **(A)** Experimental procedure. **(B)** Relative mRNA expression level in WT and *Ubr4* knockdown flies. **(C)** The overall length variation (24–120 h) of larvae. **(D)** Comparison of the length of 24-h-old larvae. **(E)** Comparison of the length of 48-h-old larvae. **(F)** Comparison of the length of 72-h-old larvae. **(G)** Comparison of the length of 96-h-old larvae. **(H)** Comparison of the length of 120-h-old larvae. a. *tub-Gal4>UAS-Ubr4-RNAi*, b. *UAS-Ubr4-RNAi*. **(I)** Larval hatchability at different time points. **(J)** Pupae hatchability at different time points. ns, P>0.05, *P<0.05, **P<0.01, ***P<0.005.

vs. 2.159 ± 0.388 mm [n = 20]; **P = 0.002, Figure 1E). At the same time, the 72-h AEL *tub-GAL4>UAS-Ubr4-RNAi* larvae had a lower length than *UAS-Ubr4-RNAi>Canton-S* (2.847 ± 0.687 mm [n = 25] vs. 3.608 ± 0.744 mm [n = 27]; ***P = 0.0004, Figure 1F). However, when the larvae developed to 24-h AEL, 96-h AEL and 120-h AEL, there was no significant difference in larval length between the *tub-GAL4>UAS-Ubr4-RNAi* and *UAS-Ubr4-RNAi> Canton-S* flies (24-h AEL: 1.007 ± 0.292 m24-h AEL: 1.007 ± 0.292 mm [n = 21] vs. 1.112 ± 0.211 mm [n = 25]; P >0.05 Figure24-h AEL: 1.007 ± 0.292 mm [n=21] vs. 1.112 ± 0.211 mm [n=25]; P>0.05, Figure 1D. 96-h AEL: 4.125 ± 0.276 mm [n = 26] vs. 4.157 ± 0.340 mm [n = 21]; P > 0.05, Figure 1G. 120-h AEL: 4.253 ± 0.417 mm [n = 23] vs. 4.258 ± 0.588 mm [n = 25]; P > 0.05, Figure 1H). These results suggested that the heterogeneity in developmental speed accounted for the difference in larval length observed in our study.

Further experiments showed that there was no significant difference in the development time from larva stage to pupa and from pupa stage to adult between the *tub-GAL4>UAS-Ubr4-RNAi* and *UAS-Ubr4-RNAi> Canton-S* groups (Figures 1I, J).

3.2 *UBR4* is essential for male fertility but not female fertility

Wild-type females (*Canton-S*) mated to *Ubr4* knockdown males (*tub-Gal4>UAS-Ubr4-RNAi*) produced significantly fewer fertilized eggs than those mated with wild-type (*UAS-Ubr4-RNAi*) males. The reduction in fertility observed in *Drosophila* could be due to several reasons, including fewer eggs being laid by mated females and/or reduced hatchability/increased mortality of these laid eggs during development (21). Therefore, to determine the cause of decreased fertility of *Ubr4* knockdown males, we counted the number of eggs laid (fertility) on day 1, day 3, day 5, day 7, and day 9. Flies crossing strategy was shown in Figure 2A. Figure 2B showed the overview of eggs numbers of Ubr4 knockdown group and control group. Over 10 days, the number of eggs produced by the females mated with *tub-GAL4>UAS-Ubr4-RNAi* males was less than in those mated with *Canton-S>UAS-Ubr4-RNAi* males (53.83 ± 18.90 [n = 6] vs. 88.00 ± 25.10 [n = 4]; *P = 0.0388, Figure 2C), on day 5 (32.50 ± 16.53 [n = 6] vs. 65.75 ± 12.18 [n = 4]; **P = 0.0090, Figure 2F) and day 9 (55.00 ± 20.26 [n = 6] vs. 88.00 ± 13.90 [n = 4]; *P = 0.05, Figure 2H). No significant difference was shown on day 1, day 3, day 7 between the tub-Gal4>UAS-Ubr4-RNAi and UAS-Ubr4-RNAi> Canton-S flies (P>0.05, Figures 2B, 2D, 2G).

However, after wild-type males (*Canton-S*) were mated to *Ubr4*-deficient females (*tub-Gal4>UAS-Ubr4-RNAi*), no significant difference was observed compared with the control (*Canton-S* males mated with *UAS-Ubr4-RNAi*) in the overall number of progenies produced over 10 days (P > 0.05, Figures 3B–H). Therefore, the *Ubr4* gene has a more important influence on male fertility than on female fertility.

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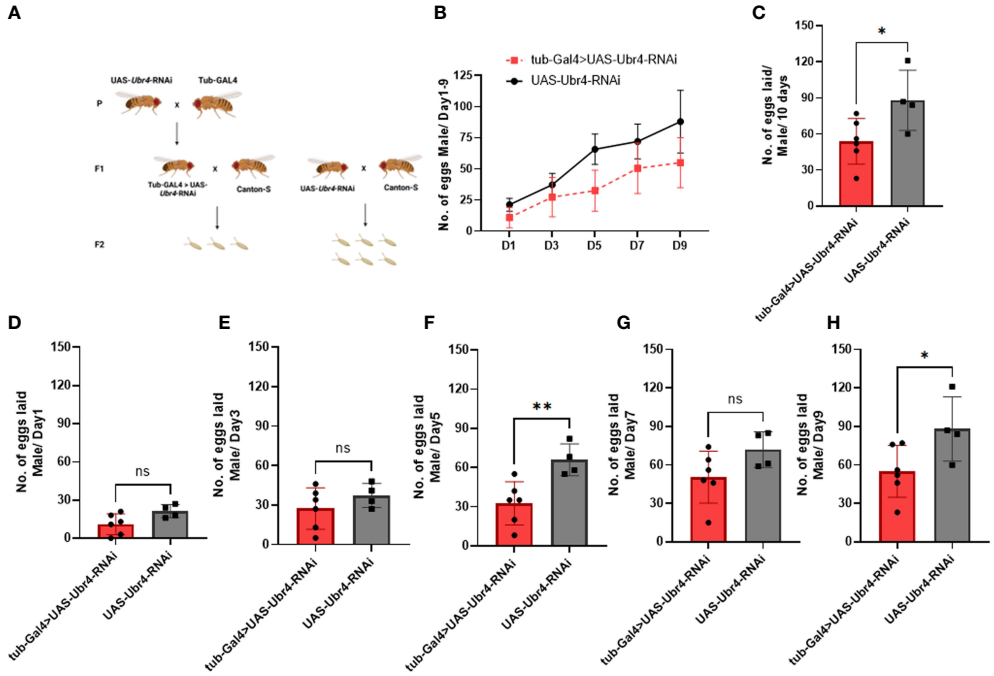

FIGURE 2
Reproductive performance of females mated to *Ubr4* knockdown males. **(A)** Experimental procedure. **(B)** The overview of eggs laid from days 1 to 9. **(C)** The overall fertility (total no. of eggs laid/10 days) of mated females. **(D)** Day 1 fertility (no. of eggs laid/day from day 1) of mated females. **(E)** Day 3 fertility (no. of eggs laid/day from day 3) of mated females. **(F)** Day 5 fertility (no. of eggs laid/day from day 5) of mated females. **(G)** Day 7 fertility (no. of eggs laid/day from day 7) of mated females. **(H)** Day 9 fertility (no. of eggs laid/day from day 9) of mated females. ns, P>0.05, *P<0.05, **P<0.01.

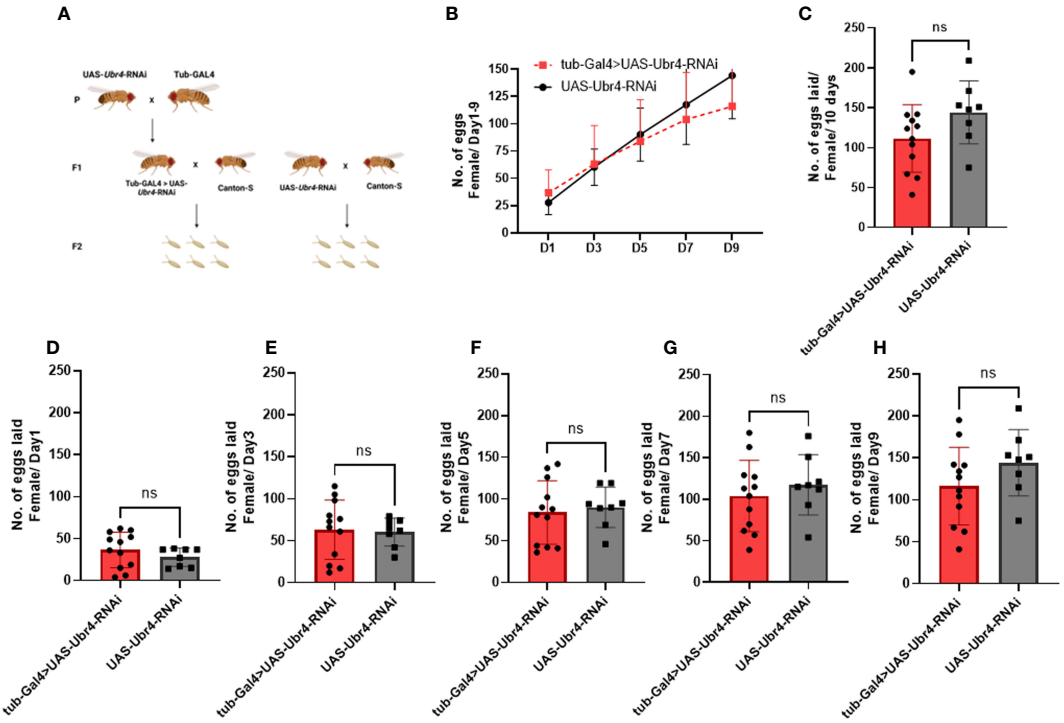

FIGURE 3
Reproductive performance of *Ubr4* knockdown females mated to wild males. **(A)** Experimental procedure. **(B)** The overview of eggs laid from days 1 to 9. **(C)** The overall fertility (total no. of eggs laid/10 days) of mated females. **(D)** Day 1 fertility (no. of eggs laid/day from day 1) of mated females. **(E)** Day 3 fertility (no. of eggs laid/day from day 3) of mated females. **(F)** Day 5 fertility (no. of eggs laid/day from day 5) of mated females. **(G)** Day 7 fertility (no. of eggs laid/day from day 7) of mated females. **(H)** Day 9 fertility (no. of eggs laid/day from day 9) of mated females. ns, P>0.05.

To determine hatchability, we focused on the number of eggs that grew into pupae (egg hatchability) and the pupae that developed into adults (pupae hatchability). Analysis of the hatchability showed no significant difference in pupa-hatching rates between the pupae from Ubr4 knockdown parents and the pupae from wild-type parents (Figure 4B). In the egg hatchability, the hatching rate of the eggs reproduced from wild-type mothers with *Ubr4* knockdown fathers is lower than that from wild-type parents (**P = 0.0090, Figure 4A), whereas the hatching rate of eggs reproduced form *Ubr4* knockdown mothers with wild-type fathers is higher than that from wild-type parents (*P = 0.0175, Figure 4A).

3.3 *Ubr4* deficiency affected testis morphology

Morphological changes in *Ubr4* knockdown flies were studied by confocal imaging. DAPI, a well-established blue-emitting fluorescent compound, was used to visualize the cell nucleus. The testis cells of the Ubr4 knockdown flies appear slightly obscure compared with wild-type flies, which required stronger laser power to be visualized (550 vs. 480). In general, the Hub cells, CySCs, GSCs, and Sp labeled by DAPI are easier to be observed whereas the spermatocytes and cyst cells are not easy to be observed. Thus, knockdown of Ubr4 may alter the ratio of cell types in the testis. However, further experiments with a cell-type-specific antibody should be used for studying which stage of germ cells or what kind of somatic cells are affected.

Furthermore, we tried to identify the number of spermatogenic cells in the germinal center of each group. The whole testes can be observed under a 20× confocal microscope, and spermatogonia at various stages were visible with 40× magnification (Figures 5A, B). The testes in the *Ubr4* knockdown group had a collapsed morphology and a disorganized internal structure compared with the control group. The number of spermatogenic cells was analyzed by ImageJ. Statistical results showed that there was a significant difference in the number of spermatogenic cells near the head of the testis (59.42 ± 10.62 [n = 12] vs. 96.62 ± 25.15 [n = 13]; ****P < 0.0001, Figure 5C).

3.4 *Pelota* was downregulated, whereas *CG11598* and *Sfp65A* were upregulated in the testis of *Ubr4*-deficient flies

To better understand the differential protein expression patterns between WT and *Ubr4*-knockdown testis, the mechanisms underlying *Ubr4*-knockdown-induced lower reproduction capacities in male *Drosophila* were explored by label-free proteomics. There were 363 differentially expressed proteins between WT and *Ubr4* knockdown *Drosophila* (Figure 6A). In comparison with WT control *Drosophila*, we found 188 upregulated proteins and 175 downregulated proteins in *Ubr4*-knockdown *Drosophila* (P < 0.05, Figure 6B). Heatmaps showed the alterations in the expression of 363 proteins (Figure 6C). Protein nodes that were upregulated (above 2.0-fold expression, red color) or downregulated (below 0.5-fold expression, blue color) were displayed using a gradient coloring scheme. Among these 363 proteins, CG11598 (orthologous to human LIPA) was the most upregulated and Sfp65A was the second, whereas Pelota (orthologous to human PELO) exhibited a significant downregulation after *Ubr4* knockdown (Figure 6C). Next, we conducted GO annotation of the 363 differential proteins affected by the knockdown of *Ubr4* to determine their function. It was suggested that the upregulation of *Sfp65A* and downregulation of *Pelota* were associated with GO terms Reproduction and Multicellular organism reproduction (Figure 6D), related to the reproductive process in *Drosophila*. *CG11598* was related to GO terms Metabolic process, Primary metabolic process, and Hydrolase

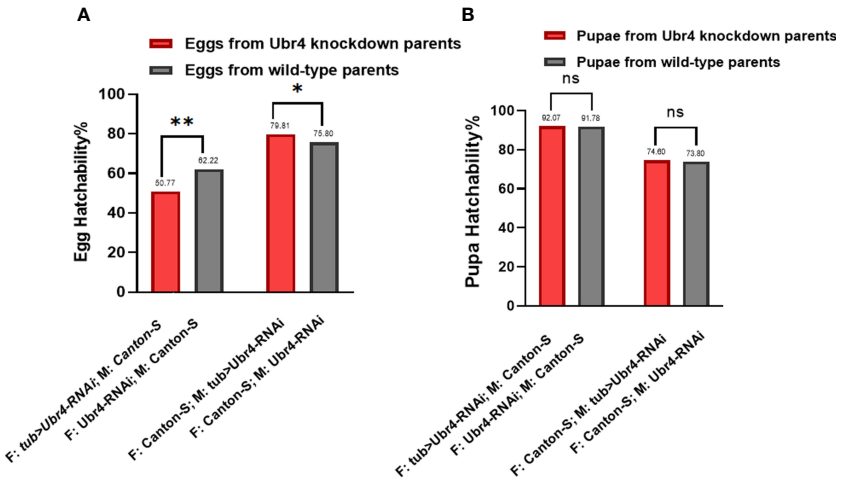

FIGURE 4
Egg hatchability and pupa hatchability. The hatchability rate was analyzed in two aspects, egg hatching rate **(A)** and pupa-to-adult rate **(B)**. Two gender groups were included: one was *Ubr4* knockdown males mated to wild females, and the other was *Ubr4* knockdown females mated to wild males. F, father; M, mother. ns, P>0.05, *P<0.05, **P<0.01.

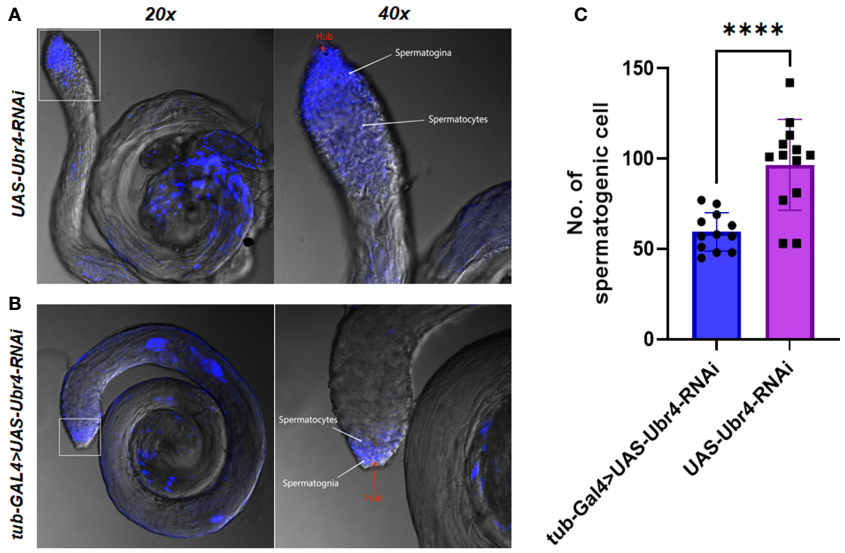

FIGURE 5
Testis morphology and spermatogenic cells To assess the effect of *Ubr4* deficiency, testes were analyzed at the ultrastructural level. Images showing the structure of WT *Drosophila* male testis **(A)** and *Ubr4* knockdown *Drosophila* **(B)**. **(C)** Quantity of spermatogenic cells in testis. ****P<0.001.

activity, which suggested that upregulation of CG11598 may reduce male fertility through biometabolic processes. The number of differential proteins was counted at the GO secondary functional annotation level, including Biologic Process (BP), Cellular Component (CC), and Molecular Function (MF). The following are related to the GOs most affected by the loss of *Ubr4*. The delta DNA polymerase complex and DNA polymerase complex were mainly enriched in CC. Regulation of endosome size, negative regulation of ERBB signaling pathway, and negative regulation of the mRNA metabolic process were significantly enriched in BP by the

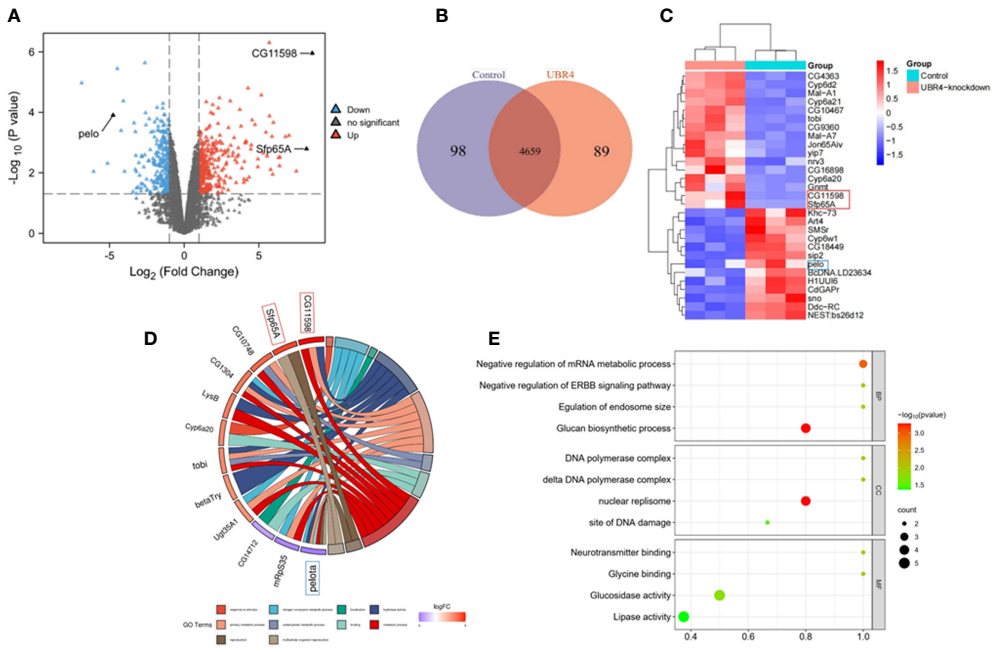

FIGURE 6
Mass spectrometry and bioinformatics analyses **(A)** Volcano plot showing the differentially expressed proteins from the WT control and the *Ubr4* knockdown group in the testis of *Drosophila*. The vertical lines mark the rate of the *Ubr4*-knockdown group compared to the control group, and the horizontal lines mark the P value. The upper right quadrants show the proteins upregulated compared to the control group, and the upper left quadrants contain downregulated compared to the control group. **(B)** Proteomics data show significant differences between WT and *Ubr4*-knockdown *Drosophila* testis in terms of upregulated and downregulated proteins. **(C)** The heatmap displays the difference in protein expression between *Ubr4*-knockdown and WT controls ($P < 0.05$). **(D)** GO enrichment analysis of the above differentially expressed proteins highlighted that the upregulation of *Sfp65A* and downregulation of *Pelota* were involved in the reproductive process. **(E)** GO enrichment analysis of key targets. It included Biologic Process (BP), Cellular Component (CC), and Molecular Function (MF).

mutant of *Ubr4*. Glycine binding, neurotransmitter binding, glucosidase activity, and lipase activity enriched in MF also showed an important interaction after knockdown of *Ubr4* (Figure 6E).

3.5 The function of *UBR4* tested and verified with bioinformatics analyses

CG11598 is orthologous to human *LIPA*, and *Pelota* is orthologous to human *PELO*. *Sfp65A* has no homologous gene in human. Therefore, this study focuses on the expression of *UBR4*, *LIPA*, and *PELO* in the cryptorchidism dataset.

Expression grouping box plots showed good between-group differences for *UBR4* (*P = 0.02) and *LIPA* (*P = 0.049) but confirmed that the between-group differences for *PELO* were not statistically significant (P = 0.06), and the up- and downregulation were consistent with the experimental results (Figure 7A). ROC curve analysis (Figure 7B) showed relatively high diagnostic efficacy for all three genes (AUC > 0.8). Single-gene GSEA predicted key genes downstream, and six results were screened (*MAPK14*, *NFKB1*, *MAPK13*, *MAPK11*, *MAPK9*, and *PIK3CD*) (Figure 7C). GO analysis showed that BP was mainly enriched in the regulation of interleukin-12 production, stress-activated protein kinase signaling cascade, and stress-activated MAPK cascade. MF is mainly enriched in protein serine/threonine/tyrosine kinase activity and MAP kinase activity. CC is predominantly enriched in the secretory granule lumen and is enriched in ficolin-1-rich granule and specific granule. KEGG was mainly enriched in Chagas disease, AGE-RAGE signaling pathway in diabetic complications, and prolactin signaling pathway (Figure 7D).

4 Discussion

Infertility is characterized by failure to establish a clinical pregnancy after 12 months of regular and unprotected sexual intercourse and is estimated to affect between 8% and 12% of reproductive-aged couples worldwide. Males are responsible for 20%–30% of infertility cases but contribute to 50% of cases overall (22, 23). Therefore, it is important to understand the mechanisms underlying fertility to improve our management of this patient population. In the present study, animal experiments indicated that *UBR4* represented a potential infertility gene that could induce infertility and abnormal testis structure in male *Drosophila*.

It is well-established that *UBR4* plays a role in the nervous system (24). To our knowledge, little study has hitherto assessed the role of *Ubr4* in the reproductive capability of *Drosophila* (25). Herein, we used bioinformatics and fly models to further confirm its important role in fertility. We provided compelling evidence that *Ubr4* deficiency could induce a decline in male reproductive

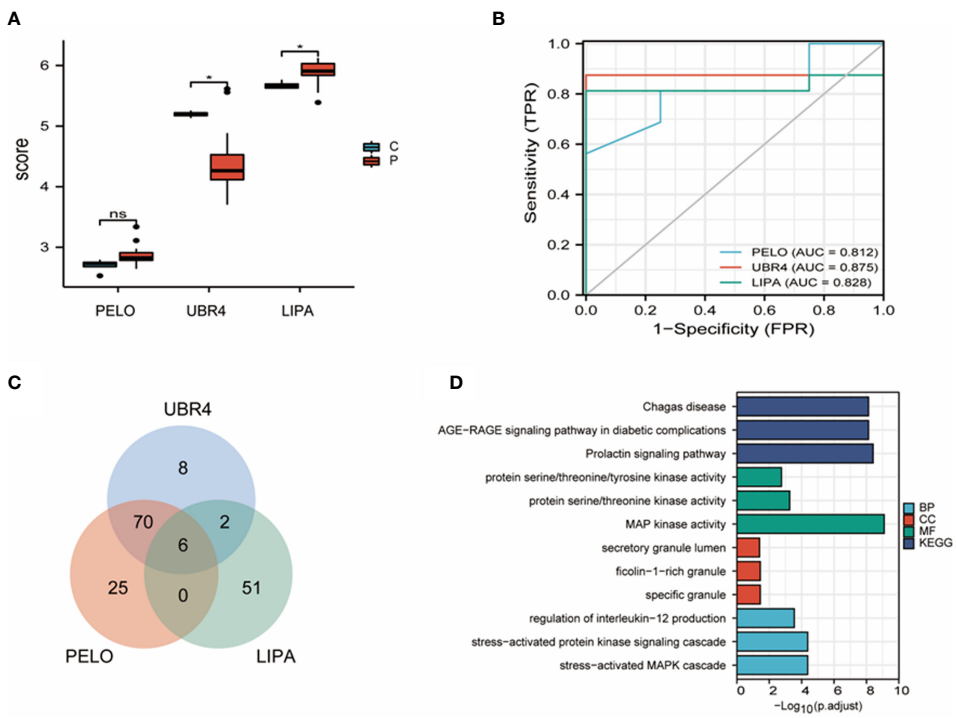

FIGURE 7
Bioinformatics analyses of *UBR4* and its related genes. **(A)** Box plot for expression control of *UBR4*, *LIPA*, and *PELO*. **(B)** Two hub genes were applied for the ROC (Receiver Operating Characteristic) evaluation in GSE16191. **(C)** Six downstream genes of the hub genes (*UBR4*, *LIPA*, and *PELO*) were selected using a Venn plot to visualize the single-gene GSEA (gene set enrichment analysis). **(D)** The GO\KEGG analysis of the hub genes and the downstream genes. ROC, receiver operating characteristic; GSEA, gene set enrichment analysis; GO, Genetic Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes. ns, P>0.05, *P<0.05.

capacity and abnormal testis structure. Intriguingly, no significant difference was observed in egg production of *Ubr4*-knockdown female *Drosophila*. As expected, similar hatchability rates were observed in both gender groups. The egg-hatching rate was significantly different between *Ubr4* knockdown and WT. The reasons might be the reduced fertility of the parents and the restricted development of the offspring. Firstly, knocking down the *Ubr4* males mated with normal females may lead to a decrease in the quality and quantity of eggs produced, which may be manifested by a decrease in egg survival and an increase in the rate of pseudo-eggs. Secondly, after *Ubr4* was knocked down, the genotypes of both offspring in the two gender groups were divided into four types, namely, (1) *tub-Gal4/+>UAS-Ubr4-RNAi/+*; (2) *tub-Gal4/+>+/+*; (3) *+/+>UAS-Ubr4-RNAi/+*; and (4) *+/+>+/+*, whereas the genotype of control groups was total *+/+>UAS-Ubr4-RNAi/+*. The egg-to-pupa hatchability can reflect the capacity of eggs to grow into pupae. Without *Ubr4* gene knockdown in the offspring of the control group, it can be explained why there was a significant difference in its egg hatchability. Indeed, differences may be observed between the *Ubr4* knockdown and the WT group if the quality of eggs cannot be ensured during production. Results showed that *Ubr4* knockdown resulted in greater inhibition of egg hatchability compared with the hatching rate of pupa-to-adult. The reasons behind this may be that the previous stages eliminated the quality of some of the poor eggs from developing into pupae. The remaining pupae, however, have sufficient capacity to hatch into adults. As previously mentioned, the offspring gene remained consistent irrespective of the parent genotype accounting for the lack of difference between both groups. It has been reported that *UBR4* encodes an E3 ubiquitination ligase (E3 enzyme), which regulates protein degradation and removes damaged or misfolded proteins from the cellular environment, thereby regulating cellular homeostasis (26). Protein error correction plays an important role in the development, reproductive inheritance, immunity, and endocrine and other important physiological processes of *Drosophila* (27–29).

It is now understood that *UBR4*, as an E3 enzyme, regulates the degradation of damaged or misfolded proteins (26). It is highly conceivable that *UBR4* gene knockdown disrupts the mechanisms that degrade erroneous proteins. We examined the protein expression in the *UBR4*-knockdown group and the WT control group to explore the mechanism behind the phenomenon. Among the GO terms that were most affected after knocking down *UBR4*, *Delta DNA polymerase complex*, *DNA polymerase complex*, and *Lipase activity* may be associated with the mechanism of male sterility after *UBR4* was knockdown. *DNA polymerase complex* has been reported to contribute to various disorders, including cancer and/or developmental defects (30). *Lipase activity* was associated with disorders of lipid metabolism, which has been reported to reduce semen quality and disrupted the blood–testis barrier integrity (31, 32). Downregulated expression of Pelo protein and upregulated Sfp65A protein was observed in the testis of *UBR4*-knockdown *Drosophila*. GO annotation indicated that Sfp65A and Pelo were associated with the reproductive process whereas CG11598 was related to biometabolic processes. Since the protein regulation ability of E3 ubiquitin ligase decreased following *UBR4* knockdown in *Drosophila*, Sfp65A protein exhibited a significant increase.

The increased proteins were likely damaged or misfolded and escaped from the regulatory process. *LIPA* encodes lysosomal acid lipase (LAL), which catalyzes the breakdown of LDL to produce free fatty acids and cholesterol (33). The study by JF-SHI showed that disruption of cholesterol homeostasis in semen affects semen quality and disrupts the blood–testis barrier, leading to reduced male fertility (31, 34).

Sfp is a non-sperm component of the ejaculate, which can increase male fitness in various aspects, encompassing sperm storage promotion, temporarily improving the female egg production rate, and decreasing female sexual receptivity (35) to ensure the quality and quantity of progeny production and reduced sperm competition (36–38). Therefore, the increase in damaged or erroneous Sfp65A proteins is associated with decreased ability to protect the normal reproductive ability of male *Drosophila*.

The Pelo protein has been documented in the mRNA surveillance pathway. Li et al. showed that Pelo forms complexes with Hbs1 to regulate multiple processes during spermatogenesis with the help of an mRNA surveillance pathway (39). Yang et al. indicated that Pelo played a significant role in silencing transposable elements (TEs) at the translation level, which was critical for genome integrity and primarily depended on Piwi proteins and associated RNAs (40). Both researchers concluded that the Pelo protein was important in stabilizing the quality of sperm and the structure of the genitals. On the other hand, the Sfp65A protein, which was significantly upregulated by inhibiting *UBR4* in *Drosophila*, has been associated with sperm development mediated by the Spliceosome pathway (41).

In the human cryptorchidism dataset (20), *UBR4*, *PELO*, and *LIPA* corresponded to the up- and downregulation of homologous genes in *Drosophila* in this experiment. This confirms that the effects of *UBR4* on male reproduction are likely to be reflected in human cryptorchidism. In terms of diagnostic efficacy, the AUC values for all three key genes were in the range of 0.810–0.880, indicating that these genes have moderate accuracy in diagnostic tests (42) and may be promising targets for the diagnosis of cryptorchidism.

To analyze the downstream genes predicting key genes, we screened six results (*MAPK14*, *NFKB1*, *MAPK13*, *MAPK11*, *MAPK9*, and *PIK3CD*) by single-gene GSEA and showed genes to be members of the IκB family, the JNK subfamily, and the MAPK family, a group of proteins that bind directly to the transcription factor NF-κB and regulate NF-κB activity, a key regulator of NF-κB activation (43, 44), and NF-κB has previously been shown to be associated with male sterility disorders (45). In GO and KEGG analyses, transitional secretion of interleukin-12 may cause abortion in women and abnormal sperm function in men (46, 47). The stress-activated protein kinase signaling cascade in BP annotation, and the stress-activated MAPK cascade and the MAP kinase activity in MF annotation, were all relative to the MAPK signaling pathway. By linking the results with the GSEA, the results suggest that activation of the MAPK signaling pathway has an important role in key genes such as *UBR4*, affecting male

sterility. According to the current study, the MAPK-JNK signaling pathway regulates a variety of important physiopathological effects such as cell proliferation, differentiation, stress, and inflammatory responses (48, 49). Importantly, MAPK signaling regulates male fertility and plays multiple roles in various biological processes in germ cells and is closely linked to processes such as germ cell apoptosis and epididymal maturation (50). More specifically, the MAP4K4-JNK signaling pathway stimulates the proliferation of human spermatogonial stem cells and inhibits apoptosis, which is closely associated with male infertility (51). Thus, the MAPK pathway consisting of these downstream genes may be a key part of the mechanism linking the action of genes such as UBR4. In future studies, we will further explore the specific mechanism of action of UBR4 in the MAPK signaling pathway.

In conclusion, we provide preliminary evidence that after *UBR4* knockdown, male *Drosophila* fertility declines due to abnormal testis structure and low egg hatchability. Nonetheless, further studies are warranted to explore the underlying mechanisms.

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 authors.

Author contributions

S-MX, J-XL, X-CC, J-DQ, and Y-LM designed the study and wrote the manuscript. C-QL, X-XZ and Y-ML performed the larva development, fertility and hatchability experiments. S-MX and J-XL performed testis dissection and analyzed the overall data. Q-WL and D-YH performed Bioinformatics analyses Q-WL and D-YH performed Bioinformatics analyses Q-WL and D-YH performed Q-WL and D-YH performed Bioinformatics analyses. 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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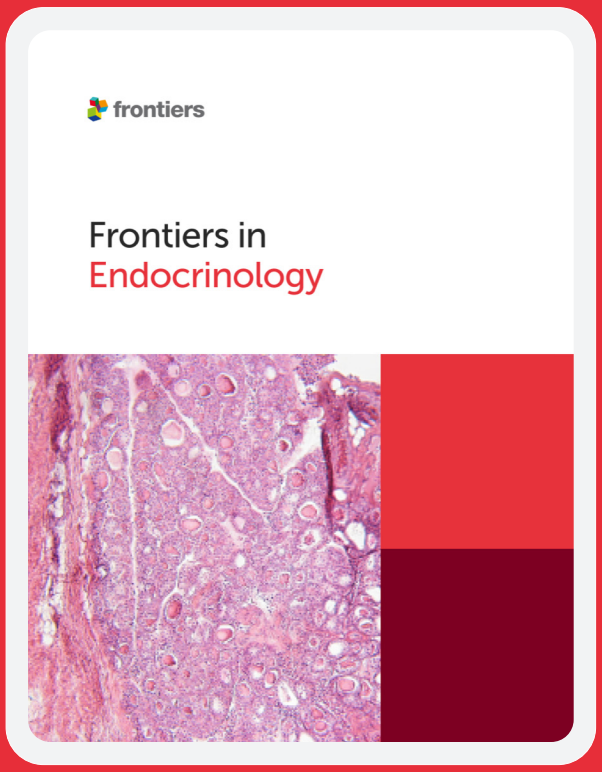

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