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Disruptors on male reproduction - emerging risk factors, volume II

Edited by
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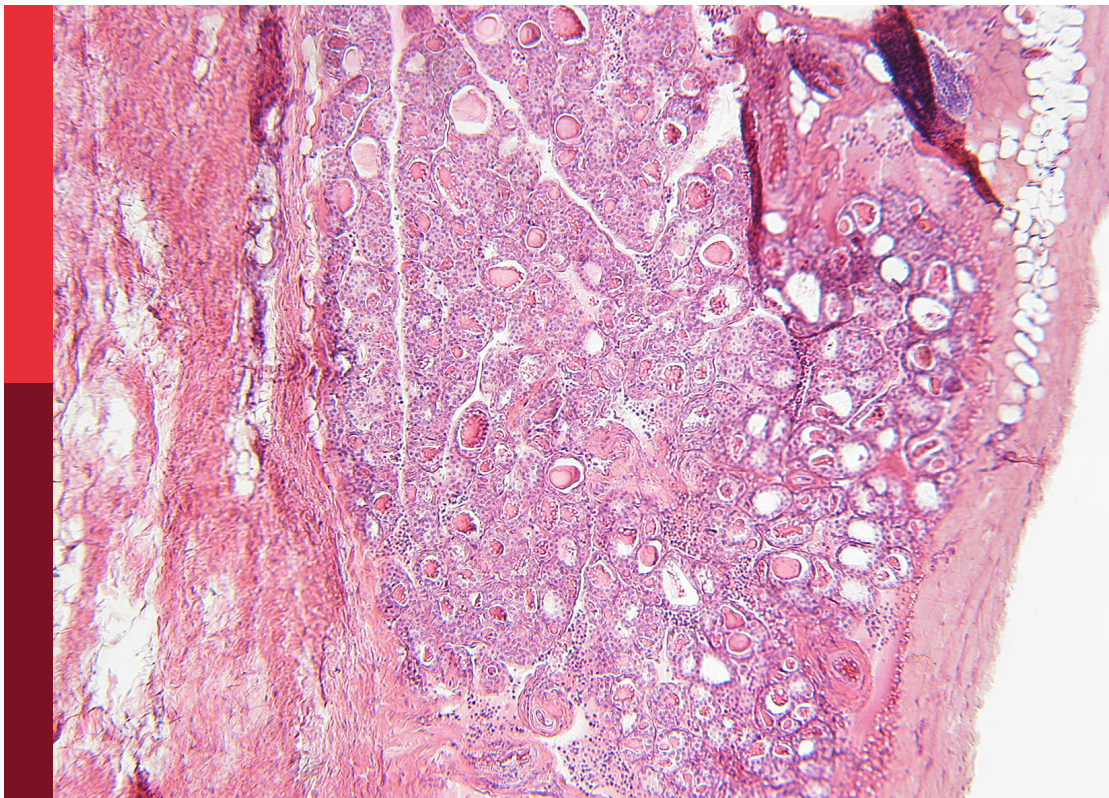

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Disruptors on male reproduction - emerging risk factors, volume II

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Table of contents

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Editorial: Disruptors on male reproduction—emerging risk factors, volume II

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KEYWORDS

disruptor, environment, male infertility, decline, sperm

Editorial on the Research Topic
Disruptors on male reproduction—emerging risk factors, volume II

Couple infertility represents a significant public issue affecting the health and financial, psychological, and social aspects of childbearing-aged couples. As reported by the World Health Organization (WHO), the number of infertile couples was 48 million in 2010 (1); thus, the current prevalence could be higher. In approximately half of these couples, a male infertility factor is identified, consistently with the presence of abnormal sperm parameters, such as abnormal number, motility, and/or morphology.

Epidemiological data indicate an increase in the prevalence of male infertility globally. The latest meta-regression analysis examining the sperm parameters of healthy subjects from all over the world reported that sperm concentration and total sperm count have halved in the last 40 years, with a higher slope after the 2000s (2). There is no apparent explanation for this evidence. Although some hypotheses have been suggested, no cause-effect relationship has been demonstrated so far. In addition, some research suggests that, despite a comprehensive diagnostic workup, there is no apparent explanation for male infertility in up to 70% of cases (3, 4), although this may be an overestimated data. Furthermore, considering the association between poor sperm quality and the greater risk of hospitalization, diabetes, cardiovascular disease, morbidity, mortality (5, 6), and cancer (7), the urgent need to better understand the etiology of male infertility and its correct treatment is easily understood.

Based on these premises, we launched this Research Topic in an attempt to collect evidence on disrupting molecules, which can – at least partially, explain the abovementioned data.

Some researchers tried to find the causes of the reduction in sperm counts in environmental pollution and its increase over the decades. Arato et al. investigated the effect of nickel oxide nanoparticles in porcine prepubertal Sertoli cells *in vitro*. They found a disruption in the cell function, proven by the altered expression and secretion of Anti-Müllerian Hormone and Inhibin B, MAPK signaling pathway, and cell viability, as supported by the increase in oxidative stress, DNA damage, and apoptosis. These

findings are interesting, considering that apoptosis of immature prepubertal Sertoli cells in childhood and adolescence can explain a reduced sperm count in adulthood since these cells lose the ability to proliferate after puberty (8).

Circadian rhythm has also been questioned as possibly being involved in male infertility. This represents an underestimated and scantily studied issue, deserving of more attention. An overview of the mechanisms by which abnormal circadian rhythm can disturb the hypothalamus-pituitary-gonadal axis is provided in the article by Li et al. Another topic on which there is scant information is the relationship between snoring and erectile dysfunction. Interestingly, Xiong et al. reported genetic evidence for the possible causal relationship between these two conditions.

Metabolic disorders have also been investigated in an attempt to understand their role in the downward trend of sperm count (9). Majzoub et al. focused on the impact of body composition on male sexual function and found a negative correlation between metabolic age, body weight, and fat composition with testosterone level and the International Index of Erectile Function 5-item score, in subjects younger than 40 years old. Tavlo et al. hypothesized the role of metformin as a reproductive toxicant, based on the available evidence already present on this drug, but also on its presence, documented globally, in freshwater and even drinking water.

Xiong et al. investigated the epigenetic profile of 36 patients with idiopathic non-obstructive azoospermia (NOA), reporting a dose-depended decrease in the global N6-metyladenosine (m6A) methylation, in patients with a higher degree of severity of the testicular histology (normal spermatogenesis, hypospermatogenesis, maturation arrest, and Sertoli-cell only syndrome). They also reported four downregulated genes that showed a significantly lower expression of m6A methylation.

Sperm DNA damage is a biofunctional marker of semen quality and it has recently been included in the latest WHO manual, due to the large number of data documenting its role in fertility. Sperm DNA fragmentation is the subject of two articles published in this Research Topic, both by Zhu et al.

Despite its increasing prevalence, management of male infertility is expensive for the patient, and patients often cannot afford the diagnostic and therapeutic workup required. The article by Wang et al. provides a feasibility analysis of incorporating infertility into medical insurance in China and underlies the challenges in undertaking assisted reproductive techniques from the patient's perspective.

Finally, research is urgently needed to understand the etiology of apparently idiopathic forms of male infertility, as well as the decline in sperm counts. This Research Topic attempts to partially undertake these aspects, although a huge amount of work still needs to be done. We are thankful to the authors who submitted their valuable research to our Research Topic.

Author contributions

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

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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ORIGINAL RESEARCH
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Genetic Evidence Supporting a Causal Role of Snoring in Erectile Dysfunction

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Background: The association between snoring and erectile dysfunction (ED) is inconsistent in multiple observational studies. To clarify the causal association of snoring on ED, we performed this two-sample Mendelian randomization study.

Materials and Methods: The single nucleotide polymorphisms (SNPs) associated with snoring were retrieved from the UK biobank cohort with 314,449 participants (117,812 cases and 196,637 controls). The summary statistics of ED were obtained from the European ancestry with 223,805 subjects (6,175 cases and 217,630 controls). Single-variable Mendelian randomization (MR) and multivariable MR were used to assess the causal relationship between snoring and ED.

Results: Snoring increases the risk of ED (Odds ratio [OR] = 3.45, 95% confidence interval [CI] = 1.68 - 7.09, $P < 0.001$) in the inverse variance weighting estimator. In sensitivity analyses, the ORs for the weighted median, MR robust adjusted profile score, and MR Pleiotropy Residual Sum and Outlier approach, MR-Egger, and maximum likelihood method are 5.70 (95% CI = 1.19 - 27.21, $P < 0.05$), 3.14 (95% CI = 1.01 - 9.72, $P < 0.05$), 3.11 (95% CI = 1.63 - 5.91, $P < 0.01$), 1.23 (95% CI = 0.01 – 679.73, $P > 0.05$), and 3.59 (95% CI = 1.07 – 12.00, $P < 0.05$), respectively. No heterogeneity and pleiotropy are observed (P for MR-Egger intercept = 0.748; P for global test = 0.997; P for Cochran's Q statistics > 0.05). After adjusting for total cholesterol, triglyceride, low-density lipoprotein, and cigarette consumption, the ORs for ED are 5.75 (95% CI = 1.80 - 18.34, $P < 0.01$), 4.16 (95% CI = 1.10 - 15.81, $P < 0.05$), 5.50 (95% CI = 1.62 - 18.69, $P < 0.01$), and 2.74 (95% CI = 1.06 - 7.10, $P < 0.05$), respectively.

Conclusion: This study provides genetic evidence supporting the causal role of snoring in ED.

Keywords: snoring, erectile dysfunction, causal estimates, Mendelian randomization, genetic evidence

INTRODUCTION

According to the definition of the National Institutes of Health (NIH) Consensus Development Panel on Impotence, erectile dysfunction (ED) refers to the inability to attain or maintain the penile erection, leading to unsatisfactory sexual intercourse (1). ED remains prevalent globally (2). As indicated by two key epidemiological surveys, the prevalence of ED ranges from 5% to 15% between 40-70 years in the Massachusetts Male Aging Study and on average, that is 30% across different age groups in the European Male Ageing Study (3, 4). It has been disclosed that ED patients have higher risks of depression, impaired self-esteem, infertility, stroke, and sub-clinical cardiovascular disease, which heavily burdens the males and further requires effective interventions to attenuate the high prevalence (5–8).

Multiple psychogenic or organic associated factors like depression, anxiety, and diabetes have been identified in the onset of ED (9). Of them, snoring is noted to be involved with ED (10). Snoring, a sort of sleep-disordered breathing, is a feature for obstructive sleep apnea (OSA), which commonly coexists with ED (11). However, the role of OSA is inconsistent across different literature. As indicated by Kalejaiye O et al. (12), males with OSA had significantly lower erectile function than the control group. According to the findings from Hanak V et al. (13), the sexual satisfaction domain score for the snorers was lower than that for the none/mild snorers. But, the levels of erectile function remained similar across different snoring categories. Additionally, in other previous studies exploring the association between OSA and ED, the results fluctuated markedly when adjusting different covariates, and even further yield opposite conclusions (14, 15). The discrepancy may be attributed to the limited sample size, cross-sectional design, and especially confounding factors. These defects cannot be overcome by the observational design and new method, such as Mendelian randomization (MR), is required to clarify the inconsistent findings, which are still absent.

MR is an epidemiological method using genetic variants to produce causal inference (16). The genetic variants are single nucleotide polymorphisms (SNPs) identified by genome-wide association studies (GWAS). When forming a zygote, the SNPs are assorted randomly regardless of postnatal confounding factors like diabetes, obesity, and hypertension (17). The random distributed SNPs are used as instrumental variables (IVs) to replace the exposures (i.e. snoring) and outcomes (i.e. ED). Therefore, a naturally formed randomized controlled trial (RCT) is imitated, which avoids reverse causality and biases from confounders, and then yields causal estimates (17). To date, no study has clarified the causal links between snoring and ED. To address this concern, we use the conventional single-variable MR (SVMR) and multivariable MR (MVMR) design to explore whether snoring is causally involved with the onset of ED.

MATERIALS AND METHODS

Data Sources of Snoring, ED, and Adjusted Covariates

The genetic estimates for snoring were derived from the white British ancestry extracted from the Neale Lab (https://gwas.mrcieu.ac.uk/datasets/ukb-a-14/). The snoring cohort included 314,449 subjects (117,812 cases and 196,637 controls). Participants were defined as a snorer according to the positive answer to a touchscreen question: "Does your partner or a close relative or friend complain about your snoring?" (18). Missing data and uninformative reports like "I don' know" and "Prefer not to answer" were excluded from the cohort.

The summary level dataset of ED was retrieved from one previous GWAS study in the European ancestry (19). By incorporating three cohorts, the combined cohort enrolled 223,805 subjects (6,175 cases and 217,630 controls), which had stronger inference power. The diagnosis of ED was based on the International Classification of Diseases version 10 (ICD-10) codes (N48.4 and F52.2), or a medical intervention history for ED like surgery or oral drugs, or self-report from the participants. Detailed information regarding the two phenotypes can be further accessed through previous publications (18, 19).

Summary-level statistics of the adjusted covariates including the low-density lipoprotein (LDL), cigarette consumption, total cholesterol (TC), and triglyceride (TG) were retrieved from previous studies (20, 21). After excluding the participants from 23&Me project, a sample size of 1.2 million individuals were subjected to detect the genetic estimates of cigarette consumption (20). To obtain the genetic estimates of lipid levels, the European population genotyped with GWAS arrays4 or Metabochip array were enrolled into analyses. A total of 188,577 participants were included (21). Detailed information regarding the ancestry, sample sizes, consortium, etc. were described in **Supplemental Table 1**.

Instrument Variable Selection

To identify SNPs closely associated with snoring, genetic instruments with genome-wide statistical significance $\geq 5 \times 10^{-8}$ were filtered. Further, to identify the independent SNPs assorted randomly during gestation, the left SNPs were then subjected to the calculation of linkage disequilibrium (LD) using the PLINK clumping approach. The LD was calculated based on 1000 Genomics European reference panel. SNPs with LD $r^2 \geq 0.001$ at a window size of 10,000 Kb were pruned. Additionally, the MR-Steiger filtering was applied to calculate the variance explained in the exposure (i.e. snoring) and the outcome (i.e. ED) and further test whether the variance explained in snoring is significantly higher than that in ED or not (22). The insignificant results indicate a reverse causal direction that the extracted SNPs may primarily affect the ED than the snoring, which should be removed. In this study, all the extracted SNPs passed the test and were not excluded. Besides, the palindromic SNPs were deleted from the extracted SNPs.

To avoid the bias from weak instrumental variables (IVs), *F*-statistics were calculated, using the following formula: *F*-statistics = (Beta/Se) ^{2}. *F*-statistics represent the strength of IVs and the mean of *F*-statistics was calculated as the overall statistics. Generally, *F*-statistics > 10 were set as the threshold of strong IVs. The *F*-statistics of all the extracted SNPs were > 10. Therefore, no SNPs were excluded in this step. Moreover, to reduce the heterogeneity and avoid the pleiotropy, radial-MR

and MR Pleiotropy Residual Sum and Outlier (MR-PRESSO) methods were used to identify the horizontal pleiotropic outliers (23, 24). No outliers were detected in this study. All the left SNPs were available in the outcome dataset. Hence, no proxy SNP was used in the MR analyses. Finally, 19 SNPs were left and used as IVs in this study. The detailed information of the IVs is displayed in **Table 1**.

Statistical Analyses

To obtain the total and direct causal estimates between snoring and ED, the SVMR, and MVMR were performed. The causal estimates were assessed using the inverse variance weighting (IVW) approach. The IVW method uses the meta-analysis technique to combine the effects from individual IVs to an overall weighted effect (25). When all the SNPs are valid IVs, this approach can produce consistent estimation, which is considered the main result in MR analyses.

The inclusion of more IVs can increase the statistical power in MR analyses, which yet may introduce the pleiotropic SNPs into analyses. The pleiotropy refers to the association between the IVs and other confounders except for the exposure (i.e. snoring in this study). This association can mediate the exposure-outcome links through other pathways, violating the basic assumption of MR and yielding biased estimates. To test and avoid the pleiotropy, several sensitivity analyses including MR-Egger, the weighed median, MR-PRESSO, and robust adjusted profile score (MR.RAPS) were performed.

MR-Egger method is adapted from Egger regression. This approach performs a weighted linear regression and introduces an intercept term into the regression function. As indicated by Bowden J (26), the MR-Egger estimator can produce unbiased estimates even when all the IVs are invalid IVs. Additionally, the distance between the introduced intercept term and zero can be adopted to quantify the directional pleiotropy. As for the weighted median estimator, it can yield consistent causal estimates when half of the IVs are valid (27). This method has greater precision in the estimates than the MR-Egger approach and better type 1 error rates than the IVW estimator. In this study, we also calculated MR.RAPS to assess the causal association between snoring and ED (28). By performing a linear model adjusting for the profile likelihood of the summary data, MR.RAPS can yield robust causal estimates. This estimator considers the weak IVs bias and remains consistent when weak IVs exist. As reported by Zhao et al., MR.RAPS displays higher statistical power than other conventional MR estimators and is statistically sound to both systematic and idiosyncratic pleiotropy (28).

Heterogeneity is another major concern in MR analyses, suggesting the possible concurrent presence of pleiotropy. To evaluate the heterogeneity between the IVs, IVW, MR-Egger, and Maximum likelihood methods were used. Cochran's Q statistic was employed to quantify the heterogeneity. Further, we also adopted the leave-one-out analysis to identify the influential IVs on the estimates. This approach excluded one IV at a time and then performed MR analysis again using the IVW method.

The statistical power to detect the difference is evaluated using an online tool (https://shiny.cnsgenomics.com/mRnd/). Under the type I error rate of 0.05, the statistical power of snoring on ED is 100%. Moreover, the overlap and bias are calculated using an online software (https://sb452.shinyapps.io/overlap/). Under the type I error rate of 0.05 and assuming the overlap proportion is 100%, the value of bias is 0.026. This indicates that the overlap of the population is less likely to bias the finding.

Metabolisms are well-known risk factors for the occurrence of ED. And the snorers are usually correlated with disordered metabolisms. To obtain the direct effect from snoring, LDL, TC, TG and cigarette consumption were considered into further MVMR analyses. For the MVMR analyses, the overlapping SNPs between snoring and adjusted factors were used as the IVs. The IVW estimator was employed to yield the direct causal effect estimates after controlling the LDL, cigarette consumption, TC, and TG, respectively.

TABLE 1 | SNPs used as genetic instruments in the Mendelian randomization analyses.

SNP	Chr	Position	A1	A2	BETA	SE	P	Palindromic	F	Gene
rs10062026	5	90052289	A	G	0.006976	0.001246	2.14E-08	FALSE	31.36	ADGRV1
rs10505911	12	24022160	A	C	-0.00796	0.001446	3.72E-08	FALSE	30.29	SOX5
rs10878271	12	65795603	C	T	0.007551	0.001244	1.28E-09	FALSE	36.84	MSRB3
rs11075985	16	53805207	A	C	-0.00738	0.001212	1.11E-09	FALSE	37.13	FTO
rs1108431	16	31054607	T	C	-0.00805	0.001237	7.61E-11	FALSE	42.36	STX4
rs12925525	16	1773914	T	G	-0.01341	0.002392	2.07E-08	FALSE	31.43	MAPK8IP3
rs13251292	8	71474355	G	A	-0.00803	0.001222	4.97E-11	FALSE	43.19	TRAM1
rs1641511	17	7559677	A	G	0.007981	0.001413	1.62E-08	FALSE	31.9	ATP1B2
rs1775550	10	9052742	A	G	0.009168	0.001533	2.21E-09	FALSE	35.78	RP11-428L9.2
rs199497	17	44866602	C	T	-0.01043	0.001648	2.48E-10	FALSE	40.05	WNT3
rs2307111	5	75003678	C	T	0.007038	0.001226	9.52E-09	FALSE	32.94	POC5
rs2614464	14	99743113	A	G	0.007823	0.001212	1.10E-10	FALSE	41.64	BCL11B
rs34811474	4	25408838	A	G	0.008518	0.001416	1.81E-09	FALSE	36.17	ANAPC4
rs592333	13	51340315	G	A	-0.00896	0.001204	1.01E-13	FALSE	55.36	DLEU7
rs61597598	2	1.57E+08	A	G	-0.01212	0.001752	4.63E-12	FALSE	47.84	LINC01876
rs62066451	17	46316540	G	A	0.016689	0.002789	2.17E-09	FALSE	35.81	SKAP1
rs7930256	11	88849434	C	T	-0.00705	0.00125	1.70E-08	FALSE	31.81	AP001482.1
rs9309771	3	77593064	G	A	0.007558	0.001202	3.22E-10	FALSE	39.54	ROBO2
rs9515311	13	1.12E+08	T	C	0.006995	0.001238	1.63E-08	FALSE	31.9	ANKRD10

All the SVMR and MVMR analyses and relevant figures were made by R 3.6.5 (R Foundation for Statistical Computing, Vienna, Austria), using the "TwoSampleMR", "RadialMR", "mr.raps", and "forestplot" packages. $P < 0.05$ (two-sided) is set as the significant threshold in statistics.

RESULTS

Causal Effect Estimates of Snoring on ED in SVMR

The causal effect estimates of snoring on ED are displayed in **Figure 1**. In **Figure 1**, the IVW estimator reveals that snoring is associated with a 3.45-fold risk of ED (95% confidence interval [CI] = 1.68 - 7.09, $P < 0.001$). In sensitivity analyses, the odds ratios (ORs) for the weighted median, MR robust adjusted profile score, and MR Pleiotropy Residual Sum and Outlier approach, MR-Egger, and maximum likelihood method are 5.70 (95% CI = 1.19 - 27.21, $P < 0.05$), 3.14 (95% CI = 1.01 - 9.72, $P < 0.05$), 3.11 (95% CI = 1.63 - 5.91, $P < 0.01$), 1.23 (95% CI = 0.01 – 679.73, $P > 0.05$), and 3.59 (95% CI = 1.07 – 12.00, $P < 0.05$), respectively (**Figure 1** and **Table 2**). As indicated in **Figure 2**, with the increase of IVs' effect on snoring, the risk of ED increases.

Additionally, in **Table 2**, the MR-Egger test detects no directional pleiotropy (Intercept = 0.0087, $P = 0.748$). The MR-PRESSO test also finds no pleiotropy (Global test $P = 0.997$). The Cochran's Q statistics are 6.42 ($P = 0.989$), 6.53 ($P = 0.994$), and 6.50 ($P = 0.994$) for the MR-Egger, IVW, and Maximum likelihood method, respectively (**Table 2**), suggesting the absence of heterogeneity. The funnel plot visualizing the heterogeneity is displayed in **Supplemental Figure 1**. The leave-one-out analysis identifies no influential IVs in the association between snoring and ED (**Supplemental Figure 2**). The estimates from each IV are visualized in **Supplemental Figure 3**.

Causal Effect Estimates of Snoring on ED in MVMR

As shown in **Figure 3**, after adjusting for TC, TG, LDL, and cigarette consumption, the ORs for ED are 5.75 (95% CI = 1.80 - 18.34, $P < 0.01$), 4.16 (95% CI = 1.10 - 15.81, $P < 0.05$), 5.50 (95% CI = 1.62 - 18.69, $P < 0.01$), and 2.74 (95% CI = 1.06 - 7.10, $P < 0.05$), respectively. The scatter plots of the SNP-snoring association against SNP-ED association are shown in **Figure 4A** (controlling for LDL), **Figure 4B** (controlling for smoking), **Figure 4C** (controlling for TC), and **Figure 4D** (controlling for TG), respectively.

DISCUSSION

Given the absence of rigorously controlled clinical trials and longitudinal prospective studies, the inconsistent association of snoring on ED is hard to be clarified. Under the framework of MR design, this study provides causal evidence that the snorers have a higher risk of ED.

The loud snoring is an indicator of OSA, which has been found to be correlated with carotid atherosclerosis, coronary heart disease, and hypertension (11, 29, 30). The majority of the cross-sectional and case-control studies reported that OSA was negatively associated with erectile function. In a cross-sectional study with 467 participants, Andersen ML et al. (31) found that males with OSA had a 2.13-fold risk of ED than the controls. Similar findings are also reported by Heruti R et al. in Israelite (32). In the biggest case-control study, Petersen M et al. recruited 308 OSA cases and 1185 controls and disclosed that OSA patients had worse general and functional sexuality than the healthy counterparts. Besides the cross-sectional design, Chen et al. revealed a 9.44-fold risk of ED in the OSA patients than the control group in a longitudinal cohort enrolling 53,335 respondents (33).

Contrary to the findings stated above, there is accumulating evidence supporting the unrelated OSA in the suffering of ED. As disclosed by Bozorgmehri S et al. (34), males with higher apnea-hypopnea index displayed similar erectile function assessed by the 5-item International Index of Erectile Function. This cross-sectional study included 2,857 American men. Additionally, in the US, Hanak V et al. recruited 827 men using the stratified random sampling method and detected no association between

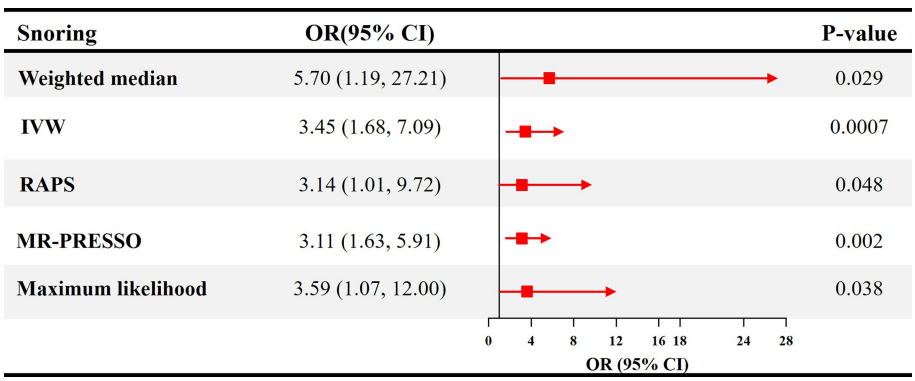

FIGURE 1 | Causal estimates of snoring on ED in SVMR. OR, odds ratio; CI, confidence interval; IVW, Inverse variance weighted method; RAPS, robust adjusted profile score; MR, mendelian randomization; PRESSO, pleiotropy residual sum and outlier; ED, erectile dysfunction; SVMR, single-variable mendelian randomization.

TABLE 2 | MR estimates from each method of the causal effect of snoring on ED.

MR method	OR	95% CI	*P* value	Cochran's Q statistic	Heterogeneity *P* value	MR-Egger intercept	Intercept *P* value
MR-Egger	1.23	0.01 - 679.73	0.949	6.42	0.989	0.0087	0.748
IVW	3.45	1.68 - 7.09	0.0007	6.53	0.994	–	–
Maximum likelihood method	3.59	1.07 - 12.00	0.038	6.50	0.994	–	–

MR, Mendelian randomization; OR, odds ratio; SE, standard error; CI, confidence interval; ED, erectile dysfunction; IVW, Inverse variance weighted method.

OSA and ED. These findings were in line with one previous study (35). The inconsistent findings may be owing to the limited sample size, study design, and more importantly, the confounders, which are addressed in our study. In addition, only a few previous studies were prospective and defined the direction of causality. Our findings indicate that snoring increases the risk of erectile dysfunction, instead of the contrary. A clear causal direction facilitates guiding clinical decision-making.

Although the causal association of snoring on ED is established, the specific molecular mechanisms still need further exploration. Vascular endothelial dysfunction in OSA patients has been noted over the years, which may be responsible for linking OSA to ED. In OSA patients, sleep fragmentation and intermittent hypoxia trigger elastic fiber disruption, fiber disorganization, and reduced endothelial nitric oxide (NO) bioavailability of the blood vessel, indicating the impairment of vascular reactivity (36, 37). Of them, NO acts as a pivotal role in mediating the relaxation of penile blood vessels and cavernous smooth muscle through the NO/cGMP pathway (38). Consequently, reduced endothelial NO bioavailability harms the penis erection and leads to ED. In clinical studies, sildenafil, an inhibitor of cGMP degradation, can improve erectile function in OSA patients with ED, with higher satisfaction than continuous positive airway pressure (CPAP) (39). However, given the high proportion of dissatisfaction in both sildenafil and CPAP groups (50% versus 75%), the therapeutic benefits may be limited. This indicates that several other pathways may also involve in the onset of ED in OSA patients. Therapy targeting different pathways should be explored in future studies.

This study has some merits and shortcomings. The main merit is the MR framework, which overcomes the endogeneity and bias from confounding factors. Given the difficulties of RCT, this study paves the way for the prevention of impotence by targeting OSA. In addition, the included samples were confined to European descent, avoiding the population architecture bias but limiting the generalizability of our findings. Moreover, there may be a partial overlap in the samples of snoring and ED, possibly leading to the over-fitting of the models and undermining the causal inference power (40). However, given the usage of strong IVs (*F*-statistics > 10) in the analyses, the bias may be minimal. Besides, in light of the binary evaluation of snoring (snorers or controls) and the lack of individual statistics, the non-linear association between snoring and ED cannot be explored (40).

In conclusion, this study provides genetic evidence supporting a causal role of snoring in the onset of ED, independent of LDL, TC, TG, and cigarette consumption. Medical interventions should be considered for snorers to attenuate the high prevalence of ED.

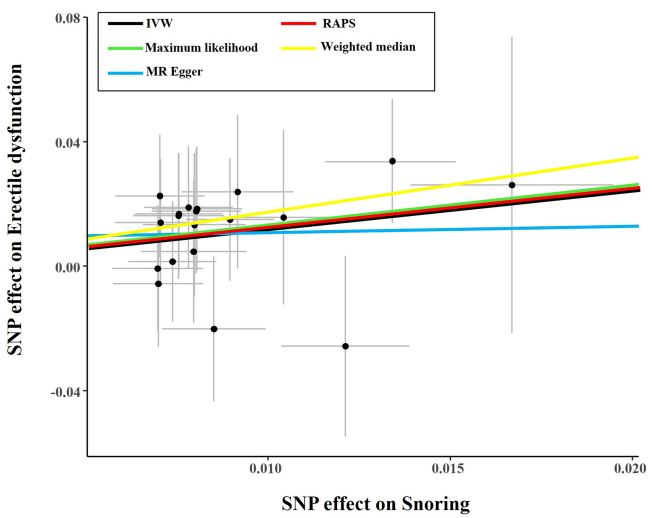

FIGURE 2 | Scatter plot of the effect size of each SNP on snoring and ED in SVMR. SNP, single nucleotide polymorphism; IVW, Inverse variance weighted method; ED, erectile dysfunction; MR, mendelian randomization; SVMR, single-variable mendelian randomization.

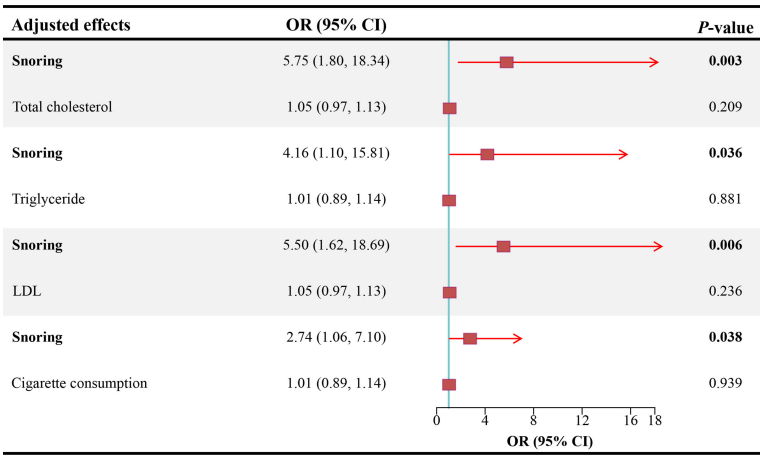

FIGURE 3 | Causal estimates of snoring on ED in MVMR. OR, odds ratio; CI, confidence interval; LDL, low-density lipoprotein; ED, erectile dysfunction; MVMR, multivariable mendelian randomization.

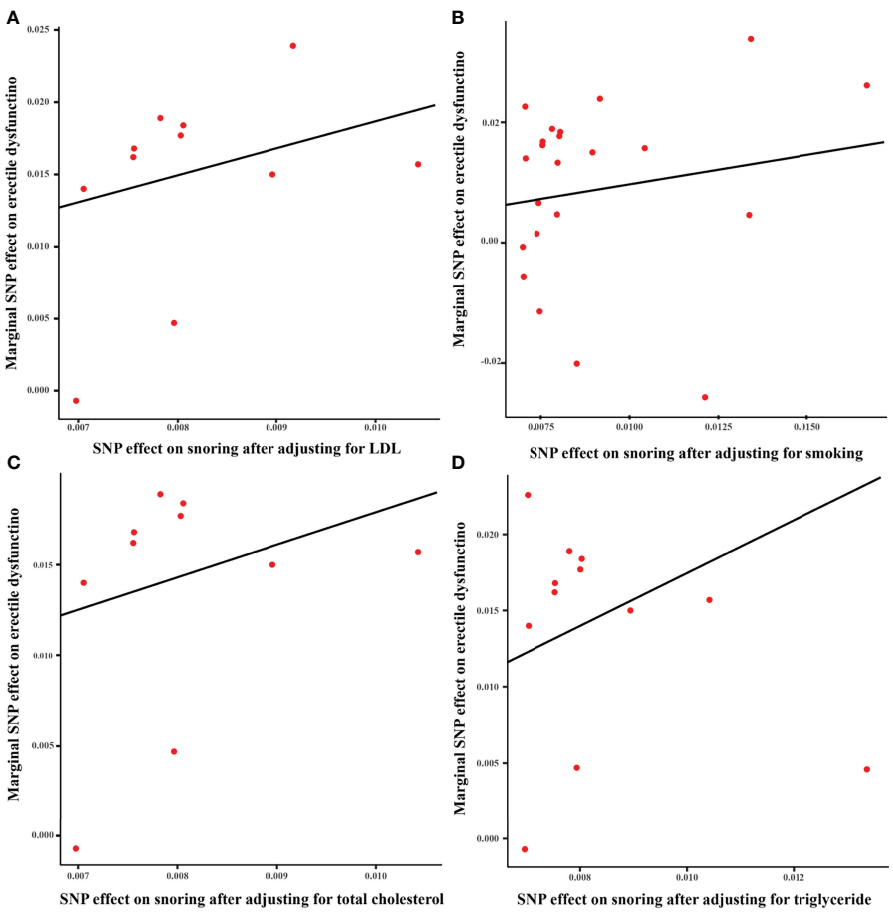

FIGURE 4 | Scatter plot of the effect size of each SNP on snoring and ED in MVMR. **(A)** Scatter plot adjusting for LDL; **(B)** Scatter plot adjusting for smoking; **(C)** Scatter plot adjusting for total cholesterol; **(D)** Scatter plot adjusting for triglyceride. SNP, single nucleotide polymorphism; LDL, low-density lipoprotein; ED, erectile dysfunction; MVMR, multivariable mendelian randomization.

CONCLUSIONS

This study provides genetic evidence supporting a causal role of snoring in ED.

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

Ethical review and approval were 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.

AUTHOR CONTRIBUTIONS

YX and XZ performed the data analyses and wrote the manuscript; WW, FZ, YZ, CW, and FQ revised the manuscript; JY participated in the study design and helped draft the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.896369/full#supplementary-material

Supplementary Figure 1 | The funnel plot in SVMR. IVW, Inverse variance weighted method; SVMR, single-variable mendelian randomization. IV, instrumental variable; SE, standard error; MR, mendelian randomization.

Supplementary Figure 2 | The leave-one-out sensitivity analyses of snoring and ED. SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval; ED, erectile dysfunction; MR, mendelian randomization.

Supplementary Figure 3 | The results of MR analyses of causal associations between each snoring SNP and ED. SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval; ED, erectile dysfunction; MR, mendelian randomization.

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Influence of sperm DNA fragmentation on the clinical outcome of *in vitro* fertilization-embryo transfer (IVF-ET)

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Purpose: To evaluate the effect of elevated sperm DNA fragmentation index (DFI) on fresh and frozen embryo transfer cycles.

Methods: A retrospective study was performed with 549 fresh embryo transfer cycles and 1340 frozen embryo transfer cycles after *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) from 2016 to 2021.

Results: The statistical results of 549 fresh embryo transfer cycles showed that the delivery rate in the normal sperm DFI group (43.9% vs. 27.1%, P = 0.014) was significantly higher than that in the abnormal sperm DFI group, and there were no significant differences in the biochemical pregnancy rate (59.0% vs. 50.8%, P = 0.232), clinical pregnancy rate (53.1% vs. 40.7%, P = 0.072), or miscarriage rate (17.3% vs. 33.3%, P = 0.098) between the two groups. The results of 1340 frozen embryo transfer cycles showed that the biochemical pregnancy rate (57.9% vs. 45.6%, P = 0.006) and clinical pregnancy rate (50.3% vs. 40.7%, P = 0.027) in the normal sperm DFI group were significantly higher than those in the abnormal sperm DFI group. The delivery rate (40.9% vs. 33.3%, P = 0.074) and miscarriage rate (18.6% vs. 18.0%, P = 0.919) were not significantly different between the two groups.

Conclusion: The increase of sperm DFI significantly reduced the delivery rate of fresh embryo transfer cycles and the biochemical pregnancy rate and clinical pregnancy rate of frozen embryo transfer cycles.

KEYWORDS

sperm DNA fragmentation, *in vitro* fertilization, intracytoplasmic sperm injection, fresh embryo transfer, frozen embryo transfer

Introduction

With the development of assisted reproductive technology (ART), new assisted reproductive methods and technologies continue to emerge. In addition to intrauterine insemination (IUI), the commonly used forms of ART include conventional *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (1). In the assessment of male infertility, traditional semen analysis still has some limitations. Conventional semen analysis cannot detect subcellular sperm dysfunction, and sperm defects may lead to multiple IUIs and the inability to achieve pregnancy. Studies have shown no significant differences in routine semen parameters between fertile and infertile men, and even semen parameters in the "normal" range may contribute to differences in pregnancy outcomes (2, 3).

During spermatogenesis, germ cells undergo mitosis and meiosis to produce haploid sperm. Sperm are highly differentiated male germ cells consisting of a head, midsection and tail. The head contains the haploid genome, which is required for successful fertilization and is transferred to the oocyte. During sperm maturation, chromatin is highly condensed, the protamine process of sperm DNA is dysregulated or some germ cells fail to undergo apoptosis, thereby escaping the programmed phagocytosis process and forming defective mature sperm, often with an increased sperm DFI (4). Lin found that an increased DFI in hyperspermia was associated with increased miscarriage rates, but the effect was not significant (5). Zini (6) and Kenned (7) reported that sperm DNA damage resulted in a significant increase in the rate of miscarriage, while Virro (8) and Dar (9) found that the sperm DFI had no significant effect on the fertilization rate. Boe-Hansen found no significant effect of sperm DFI on biochemical pregnancy, clinical pregnancy, or embryo implantation (10). Oleszczuk reported that the sperm DFI significantly affects the live birth rate, high-quality embryo rate, and miscarriage rate (11). Green reported that the sperm DFI had no significant effect on the fertilization rate, blastocyst formation rate, implantation rate, ongoing pregnancy rate, or miscarriage rate (12).

The impact of sperm DNA fragmentation on IVF-embryo transfer (ET) is still controversial. In this study, semen specimens from men in IVF-ET cycles were collected, and the sperm DFI was detected to explore the effect of sperm DNA fragmentation on the clinical outcomes of IVF-ET.

Materials and methods

Study design and population

From January 2016 to April 2021, controlled ovarian hyperstimulation (COH) was performed at the Reproductive Medical Center of Subei People's Hospital, and routine IVF/ICSI-ET was performed after egg retrieval. The exclusion criteria were as follows: (1) the woman's B-ultrasound showed severe unilateral or bilateral hydrosalpinx; (2) the woman had polycystic ovary syndrome, high blood pressure, prolactinaemia, abnormal thyroid function, diabetes and other diseases; (3) the presence of uterine fibroids (diameter ≥ 4 cm or submucosal uterine fibroids), uterine malformation, endometriosis grades I-IV, intrauterine adhesions or other gynaecological complications; or (4) there were chromosomal abnormalities in the male/female. Regarding grouping, according to the analysis results obtained with the DFIViewer software, the men were divided into a normal sperm DFI group (DFI < 30%) and an abnormal sperm group (DFI ≥ 30%) according to their sperm DFI levels.

Semen collection and routine analysis

The men abstained from sex for 2-7 days, and sperm were collected by masturbation. Routine semen processing analysis was performed according to the Laboratory Manual for Human Semen Examination and Processing, 5th Edition (13). The semen quality was analysed and recorded by using a computer-aided semen analyser (Beijing Suijia Medical Instrument) and checked manually. Freshly liquefied semen smears were air-dried and stained with a modified Pasteur method to record sperm morphology.

Analysis of sperm DNA fragmentation rate

Sperm DNA fragmentation assay (SDFA) was performed using the Sperm Chromatin Analysis (SCSA) kit (Zhejiang Cellpro Biotech Co., Ltd., Ningbo, China) strictly in accordance with the product instructions (14). The detailed analysis process was as follows. First, appropriate volume of semen were added into 0.1 mL of solution A (TNE buffer, sperm dilution) and mixed. Then, 0.2 mL of solution B (acid solution of 0.1% Triton X-100, 0.15 mol/L NaCl, and 0.08 mol/L HCl, pH 1.2) were added and mixed. After standing for 30 s, 0.6 mL of acridine orange (AO) staining solution (6 μg/ml AO, 37 mmol/L citric acid, 126 mmol/L Na_2HPO4, 1 mmol/L Na_2EDTA, 0.15 mol/L NaCl, pH 6.0) was added and mixed. After sperm were stained for 3 min, the sperm DFI was detected by a flow cytometer (FACS Calibur, BD Bioscience, San Jose, CA, USA). A minimum of 5,000 sperm were acquired, and the data were analysed by the software (DFIView 2010 Alpha11.15, CellPro Biotech, Ningbo, China).The sperm DFI was expressed as the percentage of sperm with fragmented DNA compared to the total number of sperm. The variability of the replicate DFI measures was less than 5%.

Semen optimization for IVF/ICSI

The semen was collected 2 hours before IVF, the men abstained for 2-3 days, and the semen was collected into a sterilized disposable wide-mouth collector. After checking the man's name by fingerprint identification, the sperm spots were collected and placed in a 37°C incubator for incubation and liquefaction. After liquefaction, the semen was evaluated and recorded. The density gradient centrifugation method was used to optimize the semen. The specific operation steps were as follows. (1) The gradient centrifugation medium of 80% and 40% SpermGrad (Swedish Vitrolife Company) with two different concentrations was preheated in a 37°C incubator. (2) First, 1 ml of 80% high-concentration gradient centrifuge medium was added to the sterile conical centrifuge tube with a pipette, and then 1 ml of 40% low-concentration gradient medium was slowly added on top of it while being careful not to damage the interface between the two layers of gradient solution. Then 2 ml of liquefied semen was added (adding too much semen will cause overload and affect the separation effect). According to the specific conditions of the semen, the amount of gradient centrifugation fluid was adjusted, or the number of centrifuge tubes was increased. (3) The samples were placed in a centrifuge at 300-400 × g for 15 minutes, the supernatant and gradient solution were removed, and only approximately 0.5 ml of sperm pellet was taken from the bottom. Then, 3 ml of upstream insemination solution was added, mixed well, and transferred to a Falcon 1006 centrifuge tube. (4) The tube was placed into the centrifuge at 300-400 × g and centrifuged for 5 minutes. The supernatant was removed, the sperm precipitate that was visible at the bottom of the tube was obtained, and 0.5 mL of upstream insemination solution (IVF solution) was added. (5) The Falcon 1006 centrifuge tube was tilted at an angle of 30-45 degrees and placed in a 37°C, 6% CO_2-saturated humidity incubator for upstream treatment. After 40 minutes, the upper sperm suspension was aspirated into another clean Falcon 1006 centrifuge tube to evaluate IVF use.

IVF-ET

Drug-based ovarian stimulation was performed to stimulate follicle development until two or more follicles reached an average diameter of 18 mm. Then, a trigger was applied to induce final maturation of the developing oocytes, and approximately 36 hours after the trigger, oocytes were retrieved under transvaginal ultrasound guidance. On the day of egg retrieval, routine IVF was performed, and rescue ICSI (RICSI) was performed if no fertilization or low fertilization occurred. Progression through the cleavage stage was regularly monitored every day. After exclusion of transfer contraindications, ET or whole-embryo freezing was performed according to the condition of the woman and the embryos. Frozen embryo transfer was performed after frozen embryos were thawed. A blood test was performed 14-16 days after transplantation to measure the β-hCG level in peripheral blood to determine whether a biochemical pregnancy (more than 5.0 mIU/ml is diagnosed as biochemical pregnancy) was present. Luteal support was given in cases of biochemical pregnancy, and the presence of clinical pregnancy was determined 4-5 weeks after transplantation.

Data statistics and analysis

Statistical analysis was performed using IBM SPSS Statistics 22.0 version 25 (IBM Corp., Armonk, NY, USA). Categorical variables were presented as frequencies and percentages, whereas continuous variables were reported as the means ± standard deviations (SDs) or as the medians and interquartile ranges (IQRs, 25th-75th percentile). The normality of the distribution of the variables was determined using the Kolmogorov-Smirnov test. Normally distributed data were expressed as the means and standard deviations, while the medians and IQRs were used for nonnormally distributed data. Correlation analysis was performed by the Pearson method or Spearman method The chi-square or Fisher's exact test was used to compare categorical variables. $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered extremely significant.

Results and analysis

General statistics

A total of 1638 IVF/ICSI cycles were included in this study. The average age of the women was 30.56 years, the average age of the men was 31.79 years, and the average infertility period was 3.46 years. Among them, there were 1329 cases of conventional IVF cycles, 218 cases of ICSI cycles, 89 cases of IVF-RICSI cycles, and 2 cases of IVF-ICSI cycles. The Kolmogorov-Smirnov (K-S) test results were showed that the data did not obey a normal distribution ($P < 0.01$) (Table 1).

Comparison of related parameters between the normal group and abnormal group in fresh embryo transfer cycles

A total of 549 fresh ET cycles in the IVF cycle were selected and grouped by the sperm DFI value, 490 cases were in the normal sperm DFI group (89.3%), and 59 cases were in the

TABLE 1 Basic clinical data of the *in vitro* fertilization cycles.

	Median	Mean	Bias	Kurtosis	K-S *P* value
Female age (years)	30.00 (27.00-33.00)	30.56 ± 4.49	0.867	0.506	<0.001**
Male age (years)	31.00 (28.00-34.00)	31.79 ± 5.39	1.385	2.508	<0.001**
Infertility years	3.00 (2.00-4.50)	3.46 ± 2.62	2.236	7.816	<0.001**
Female BMI	22.30 (20.20-24.50)	22.56 ± 3.33	1.283	6.031	<0.001**
Male BMI	24.73 (22.46-27.36)	24.98 ± 3.82	0.482	3.263	0.001**
Abstinence days	4.00 (3.00-5.00)	4.16 ± 2.13	6.665	93.055	<0.001**
Semen volume (ml)	3.80 (2.70-5.10)	4.17 ± 2.01	0.894	0.839	<0.001**
Sperm concentration (10^6/ml)	65.30 (35.51-102.0)	71.54 ± 47.65	1.374	6.205	<0.001**
Sperm motility (%)	58.00 (42.98-72.00)	56.14 ± 20.59	-0.466	-0.283	<0.001**
Sperm progressive motility (%)	48.00 (34.00-61.00)	46.86 ± 19.02	-0.317	-0.483	<0.001**
Sperm nonprogressive motility (%)	7.00 (4.00-14.00)	9.27 ± 7.10	1.378	4.664	<0.001**
Sperm immotility (%)	42.00 (28.00-57.11)	43.80 ± 20.37	0.455	-0.297	<0.001**
DFI (%)	14.79 (9.60-22.30)	17.50 ± 11.58	1.766	4.540	<0.001**
HDS (%)	6.05 (4.25-8.72)	7.24 ± 4.94	3.064	16.567	<0.001**

*(P < 0.01), **P < 0.01.

abnormal sperm DFI group (10.7%). The sperm concentration (P = 0.014), sperm motility (P < 0.001), and the percentage of sperm showing forward motility (P < 0.001) in the normal sperm DFI group were significantly higher than those in the abnormal sperm DFI group, and the abstinence days (P = 0.002) and sperm immobility percentage (P < 0.001) were significantly lower than those in the abnormal sperm DFI group. There were insignificant differences in female age (P = 0.222), male age (P = 0.208), infertility years (P = 0.941), female BMI (P = 0.917), male BMI (P = 0.204), endometrial thickness (P = 0.194), sperm high-staining HDS rate (P = 0.433), semen volume (P = 0.740), and sperm nonprogressive motility percentage (P = 0.056) between the two groups (Table 2).

Comparison of clinical outcomes between the normal and abnormal sperm DFI group in fresh embryo transfer cycles

Among the 549 cycles of fresh ET, there were 490 cases of normal sperm DFI, 289 cases of biochemical pregnancy (59.0%), 260 cases of clinical pregnancy (53.1%), 215 cases of childbirth (43.9%), and 45 cases of miscarriage (17.3%). There were 59 cases of abnormal sperm DFI, 30 cases of biochemical pregnancy (50.8%), 24 cases of clinical pregnancy (40.7%), 16 cases of childbirth (27.1%), and 8 cases of miscarriage (33.3%). The results showed that the biochemical pregnancy rate (59.0% vs.

TABLE 2 Comparison of related parameters between the normal DFI group and abnormal DFI group in fresh embryo transfer cycles.

	Normal group (DFI<30%)	Abnormal group (DFI≥30%)	Z value	*P* value
Abstinence days	4.00 (3.00-5.00)	4.50 (3.00-6.00)	-3.095	0.002**
Semen volume (ml)	3.50 (2.50-4.70)	3.35 (2.08-7.29)	-0.332	0.740
Sperm concentration (10^6/ml)	66.70 (37.18-101.75)	51.25 (16.63-94.25)	-2.466	0.014*
Sperm motility (%)	57.00 (44.00-70.25)	33.50 (22.00-50.00)	-5.390	<0.001**
Sperm progressive motility (%)	50.00 (36.75-61.25)	27.00 (19.00-42.00)	-5.450	<0.001**
Sperm nonprogressive motility (%)	5.00 (3.00-8.00)	4.00 (2.00-8.00)	-1.913	0.056
Sperm immotility (%)	43.00 (30.00-57.00)	66.50 (50.00-78.00)	-5.372	<0.001**
HDS (%)	6.22 (4.37-8.86)	6.60 (4.19-10.15)	-0.784	0.433
Female age (years)	30.00 (28.00-33.00)	30.00 (28.00-35.00)	-1.221	0.222
Male age (years)	31.00 (28.00-34.00)	31.00 (28.00-35.00)	-1.260	0.208
Infertility years	3.00 (2.00-4.00)	3.00 (2.00-4.02)	-0.074	0.941
Female BMI	22.30 (20.10-24.50)	22.30 (20.88-25.00)	-0.104	0.917
Male BMI	25.02 (22.53-27.68)	24.22 (21.96-26.31)	-1.269	0.204
Intimal thickness (mm)	12.00 (10.00-14.00)	11.80 (9.60-14.00)	-1.299	0.194

*P < 0.05, **P < 0.01.

50.8%, $P = 0.232$) and clinical pregnancy rate (53.1% vs. 40.7%, $P = 0.072$) of the normal sperm DFI group were higher than those of the abnormal sperm DFI group and that the abortion rate (17.3% vs. 33.3%, $P = 0.098$) was lower that of the normal sperm DFI group than in the abnormal sperm DFI group; however, the difference was not statistically significant. Additionally, the delivery rate (43.9% vs. 27.1%, $P = 0.014$) was significantly high in the normal sperm DFI group than in the abnormal sperm DFI group (Table 3).

Comparison of related parameters between the normal group and abnormal group in frozen embryo transfer cycles

A total of 1340 frozen ET cycles were selected and grouped by the sperm DFI value. There were 1190 cases (88.8%) in the normal sperm DFI group and 150 cases (11.2%) in the abnormal sperm DFI group. There were statistically significant differences in female age ($P = 0.041$) and male age ($P = 0.011$) between the two groups, but the differences were not significant. Additionally, there was no significant difference in endometrial thickness ($P = 0.786$), number of transferred embryos ($P = 0.353$), sperm high-staining (HDS) rate ($P = 0.76$), or infertility years ($P = 0.143$) between the two groups (Table 4).

Impact of the sperm DFI on clinical outcomes in frozen embryo transfer cycles

Among 1340 frozen ET cycles, there were 1190 cases of normal sperm DFI, 689 cases (57.9%) of biochemical pregnancy, 598 cases (50.3%) of clinical pregnancy, 487 cases (40.9%) of delivery, and 111 cases (18.6%) of miscarriage. In the abnormal sperm DFI group, there were 69 cases (45.6%) of biochemical pregnancy, 61 cases (40.7%) of clinical pregnancy, 50 cases (33.3%) of delivery, and 11 cases (18.0%) of miscarriage. The statistical results showed that in the normal sperm DFI group, the biochemical pregnancy rate (57.9% vs. 45.6%, $P = 0.006$) and clinical pregnancy rate (50.3% vs. 40.7%, $P = 0.027$) were significantly higher than those of the abnormal sperm DFI group, and the delivery rate was higher than that of the abnormal sperm DFI group (40.9% vs. 33.3%, $P = 0.074$); however, the difference was not statistically significant, and the abortion rate of the normal sperm DFI group (18.6% vs. 18.0%, $P = 0.919$) was not significantly different from that of the abnormal DFI group (Table 5).

TABLE 3 Comparison of pregnancy outcome rates between the normal and abnormal sperm DFI group in fresh embryo transfer cycles.

Pregnancy outcome	Normal group (DFI<30%)	Abnormal group (DFI≥30%)	χ2	P value
Biochemical pregnancy	59.0% (289/490)	50.8% (30/59)	1.431	0.232
Clinical pregnancy	53.1% (260/490)	40.7% (24/59)	3.234	0.072
Delivery	43.9% (215/490)	27.1% (16/59)	6.068	0.014*
Abortion	17.3% (45/260)	33.3% (8/24)	2.737	0.098

*P < 0.05, **P < 0.01.

Discussion

An increasing number of studies have found that the sperm DFI is closely related to semen parameters. In the current study, the analysis of 1638 IVF/ICSI cycles showed that the sperm DFI was significantly positively correlated with male age, indicating that the degree of sperm DNA fragmentation increased with age (Supplementary Table 1). This is consistent with the findings of Bellver (15), Ghanbarzadeh (16), Zhang (17), Gonzalez (18), Lu (19) and Belloc (20). The sperm DFI was significantly and positively correlated with abstinence days, semen volume, immotile sperm percentage, sperm high-staining HDS percentage and other parameters, suggesting that the increase in abstinence days may increase semen volume, but the immotile sperm percentage and sperm high-staining HDS percentage may increase at the same time. The expression of factors promoting sperm apoptosis leads to an increase in the sperm DFI value. The sperm DFI is significantly and negatively correlated with parameters such as sperm concentration, sperm motility, forward motility sperm percentage, and nonprogressive motility sperm percentage. Sperm concentration reflects the spermatogenic function of the testis to a certain extent. When spermatogenesis is in good condition, sperm DNA damage is reduced, and sperm motility, the percentage of motile sperm and the percentage of nonforward motile sperm all reflect the state of sperm to a certain extent. Motile sperm DNA integrity is good, and the sperm DNA fragmentation rate is low. Studies have found that the sperm DFI is positively correlated with obesity, and obesity-related abnormal lipid metabolism and reproductive function-altered hormones may lead to decreased sperm quality (21). This study found that the sperm DFI was negatively correlated with male BMI, which was inconsistent with the results of studies by Fariello (22), Tolouei (23), Ferigolo (24) and others showing that obesity led to an increased the sperm DFI. There was no significant correlation between sperm DFI and infertility years, suggesting that infertility years can be influenced by many factors. Studies with larger sample sizes may be needed to investigate the relationship between male BMI and the sperm DFI.

According to different sperm DFI values, groups were assembled to study the effect of the sperm DFI on the clinical outcome of IVF-fresh ET in ART. There were 549 fresh ET cycles including 490 cases (89.3%) in the normal sperm DFI group and 59 cases in the abnormal group (10.7%). The normal DFI group and abnormal group were similar in terms of female age, male age, years of infertility, female BMI, male BMI,

TABLE 4 Comparison of related parameters between the normal DFI group and abnormal DFI group in frozen embryo transfer cycles.

	Normal group (DFI<30%)	Abnormal group (DFI≥30%)	Z value	*P* value
Sperm DFI (%)	13.39 (9.25-18.7)	39.85 (32.32-46.82)	-19.983	0.000**
Intimal thickness (mm)	10.6 (9.0-12.0)	10.8 (9.45-12.3)	-0.272	0.786
Embryo transfer number	2.0 (1.0-2.0)	2.0 (1.0-2.0)	-0.929	0.353
HDS (%)	5.72 (4.10-8.58)	6.63 (4.15-10.40)	-1.772	0.76
Female age (years)	30.0 (28.0-34.0)	31.0 (28.0-34.0)	-2.041	0.041*
Male age (years)	31.0 (29.0-35.0)	32.0 (29.5-33.5)	-2.557	0.011*
Infertility years	3.08 (2.0-5.0)	4.25 (3.0-6.0)	-1.464	0.143

*P < 0.05, **P < 0.01.

TABLE 5 Comparison of pregnancy outcome rates between normal and abnormal sperm DFI in frozen embryo transfer cycles.

Pregnancy outcome	Normal group (DFI<30%)	Abnormal group (DFI≥30%)	χ2	*P* value
Biochemical pregnancy	57.9% (689/1190)	46.0% (69/150)	7.667	0.006**
Clinical pregnancy	50.3% (598/1190)	40.7% (61/150)	4.897	0.027*
Delivery	40.9% (487/1190)	33.3% (50/150)	3.196	0.074
Abortion	18.6% (111/598)	18.0% (11/61)	0.010	0.919

*P < 0.05, **P < 0.01.

endometrial thickness, semen volume, sperm high-staining HDS rate, and nonprogressive motile sperm percentage, among others. The lack of significant differences in these parameters suggests that there were no significant differences in the general data between the normal and abnormal groups in this study, reducing the influence of other factors on clinical outcomes. The sperm concentration, sperm motility, and percentage of forward motile sperm in the normal DFI group were significantly higher than those in the abnormal DFI group, and the percentage of immotile sperm and abstinence days in the normal DFI group were significantly lower than those in the abnormal DFI group. The results were consistent with Bieniek's findings (25). Comparisons of the clinical outcomes between the normal and abnormal DFI groups showed that in the fresh ET cycle, the normal DFI group had a higher biochemical pregnancy rate (59.0% vs. 50.8%, $P = 0.232$) and clinical pregnancy rate (53.1% vs. 40.7%, $P = 0.072$) than the abnormal DFI group, but the difference was not significant; however, the delivery rate (43.9% vs. 27.1%, $P = 0.014$) was significantly higher in the normal DFI group than in the abnormal DFI group. Additionally, the abortion rate was lower than that in the abnormal group (17.3% vs. 33.3%, $P = 0.098$), but the difference was not significant.

Regarding the effect of the sperm DFI on the frozen ET cycles, a total of 1340 cases were included in this study. There was no statistically significant difference between the normal sperm DFI group and the abnormal sperm DFI group in terms of years of infertility, endometrial thickness, or the number of transferred embryos. There were statistically significant differences with the abnormal DFI group in terms of female age (30 vs. 31, $P = 0.041$) and male age (31 vs. 32, $P = 0.011$), but the difference was not large. Regarding the clinical outcomes, the biochemical pregnancy rate (57.9% vs. 46.0%, $P = 0.006$) and clinical pregnancy rate (50.3% vs. 40.7%, $P = 0.027$) of the normal DFI group were significantly higher than those of the abnormal group. The delivery rate (40.9% vs. 33.3%, $P = 0.074$) was higher in the abnormal DFI group, but the difference was not significant. Additionally, the abortion rate (18.6% vs. 18.0%, $P = 0.919$) of the normal DFI group was not significantly different from that of the abnormal group.

The results of this study showed that an abnormal sperm DFI in fresh embryo transfer cycles led to a significant decrease in the delivery rate, suggesting that a high sperm DFI will lead to abnormal embryo development, resulting in embryo or foetal loss before delivery. The differences in frozen embryo transfer cycle delivery rates were not significant, which may suggest that the effect of freezing damage on clinical outcomes should also be taken into account (e.g., the effect of freezing damage may be greater than the effect of sperm DFI on embryos at the beginning of fertilization). In the cryo-resuscitation cycle analysis, the biochemical pregnancy rate and clinical pregnancy rate were significantly different between the two groups, suggesting that the effect of the sperm DFI is significant between the embryo implantation and clinical pregnancy stages; however, the effect is not significant in delivery stages, indicating that once the embryo reaches a status of clinical pregnancy, the effect of sperm DFI is no longer significant. This information is helpful for guiding clinical practice. Studies have shown that the sperm DFI of men from couples experiencing habitual abortion is significantly higher than that of men without habitual abortion or men

who are fertile (26–28). The effect of the sperm DFI on pregnancy outcomes in IVF-fresh ET suggests that paternal genes may begin to play a major role in later embryonic stages. The impact of the sperm DFI on IVF clinical outcomes is inconsistent. Selvam found that sperm DNA integrity may affect the outcome of conventional IVF-assisted pregnancy by affecting embryo quality (29). Zhang reported that the sperm DFI can be used as an indicator for evaluating pregnancy outcomes of ART-assisted pregnancy, and is one of the outcome predictors (30), but other results in the literature do not reveal a significant effect, making the sperm DFI not instructive for clinical practice (31). The results of this study suggest that although the semen on the egg retrieval day in the IVF-ET process is optimized by gradient centrifugation and/or upstream methods, the sperm is optimized, but the excessive DNA damage of the sperm will not only lead to a decrease in sperm quality but also have a significant effect on the delivery rate of fresh ET cycles and the biochemical and clinical pregnancy rates of frozen ET cycles.

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 studies involving human participants were reviewed and approved by Northern Jiangsu People's Hospital ethics committee(2021ky068). The patients/participants provided their written informed consent to participate in this study, and the human tissues were obtained with informed consent. The patients/participants provided their written informed consent to participate in this study.

Author contributions

HC and FL conceived the idea. CZ, FC and SZ collected the data and wrote the manuscript. HS, YJ, XW, CY and YS edited and revised the manuscript. ND,TX and KL checked the data. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.945242/full#supplementary-material

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Correlations between elevated basal sperm DNA fragmentation and the clinical outcomes in women undergoing IUI

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Objective: This study aimed to explore the impact of the sperm DNA fragmentation index (DFI) on the clinical outcomes in women undergoing artificial insemination by husband intrauterine insemination (AIH-IUI).

Methods: In this retrospective study, the value of sperm DFI was detected by sperm chromatin structure assay (SCSA) in a semen analysis collected before fertility treatment (basal DFI) in 1,500 IUI cycles at the infertility clinic of Northern Jiangsu People's Hospital Reproductive Medicine Center from Jan 2016 to April 2021. Receiver operating characteristic (ROC) curves were used to calculate the cut-off value for the clinical outcomes of IUI, including the biochemical pregnancy rate, clinical pregnancy rate, delivery rate, and live birth rate, and multivariate logistic regression was conducted to analyse the risk factors for clinical outcomes after IUI.

Result: In 1,500 IUI cycles, the results showed that there were no statistically significant differences between the normal DFI group and the abnormal DFI group in biochemical pregnancy rate (14.41% vs. 11.3%, P = 0.386), clinical pregnancy rate (12.9% vs. 10.5%, P = 0.433), delivery rate (11.0% vs. 8.9%, P = 0.456), live birth rate (10.9% vs. 8.9%, P = 0.484) or pregnancy loss rate (14.6% vs. 15.4%, P = 1.000).

Conclusion: Sperm DFI alone may have limited predictive power for IUI clinical outcomes.

KEYWORDS

AIH, IUI, sperm DNA fragmentation, SCSA, ROC curve

Introduction

The process of spermatogenesis is complex, and multiple factors may lead to dysfunction of spermatogenesis, which ultimately leads to fertilization failure (1). Sperm DNA integrity is crucial for fertilization and the development of healthy offspring, and more and more reports emphasize the direct relationship between sperm DNA damage and male infertility (2). Sperm DFI can reflect the integrity of sperm DNA, and is an important indicator to assist in the evaluation of semen quality after the traditional semen analysis (3, 4). With the continuous development of science and technology, many new technologies have been applied to the examination of sperm DFI in clinical practice, including the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, Comet assay, SCSA (sperm chromatin structure assay), SCD (sperm chromatin dispersion) test, etc. (2, 5). Many studies show that high sperm DFI is associated with fertilization failure (6), delay embryonic development (7), lower high-quality blastocyst formation (8) and recurrent pregnancy loss (RPL) (9).

Infertility has become an important reproductive health problem in recent years, afflicting approximately 15% of couples at reproductive age worldwide (10). Infertility has been a neglected health issue for a long time partly because of the one-child policy in mainland China. A reproductive health survey found that the prevalence of infertility was approximately 15.5% (11). By the end of 2019, there were 517 assisted reproductive centers and 27 human sperm banks in mainland China. China's ART cycles exceeded 1 million in 2016 (12) and reached 1.15 million in 2017 (13).

Artificial insemination (AI) refers to the technology of injecting the optimized sperm from the husband or the donor into the female reproductive tract so that the sperm and the egg are naturally combined to obtain pregnancy. With the development of artificial insemination as early as more than 200 years ago, human beings began to explore artificial insemination technology (14, 15). The first documented application of artificial insemination was presented in London in the 1770s by John Hunter (16). In 1954, Bunge and Sherman in the United States reported for the first time that frozen semen artificial insemination resulted in pregnancy, and the development of artificial insemination technology has also entered a new stage (17). The successful application of sperm freezing technology provides conditions for the preservation of male fertility and the storage and transportation of donated semen, and artificial insemination technology for sperm has been applied on a large scale. Since artificial insemination technology is closer to natural conception, it has the advantages of noninvasiveness, simplicity and convenience, making most patients more acceptable and more compliant, and it is also the preferred adjuvant treatment in clinical practice (18, 19). Intrauterine insemination (IUI) is safer and more cost-effective in clinical practice than other ARTs (20).

Although IUI has experienced a long time in clinical practice, the research on the influence of sperm on it is still ongoing. Many studies have found that the biochemical pregnancy rate, clinical pregnancy rate, and delivery rate of IUI in the high DFI group are lower than those of the normal sperm DFI group (21, 22), and other studies have found that the sperm DFI has no effect on IUI clinical pregnancy (18, 23). The effects of sperm DFI on the clinical outcome of IUI are still controversial. In this retrospective study, the sperm DNA fragmentation was detected in raw semen of men with IUI cycles to investigate the effect of sperm DNA fragmentation on the clinical outcome of IUI. This will provide a reference for the clinical application of sperm DFI in IUI.

Materials and methods

Study population

A total of 4,499 male semen samples were collected from January 2016 to April 2021 in the Reproductive Center of Subei People's Hospital, and 1,500 cycles of clinical cases of couples were treated by IUI. Inclusion criteria: 1) Infertile couples completed all previous examinations, no abnormality in chromosomal examination, and the woman's fallopian tubes were unobstructed (at least one side was unobstructed); 2) The female had dominant follicle development and ovulation; 3) Male sexual dysfunction, mild oligospermia and so on. Regarding grouping, men were divided into the normal sperm DFI group (DFI < 30%) and the abnormal sperm group (DFI ≥ 30%) according to their sperm DFI levels (5, 22). The clinical data of patients was collected, including age, body mass index (BMI), infertility duration, etc. All patients signed an informed consent form related to IUI and the studies involving human participants were reviewed and approved by hospital ethics committee (2021ky068).

IUI was performed in natural cycles if infertile women with regular menstruation and normal ovulation. For those with ovulation disorders, abnormal follicular development or prolonged menstrual cycle, after vaginal ultrasound examination on the third to fifth day of menstrual cycle, oral clomiphene (CC) or letrozole (LE) alone or in combination with gonadotropins or gonadotropins alone were used to stimulate ovaries to induce ovulation. From the 8th day of menstrual cycle, the growth of follicles was dynamically monitored under vaginal B-ultrasound. When follicles with a diameter of about 18mm appeared, 5,000-10,000 IU of human chorionic gonadotropin (hCG) was injected intramuscularly to induce ovulation, and IUI was carried out 36-42 hours after hCG injection.

Collection semen and routine analysis

The men were abstinent for 2-7 days, and sperm were collected by masturbation. Routine semen processing analysis was performed according to the Laboratory Manual for Human Semen Examination and Processing, 5th Edition (24). The semen quality was analyzed and recorded by using a computer-aided semen analyzer (Beijing Suijia Software Co., Ltd).

Analysis of sperm DNA fragmentation index

Sperm DNA fragmentation assay (SDFA) was performed using the sperm chromatin analysis (SCSA) kit (Zhejiang Cellpro Biotech Co., Ltd., Ningbo, China) in strict accordance with the product instructions (5, 25). The detailed analysis process was as follows. First, an appropriate volume of semen was added to 0.1 ml of solution A (TNE buffer, sperm dilution) and mixed. Then, 0.2 ml of solution B (acid solution of 0.1% Triton X-100, 0.15 mol/L NaCl, and 0.08 mol/L HCl, pH 1.2) was added and mixed. After standing for 30 s, 0.6 ml of acridine orange (AO) staining solution (6 μg/ml AO, 37 mmol/L citric acid, 126 mmol/L Na_2HPO_4, 1 mmol/L Na_2EDTA, 0.15 mol/L NaCl, pH 6.0) was added and mixed. After the sperm were stained for 3 min, the sperm DFI was detected by a flow cytometer (FACS Calibur, BD Bioscience, San Jose, CA, USA). A minimum of 5,000 sperm were acquired, and the data were analyzed by using the software (DFIView 2010 Alpha11.15, CellPro Biotech, Ningbo, China). Total %DFI is Medium + High level of DNA fragmentation. The sperm DFI was expressed as the percentage of sperm with fragmented DNA compared to the total number of sperm. The variability of the replicate DFI measures was less than 5%.

Semen optimization for IUI

The semen was collected 2 hours before IUI, the men abstained for 2-7 days, and semen was collected into a sterilized disposable wide-mouth collector. After checking each man's name by fingerprint identification, the sperm spots were collected and placed in a 37°C incubator for incubation and liquefaction. After liquefaction, the semen was evaluated and recorded. The density gradient centrifugation method was used to optimize the semen. The specific operation steps were as follows: 1) The gradient centrifugation medium of 80% and 40% SpermGrad (Swedish Vitrolife Company) with two different concentrations was preheated in a 37°C incubator. 2) 1 ml of 80% high-concentration gradient centrifuge medium was added to the sterile conical centrifuge tube with a pipette, and then 1 ml of 40% low-concentration gradient medium was slowly added on top of it while being careful not to damage the interface between the two layers of gradient solution. Then, 2 ml of liquefied semen was added. According to the specific conditions of the semen, the amount of gradient centrifugation fluid was adjusted, or the number of centrifuge tubes was increased. 3) The samples were placed in a centrifuge at 300-400 × g for 15 minutes, the supernatant and gradient solution were removed, and only approximately 0.5 ml of sperm pellet was taken from the bottom. Then, 3 ml of upstream insemination solution was added, mixed well, and transferred to a Falcon 1006 centrifuge tube. 4) The tube was centrifuged at 300-400 × g for 5 minutes. The supernatant was removed, the sperm precipitate that was visible at the bottom of the tube was obtained, 0.5 ml of upstream insemination solution (IVF solution) was added, and the sperm suspension was evaluated and prepared for IUI use. The sperm DFI were tested before sperm preparation.

Intrauterine insemination method

The patient was in the lithotomy position after emptying the bladder, washed the vulva with normal saline, and wiped the vagina, cervix, and fornix with cotton swab. A 1 ml syringe was connected with a disposable artificial insemination tube (COOK Company), 0.5 ml of sperm suspension was carefully and gently placed in the uterine cavity through the cervix and about 1cm above the uterine cavity, and then the artificial insemination tube was slowly removed after a short stay. After the operation, the patient was instructed to raise the hip at an angle of approximately 30 degrees until 15-30 minutes of observation in bed, and then leave if there was no special discomfort.

Follow-up of pregnancy outcomes

A blood test was performed 14-16 days after IUI to measure the β-hCG level in peripheral blood to determine whether a biochemical pregnancy (more than 5.0 mIU/ml is diagnosed as biochemical pregnancy) was present. The intrauterine pregnancy sac was observed by vaginal ultrasonography between the fourth week and fifth week after IUI. Luteal support (progesterone 20 mg to be taken orally every day) was given in the stimulation cycles starts from 48 hours after IUI until clinical pregnancy if β-HCG positive. Pregnancy loss included miscarriage, ectopic pregnancy and stillbirth (26). Biochemical pregnancy rate = number of biochemical pregnancy cycles/artificial insemination cycles *100%; Clinical pregnancy rate = number of clinical pregnancy cycles/number of artificial insemination cycles *100%; Delivery rate = number of delivery cycles/number of artificial insemination cycles *100%; Live birth rate = number of live birth cycles/number of artificial insemination cycles *100%; Pregnancy loss rate = number of

pregnancy loss cycles/number of clinical pregnancy cycles *100%.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22.0 version 25 (IBM Corp., Armonk, NY, USA). Categorical variables are presented as frequencies and percentages, whereas continuous variables are reported as the means ± standard deviations (SDs) or as the medians and interquartile ranges (IQRs, 25th-75th percentile). The normality of the distribution of the variables was determined using the Kolmogorov-Smirnov (K-S) test. Normally distributed data were expressed as the means and SDs, while the medians and IQRs were used for nonnormally distributed data. Groups were compared with student's t-test or Mann–Whitney U-test as appropriate. Correlation analysis was performed by the Pearson method or Spearman method. The chi-square or Fisher's exact test was used to compare categorical variables. The odds ratios and their 95% confidence intervals (ORs, 95% CIs) were calculated to show the associations between each predictor and the risks for clinical outcomes. The receiver operating characteristic curve (ROC) and the area under the curve (AUC) were calculated by MedCalc version 17 (MedCalc Software, Mariakerke, Belgium). The cut-off point of the ROC was also calculated to obtain the sensitivity and specificity of the model. All tests were two-tailed, $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered extremely significant.

Results

A total of 4,499 semen samples were collected from outpatients, for a total of 1,500 IUI cycles, including 208 cycles of biochemical pregnancy (13.9%), 191 cycles of clinical pregnancy (12.7%), and 163 cycles of childbirth (10.9%). There were 161 cycles of live birth (10.7%, including 2 cycles of twin pregnancy, with a multiple birth rate of 1.2%) and 28 cycles of miscarriage (14.7%). The specific statistical analysis results are as follows.

Correlation analysis between sperm DFI and semen routine parameters

A total of 4,499 male semen samples were collected from outpatient clinics, and sperm DFI was detected. The Kolmogorov-Smirnov (K-S) test results showed that the data did not obey a normal distribution ($P < 0.001$), except for the sperm specific movement parameter of beat-cross frequency (BCF) (Supplementary Table 1). The correlation analysis showed that the sperm DFI was positively correlated with sperm immotility percentage (r = 0.451, $P < 0.001$), male age (r = 0.140, $P < 0.001$), semen volume (r = 0.089, $P < 0.001$), abstinence days (r = 0.07, $P < 0.001$) and percentage of sperm DNA high stainability (HDS) (r = 0.171, $P < 0.001$), and negatively correlated with sperm concentration (r = -0.330, $P < 0.001$), sperm progressive motility percentage (r = -0.465, $P < 0.001$), sperm nonprogressive motility percentage (r= -0.08, $P < 0.001$), and sperm specific motility parameters (curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), mean angular displacement (MAD), straightness (STR), amplitude of lateral head displacement (ALH), linearity of movement (LIN), wobble (WOB) and beat cross frequency (BCF)) (all *Ps* < 0.001) (Table 1).

Comparison of general data between the normal and abnormal sperm DFI groups in IUI cycles

A total of 1,500 IUI cycles were divided into the normal DFI group (DFI < 30%) and the abnormal sperm DFI group (DFI ≥ 30%) according to the diagnostic criteria of the sperm DFI; 1,376 cases were in the normal group (91.7%), and 124 cases (8.3%) were in the abnormal group. Statistical analysis results showed that there was no significant difference between the normal DFI group and the abnormal sperm DFI group in basic data such as infertility duration (2.1 vs. 3.0, $P = 0.601$), female age (28.0 vs. 29.0, $P = 0.133$), male BMI (24.8 vs. 24.2, $P = 0.851$), and sperm HDS percentage (6.2% vs. 5.9%, $P = 0.755$). There was a statistically significant difference in male age (30.0 vs. 30.5, $P = 0.022$) between the normal DFI group and the abnormal sperm DFI group (Table 2).

Comparison of clinical outcomes between the normal and abnormal sperm DFI groups in IUI cycles

Among the 1,500 IUI cycles, there were 1,376 cases of normal sperm DFI, 194 cases of biochemical pregnancy (14.1%), 178 cases of clinical pregnancy (12.9%), 152 cases of delivery (11.0%), 150 cases of live birth (10.9%), and 28 cases of pregnancy loss (15.7%, including 20 cases of miscarriage, 6 cases of ectopic pregnancy and 2 cases of stillbirth). There were 124 cases of abnormal sperm DFI, 14 cases of biochemical pregnancy (11.3%), 13 cases of clinical pregnancy (10.5%), 11 cases of delivery (8.9%), 11 cases of live birth (8.9%), and 2 cases of miscarriage (15.4%). The results showed that there were no statistically significant differences between the normal sperm DFI group and the abnormal sperm DFI group in the biochemical pregnancy rate (14.1% vs. 11.3%, $P = 0.386$), clinical pregnancy rate (12.9% vs. 10.5%, $P = 0.433$), delivery rate (11.0% vs. 8.9%, $P = 0.456$), live birth rate (10.9% vs. 8.9%, $P = 0.484$), or pregnancy loss rate (15.7% vs. 15.4%, $P =$

TABLE 1 Correlation between sperm DFI and semen conventional parameters.

Variable	ρ value	*P* value
Male age (years)	0.140	<0.001**
Semen volume (ml)	0.089	<0.001**
Abstinence days (days)	0.070	<0.001**
Sperm concentration (10^6/ml)	-0.330	<0.001**
Sperm progressive motility (%)	-0.465	<0.001**
Sperm nonprogressive motility (%)	-0.08	<0.001**
Sperm immotility (%)	0.451	<0.001**
Sperm high DNA stainability (%)	0.171	<0.001**
VCL (μm/s)	-0.440	<0.001**
VSL (μm/s)	-0.429	<0.001**
VAP (μm/s)	-0.460	<0.001**
BCF (times/s)	-0.439	<0.001**
MAD (degree)	-0.446	<0.001**
STR	-0.448	<0.001**
LIN	-0.423	<0.001**
WOB	-0.475	<0.001**
ALH (μm)	-0.418	<0.001**

VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average pathway velocity; BCF, beat cross frequency; MAD, mean angular displacement; STR, straightness (VSL/VAP); LIN, linearity of movement (VSL/VAP); WOB, wobble (VAP/VCL); ALH, amplitude of lateral head displacement. **P<0.01.

1.000) (Table 3). Further subgroup classification comparison (715 natural cycles and 785 stimulated cycles), there were no statistically significant differences in clinical outcomes between the normal and abnormal sperm DFI groups (Supplementary Table 2).

Prediction of sperm DFI on clinical outcomes after IUI

Receiver operating characteristic (ROC) curves were constructed to assess the effectiveness of sperm DFI in predicting the clinical outcomes of IUI. A clinically acceptable threshold was calculated when sensitivity plus specificity were maximum. The AUC of the sperm DFI for predicting biochemical pregnancy was 0.537 (95% CI: 0.551-0.562, *P* = 0.077). The cut-off value of the ROC was 16.75%, which had the best sensitivity of 69.23% and specificity of 40.63%. The AUCs of the sperm DFI for predicting clinical pregnancy, delivery and live birth were 0.531 (95% CI: 0.506-0.557, *P* = 0.148), 0.533 (95% CI: 0.507-0.559, *P* = 0.150), and 0.532 (95% CI: 0.507-0.558, *P* = 0.166), respectively (Table 4). The ROC curves showed that the sperm DFI was not valuable in predicting pregnancy for patients after IUI (all areas under the ROC curve of clinical outcomes below 54%). According to the results of the multivariable logistic regression analysis, the impact of sperm DFI (OR = 0.986, 95% CI 0.968-1.004, *P* = 0.118) on clinical outcomes of IUI was limited despite the removal of some confounding factors (Supplementary Table 3).

Discussion

With the improvement of people's education level, changes in lifestyle, live environment, fertility concepts, and the aging of the social population, the number of births has declined in China. China proposed the implementation of the two-child fertility policy for couples where either the husband or the wife is from a single-child family in 2013, and a universal two-child policy was implemented in 2016 and then to the three-child policy proposed in 2021 (27, 28). It shows that the state hopes to promote the growth of the birth population. On the other hand, the increase in the number of assisted reproductive institutions approved by the state can alleviate the negative impact of 'cannot birth', but the solution to the problem of infertility still needs the advancement of assisted reproductive technology. The optimized sperm was sent into the woman's uterine cavity through IUI to achieve the process of natural fertilization, pregnancy and childbirth, which is one of the commonly used assisted reproductive technologies, and the largest comprehensive analysis integrating success, risks and costs shows that IUI is safer and more cost-effective than other ART treatments (19, 20). IUI pregnancy rates have been reported to be mixed and varied widely, ranging from 8% to 22% (29). There are many factors affecting artificial insemination results, and most studies focus on patient age (30), ovarian function and egg quality (31, 32), infertility duration, ovulation induction (33, 34),

TABLE 2 Comparison of general data between the normal DFI group and abnormal DFI groups in IUI cycles.

Variable	Normal group (n=1376)	Abnormal group (n=124)	Z	*P* value
Sperm DFI (%)	13.7 (9.8-18.8)	35.9 (32.9-46.4)	-18.467	0.000**
Infertility duration (years)	2.1 (2.0-3.23)	3.0 (2.0-3.08)	-0.523	0.601
Male age (years)	30.0 (28.0-32.0)	30.5 (28.0-32.0)	-2.296	0.022*
Female age (years)	28.0 (27.0-31.0)	29.0 (27.0-31.0)	-1.503	0.133
Male BMI	24.8 (22.3-27.4)	24.2 (23.2-16.8)	-0.188	0.851
Sperm high DNA stainability(%)	6.2 (4.1-8.8)	5.9 (4.3-10.9)	-0.312	0.755

DFI, DNA fragmentation index; BMI, Body mass index. *P<0.05, **P<0.01.

10.3389/fendo.2022.987812

TABLE 3 Comparison of clinical outcomes between the normal and abnormal sperm DFI groups in IUI cycles.

Variable	Normal group(DFI<30%)	Abnormal group(DFI≥30%)	χ2	*P* value
Biochemical pregnancy	14.1% (194/1376)	11.3% (14/124)	0.751	0.386
Clinical pregnancy	12.9% (178/1376)	10.5% (13/124)	0.616	0.433
Delivery	11.0% (152/1376)	8.9% (11/124)	0.556	0.456
Live birth	10.9% (150/1376)	8.9% (11/124)	0.489	0.484
Pregnancy loss	15.7% (28/150) #	15.4% (2/13)	0.000	1.000

#: Pregnancy loss including 10 cases of miscarriage, 6 cases of ectopic pregnancy and 2 cases of stillbirth.

endometrial thickness (35), and the number of inseminations. As women age, fertility declines significantly, and the proportion of early miscarriage and chromosomal abnormalities increases significantly (36, 37). Although there are existing methods such as sperm concentration, motility, and morphology to evaluate male fertility (38), these parameters are not standardized to a high degree and are subjective (39). There is controversy about the clinical significance of sperm DFI detection indicators for IUI (40).

In this study, the sperm DFI data of 4,499 sperm samples were tested for normality, showing a skewed distribution. Correlation analysis results showed that the sperm DFI was positively correlated with the man's age, semen volume, abstinence days, and immotile sperm percentage; and negatively correlated with nonprogressive motility percentage, sperm concentration, sperm progressive motility percentage, and the specific motility parameters of sperm (VCL, VSL, VAP, BCF, ALH, MAD, LIN, STR and WOB). Sperm specific motility parameters are negatively correlated with sperm DFI, which is consistent with the results of Le et al. (41). There are significant correlations between routine semen parameters and sperm function parameters, which are both indicators of sperm quality, but the focus of detection was different (42). The percentage of sperm HDS is another index in the process of sperm DFI detection by SCSA method, which reflected the immaturity of the sperm nucleus and has been proposed to be due to a sub-optimal histone to protamine ratio that affects sperm nucleus compaction and therefore makes it susceptible to DNA damage (5). The sperm DFI was positively correlated sperm HDS percentage. These results suggest that the occurrence of human sperm functional defects is not a single reason and may be multifactorial. There is a significant correlation between sperm DFI and sperm motility, that is, the risk of abnormal sperm function is higher in low-quality sperm, which may have a common mechanism with the two abnormal phenotypes. Therefore, this study supports sperm DFI as a supplement to routine semen analysis. The positive correlation between sperm DFI and age is consistent with the results of Moskovtsev et al., who found that sperm DFI increased linearly with increasing male age (43–47). Fertility among older men is increasing worldwide, especially with the liberalization of China's second-child birth policy, and a large number of couples over the age of 40 are trying to use assisted reproductive technology to achieve fertility. A comprehensive analysis of semen quality should be carried out to fully assess male fertility.

This study analyzed the association of sperm DFI with clinical outcomes in 1,500 IUI cycles. According to the diagnostic criteria of sperm DFI, they were divided into the abnormal sperm DFI group and the normal sperm DFI group, of which 124 cases were abnormal (8.3%) and 1,376 cases were normal (91.7%). Statistical analysis results showed that there was no statistically significant difference in basic data, such as infertility duration, female age, male BMI, and sperm HDS percentage between the two groups. There was a statistically significant difference in male age between the two groups, but the difference was not large. Statistical analysis and comparison showed that the normal sperm DFI group had a higher biochemical pregnancy rate, clinical pregnancy rate, delivery rate and live birth rate than the abnormal sperm DFI group, but there were no significant statistical differences. This result is consistent with Yang et al. (23) and different from Bungum et al.

TABLE 4 ROC curve analysis of the sperm DFI for IUI pregnancy.

	Area underROC curve	SE	95% CI	Cut-off value(%)	Sensitivity(%)	Specificity(%)	*P* value
Biochemical pregnancy	0.537	0.021	0.551-0.562	16.75	69.23	40.63	0.077
Clinical pregnancy	0.531	0.022	0.506-0.557	15.42	62.30	46.45	0.148
Delivery	0.533	0.023	0.507-0.559	15.42	63.19	46.37	0.150
Live birth	0.532	0.023	0.507-0.558	15.42	62.73	46.30	0.166

ROC, receiver operating characteristic; CI, confidence interval.

(22), which may be related to the population. With regard to the sperm DFI, we observed an optimum cut-off point of 16.75% for IUI biochemical pregnancy and 15.42% for clinical pregnancy, delivery and live birth, but they were not significant. Logistic regression analysis showed little prognostic value in predicting clinical outcomes after IUI. Therefore, the effect of sperm DFI on IUI clinical outcomes needs to be studied in larger samples.

Sperm DNA as a carrier of paternal genetic information, plays an important role in fertilization and embryonic development (48). Sperm DFI can reflect the integrity of sperm DNA and is an important indicator to assist in the evaluation of semen quality after the traditional semen analysis (3, 4). Oguz et al. compared the effects of two commonly used sperm preparation methods (swim-up and gradient technique) on sperm DFI through SCD method, and the result showed that gradient method has no statistically significant reduction in the DNA fragmented sperm rate after preparation as compared to basal rates (49). This study results showed that the elevated of basal sperm DFI had no significant impact on the clinical outcomes of IUI, which may be related to the reduction in the DNA fragmented sperm rate during sperm preparation, although there is no significant statistical difference before and after gradient centrifugation. The molecular mechanism of sperm DNA fragmentation and its impact on IUI need to be further studied.

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 Northern Jiangsu People's Hospital ethics committee (2021ky068). The patients/participants provided written informed consent to participate in this study, and human tissues were obtained with informed consent.

Author contributions

HC, TX and FL conceived the idea. CZ and SZ wrote the manuscript. FC, HS, YJu and XW analyzed the data. CY, YS, YJi, YP, ND and KL edited and revised the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of interest

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

Supplemetary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.987812/full#supplementary-material

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Feasibility analysis of incorporating infertility into medical insurance in China

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In recent years, the incidence of infertility has been increasing gradually, while the natural rate of population growth is declining or even at zero growth. China is observed to enter a depth of aging society, leading to more severe infertility. Infertility patients face many predicaments, and many unreasonable behaviors existed in seeking medical diagnosis and treatment, of which the main influencing factor is economic condition. In China, Beijing has taken the lead in providing medical insurance for 16 assisted reproductive technology items. Assuming that all infertile couples with the option of assisted reproduction are treated, there would be a huge market gap. The reimbursement rate can be adjusted based on some factors within the affordable range of the medical insurance fund. Progress on infertility coverage in other countries was also reviewed. This paper cited the data of medical insurance funds in China in the recent 4 years as a reference. Based on the data, it is not currently able to cover all the costs of infertility diagnosis and treatment during the research period, but it is feasible to access selective reimbursement and subsidies for those in particular need as well as to develop some commercial insurances. There is a big gap in the application of assisted reproductive technology between China and developed countries. More comprehensive and constructive policies should be formulated countrywide to standardize the market. Assisted reproduction-related technologies and acceleration of the domestic medical apparatus and instrument replacement should be improved to reduce the cost.

KEYWORDS

assisted reproductive technology, feasibility, *in vitro* fertilization, infertility, medical insurance

Abbreviations: ART, assisted reproductive technology; IVF, *in vitro* fertilization; IVF-ET, *in vitro* fertilization and embryo transfer; ICSI, intracytoplasmic sperm injection; IUI, intrauterine insemination.

Introduction

Over the past three decades, the age of first marriage for Chinese women has been rising, while the average fertility rate is falling sharply. According to the National Bureau of Statistics, the natural population growth rate in 2021 will be 0.34‰, which is lower than that of 2020 (1.45‰) and 2019 (3.34‰). Although the fertility rate has recovered after the comprehensive implementation of the two-child policy, the overall fertility rate is still far below the replacement level (1). The decline is mainly caused by two factors: lack of will and ability. Childbearing costs continue rising, which significantly reduce the social fertility willingness. At the same time, a pretty big contradiction exists between the high infertility rate and the insufficient availability of technology and equipment. The ability to increase the supply of medical services for the unfertile people with childbearing desires still required more improvements. "If the government subsidizes the treatment, it will increase the chances for more people to conceive children." Gao Li, a deputy to the National People's Congress, submitted a proposal to include infertility and assisted reproductive technology (ART) in the national medical insurance.

There is no doubt that the inclusion of the diagnosis of infertility and ART into medical insurance coverage would be a significant move to benefit more people. To a certain extent, it may overcome some infertile families' urgency and then increase the fertility rate across the whole nation. Considering that socio-economic affordability is limited, it is necessary to comprehensively decide whether and how to include the diagnosis and treatment costs into medical insurance reimbursement. Although the reform of medical insurance has been completed, more investigations and policy implementations are needed to fill the gaps between the current coverage, reimbursement level, and people's warranted needs.

Review of existing studies

The high incidence of infertility in China

Infertility refers to both men and women having the desire to conceive children, cohabiting for more than 12 months with normal sex life, and have no contraceptive measures without pregnancy. Female infertility is usually secondary to reproductive tract infection. Overall, 50% of infertility is due to men. Individually, 20 to 30% of infertility occurs solely due to male factors (2). Clinical investigation showed that the prevalence of infertility was climbing year by year and the patients trended to be younger, which was affected by diverse factors such as childbearing age, occupation, drug abuse, environmental pollution, sexual infection, and living habits. Li et al. conducted stratified and random sampling analyses and found that the smoking rate of male patients was 67.5%, and 69.70% of women had a history of induced abortion or spontaneous abortion (3). It is worth noticing that the causes and risk factors of male infertility are not identified at the population level despite a bunch of comprehensive research (4). Most of the current research is limited to individual institutions or a smaller sample size like specific city, district, and county investigations. Data from the 2015 China Infertility Survey Report showed that the incidence of infertility in China had reached 15%. In 2017, Zhou et al. conducted a population-based epidemiological survey of infertility in eight provinces in China and found that the prevalence rate of infertility was 15.5% among couples living together for more than 1 year and the wife was 20–49 years old, which reached 25% after excluding couples without fertility desire. Only 55% of infertile couples seek medical service (5) and about 20% of them were qualified for the treatment of ART.

According to demographic statistics, women aged 15 to 49 are generally defined as women of childbearing age. Due to the severe aging trend in the whole society of China, women aged 45 to 49 were considered the major proportion of women of childbearing age (19.7%), and women of non-optimal childbearing age (over 35 years old) accounted for 50.6%. It is predicted that the arithmetic fertility rate of women aged 15–49 in urban, town, and rural areas will continue to decline from 25.35, 33.18, and 41.27‰ in 2010 to 5.37, 18.34, and 12.03‰ in 2050, respectively (6).

Male infertility and overall male health

It is estimated that male factors cause about 50% of infertility, but male factors rarely cause attention to couples' infertility (7). Male factors must be fully considered in infertility treatment. According to spermatogenesis, male infertility can be divided into congenital, acquired, and idiopathic factor-related diseases (8). Age-standardized male infertility rate increased annually by 0.291% in the last three decades (9). Sperm counts have dropped over 50% in the past few decades (10, 11). Congenital factors include chromosomal abnormalities, cryptorchidism, and absence of vas deferens. Varicocele is the most common epigenetic factor, with a prevalence of 40%. Urogenital infections are also frequent. Idiopathic risk factors include exposure to tobacco, alcohol, drugs, or occupational productive toxins as well as an unhealthy diet (9). Moreover, 15% of adult men have clinical varicocele, 35% present infertility, and up to 81% may have secondary infertility (12). The infertile male should undergo timely semen analysis, physical examination, hormonal evaluation, gene testing, and imaging as well as organic surgery (7). Correcting sperm defects should be included in the management practices of infertile couples,

such as severe oligospermia leading to a low success rate of intracytoplasmic mirror injection (13). At present, it is not ruled out that COVID-19 will damage the male reproductive system and lead to infertility (14).

There is growing evidence that impaired male fertility is a potential predictor of impaired male health status, but the cause of the relationship is currently unclear (15). As mentioned in the previous paragraph, male infertility deserves more attention and is becoming a significant predictive biomarker of overall male health and survival. Some scholars conducted a comprehensive study on male infertility in the past three decades, revealing that male infertility is associated with cardiovascular disease, cancer, chronic disease, and even mortality to varying degrees (16).

Male hypogonadism is a hyposexual disease caused by the reduction of androgen or the failure of androgen function, and it is very likely to cause male infertility. Clinical and subclinical hypogonadism (16, 17) is associated with morbidity and mortality from cardiovascular diseases. Male infertility may be an independent predictor of cardiovascular diseases (18), such as hypertension, heart disease, and peripheral vascular disease. The incidence of cardiovascular disorders in infertile men, especially varicocele patients, is higher than that in men having children (19). The infertile male patients have been found to have more comorbidities like diabetes mellitus, pulmonary diseases (chronic bronchitis, emphysema, allergic bronchitis, asthmatic bronchitis, and so on), connective tissue disorders (systemic lupus erythematosus, rheumatoid arthritis, and so on), peptic ulcer, and liver diseases (chronic hepatitis, cirrhosis of liver, and alcoholic cirrhosis) than fertile male (20). Male infertility can be a predictor of chronic comorbidity diseases (21). Charlson comorbidity index can evaluate the comorbidity burdens utilizing integration (22). The coexistence of comorbidity may be attributed to the common mechanism with male infertility, or the comorbidity harms a patient's fertility directly (21). In retrospective studies, infertile men, especially those with azoospermia, are at a high risk of cancer—including a proven risk of non-Hodgkin's lymphoma and a well-studied risk of testicular cancer (16, 23). Adenocarcinoma is very controversial in the correlation between male infertility and future cancer risk (23, 24). Genetic alterations such as disruption of the MLH1 gene may account for the potential association (25). Men with azoospermia had the highest risk of death among infertile men (26). When more than one semen parameters were abnormal, the risk of death was more than twofold (27).

Effects of semen parameters on assisted reproduction outcomes

Semen parameters include semen volume, quality, density, motility, morphology, biochemical analysis, cell count, anti-sperm antibody testing, *etc.* Abnormal semen parameters such as sperm without acrosomes or positive anti-sperm antibodies will lead to male infertility under natural conditions (28). The semen's concentration, motility, or morphology may have no association with the pregnancy rate of ART according to present investigations. To some extent, it may mean that ART overcomes impaired semen quality. However, whether semen parameters can affect other ART outcomes, such as fertilization, implantation, live birth, and perinatal health, is calling for further research in the administration of ART (29). A study conducted in China revealed that even if morphologically normal sperm was less than 4%, the ART clinical outcomes still stayed, while the fertilization success rate fell (30). A comprehensive study utilizing meta-analyses on the recent 20 years of teratozoospermia found no correlation between sperm morphology and pregnancy rate. However, case studies of oligozoospermia and asthenospermia found that the sperm concentration's decrease or asthenospermia severity's increase caused the corresponding pregnancy rates to decrease (29, 31). The meta-analysis showed that implantation rate, pregnancy rate, and live birth rate were influenced negatively by sperm DNA damage in *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) cycles (32). However, the pregnancy rate was not significant after bias adjustment. At present, studies on the effect of semen quality on ICSI are less than those on IVF, and some studies show that ICSI is more able to overcome sperm damage and achieve a successful pregnancy (29, 32).

Infertile families faced with predicaments and psychological pressure from the low level of medical security

Infertility greatly bothered the related couples and their families—for example, due to the traditional cultural influence of blood inheritance, women are relatively under more tremendous pressure to bear children. Infertility treatment might sometimes gain some adverse influences on the relationship between husband and wife, which is embodied in "decreased sexual quality of husband and wife" (27.4%) and "couples often have conflicts" (24.1%) as well as "physical or verbal violence by her husband" (4.5%) (33). Additionally, long-term and complex treatments could also affect the harmony between couples, leading to the destruction of family stability.

The study conducted by Li et al., based on the fertility-related stress scale, indicated that infertility-related examination, IVF, and *in vitro* fertilization embryo transfer (IVF-ET) treatment would increase the stress levels of patients. In particular, the uncertainty of the treatment outcome increased the psychological burden for the patients (34). Batool Rashidi et al. investigated patients who were planning to undergo IVF or ICSI treatment by the SF-36 questionnaire and found that women scored lower than men as women were more frequently blamed for infertility (35). As a result, the complicated examination and treatment process forced more

female patients to quit or pause their jobs, and the increased economic burden also induced psychological pressure. Wang et al. showed that anxiety and depression were detected in 45.68 and 50.62% of the patients with advanced age. In addition, some socio-demographic characteristics (*e.g.*, educational level) were argued to be related to infertile women's health (36). These negative emotions significantly influenced the quality of life and reproductive outcome (37). Yu et al. found that the greater the cost to the patients' own time and career, the greater their anxiety (38).

Infertility patients' unreasonable behaviors in seeking medical diagnosis and treatment

Lv et al. showed that 42% of infertility patients never went to the hospital. The top reason for not seeking treatment in hospitals was financial status, which accounted for 23% (39). An investigation showed that standardization of examination and treatment costs can encourage infertile men to deal with stress (7). The survey by Wang et al. identified that 92.4% of patients continued their treatment with 39.7% success of pregnancy, and 55.1% patients visited two or three hospitals with 47.1% success of pregnancy. Only 70% continued the treatment with the same doctor (40). A study also found that men who voluntarily received infertility treatment could downgrade the intensity of intervention that couples need (41). It is reported that among 18–27% of infertility couples, the male partner did not undergo an infertility evaluation (42). In rural areas, patients may tend to be credulous about unscientific methods with unsystematic treatments. Moreover, income is considered to be an important determinant of access to healthcare, especially in countries that lack universal health insurance and relied mainly on private health insurance. Clinical studies have found that patients without health insurance had fewer hospital days and received more inappropriate treatments (43).

Cost of ART and medical insurance policies

Many people opt for ART after trying medications and surgery for organic causes. At present, ART has become the primary method of infertility treatment. However, assisted reproduction is a medical vertical segmentation industry that has a relatively low degree of marketization and maturity throughout the medical industry. Since the first IVF birth in 1978 (44), ART has been used in the clinic for more than 40 years. ART has made an outstanding contribution to improving fertility outcomes and increasing birth rates worldwide with a series of developments (Figure 1).

According to Qiu et al., the costs of infertility treatment were mainly concentrated in "30,000–200,000 yuan". Specifically, "50,000–100,000" accounted for 31.2%. In addition, 93.3% of patients said that the treatment had impacted the family finances to some degree, of which 17.6% took on debt. Moreover, 31.1% of patients claimed that their daily expense was greatly reduced (33, 34). Furthermore, in a notice on regulating and adjusting the prices of some medical services, Beijing regulated the prices of 16 assisted reproductive technologies, including 2,400 yuan for vaginal oocyte collection, 3,346 yuan for intracytoplasmic sperm injection 1,287 yuan for intrauterine artificial insemination, and 2,300 yuan for embryo transfer. Sperm separation resuscitation, selection, and centrifugation cost an average of 800 yuan per type, and *in vitro* fertilization–embryo culture need 1,566 yuan per day. The whole process is expensive, and if the parents have a single gene disease and need embryo testing, it will cost at least 6,000 to 7,000 yuan more.

Promoting the positive interactions among the medical insurance policy, price policy, medical service supply system, and payment mechanism can play the incentive and constraint role of the medical insurance payment on the professional behavior of doctors. This could result in accelerating the establishment of compatible resource allocation mechanisms among medical service providers, demanders, and medical insurance providers (45).

Challenges for assisted reproduction centers and technology

The reproductive center integrates clinical and scientific research and teaching. Due to the fierce competition in the medical market, the legal system of assisted reproduction is not perfect. At present, there are still some illegal assisted reproduction institutions, malpractice by clinicians, and inappropriate use of new techniques. To tackle these threats, establishing a robust regulatory framework is an urgent need (46).

At present, ART and its application still have some controversial and unclear aspects. Impaired pregnancy outcomes in women using ART may be related to thin endometrium, especially on ovulation triggering days. Thin endometrium may raise the risk of female hypertensive disorders during pregnancy and infants who are small for their gestational age or with decreased birth weight (47). For embryo transfer patients with impaired ovarian reserve, the anti-mullerian hormone cannot predict clinical pregnancy (48). Paternal childbearing age as well as the use of ART influences the later generations' bone mineral density (49). Recurrent implantation failure is very challenging in the clinical application of ART. Certain lymphocytes in serum have been identified as potential biomarkers, but further research is needed.

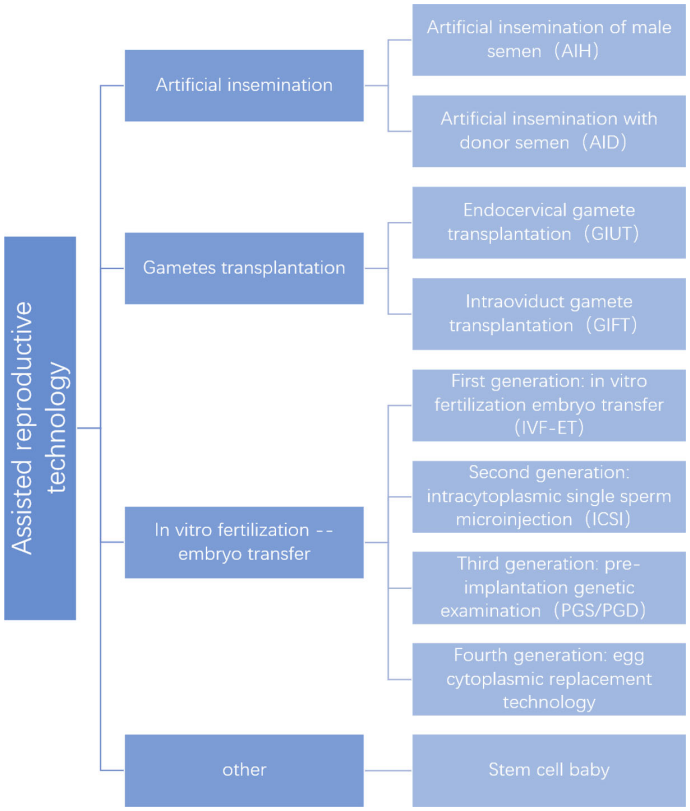


FIGURE 1
Assisted reproduction technology classification.

The development status of ART in various countries worldwide

While the use of ART is increasing globally, there are wide disparities in access to treatment among different countries. It is estimated that at least 180 million couples suffered from infertility in developing countries (50). Daar and Merali divided the consequences of infertility and childlessness into seven levels——only a few developing countries will reach the third level (social violence and social isolation). It is estimated that access to IVF procedures is 100% in Belgium, while it is 1.1% in Africa (50). However, in developed countries, the situation is much more advanced—for example, in Finland, private clinics can provide over 60% of IVF treatments (51). Live birth rate per cycle is highest in the USA (28.4%), followed by Canada (23.9%), UK (22.8%), Scandinavia (21.5%), Japan (13.7%), and Australia (17.6%). While multiple birth rate per delivery in the USA is also the highest (34.2%), Japan has the lowest multiple pregnancy rate of the six countries covered (17%) (52). Denmark has the highest ART cycles per million in 14 countries across Europe. The average number of ART in Europe is 1,022 per million, while that of the USA and Israel is 395 and 3,000 in 2003, respectively (46).

The number of ART units varies considerably among different countries, and some factors like GDP are thought to have a close relationship with it. There are two to three ART units per million in Scandinavian countries, one to 1.5 in Western European countries, and less than one in the former Soviet Union (46). For the patients' best interests, some doctors would recommend some cross-border reproductive facilities for the couples' need (53).

Reimbursement of infertility in various countries worldwide

The cost per live birth of IVF was highest in the USA and the UK ($41,132 and $40,364, respectively) and lowest in Scandinavia and Japan ($24,485 and $24,329, respectively) (52). The financial burden for the patients in Belgium was minimal based on the reimbursement policy of six IVF cycles per patient (50). The American Society of Reproductive

Medicine reported that the average cost for a process of IVF is $12,400.18 in the USA (54), $8,500 in Canada, $6,534 in the UK, $5,645 in Australia, $5,549 in Scandinavia, and $3,956 in Japan (52). Smith et al. found that the average cost was $30,274 for IVF treatment and $7,704 for intrauterine insemination at a single institution. Besides this, women who received only medications were reported to have charges of $1,403 (55). In male infertility treatment, 64% of the out-of-pocket expenses are more than $15,000, whereas 16% are over $50,000 (56).

In addition, reimbursement for infertility treatment has become a state-mandated insurance coverage in the USA. Arkansas, Maryland, and Rhode Island offered a range of up to $15,000, $100,000, and $100,000 over a lifetime. In Hawaii, Connecticut, New Jersey, and Illinois, reimbursement is good for one, two, four, and six IVF cycles, respectively. Massachusetts, Montana, New York, Ohio, and West Virginia did not set a maximum reimbursement rate or limit the number of IVF cycles (54), but it is worth noting that, when examining IVF results, states with reimbursement were found to have lower rates of live births per cycle (57). Moreover, in any country, the total cost of IVF did not exceed 0.25% of healthcare expenditure (52). Furthermore, ART legislation also varied considerably across Europe (20). National health insurance covered 60% of doctors' fees with part of examinations, and 50% of the drug costs were reimbursed by the Social Insurance Institution in Finland (51). Since the German healthcare modernization law was introduced in 2004, which induced the original 100% coverage for up to four cycles, no extensive age restriction has been cut to 50% reimbursement limited to three cycles and strict age limitation (46).

Feasibility analysis of incorporating infertility into medical insurance

Improve patients' compliance

As a fundamental civil right, the reproductive right should be guaranteed. A survey by Merck found that nearly half of the patients who went to reproductive centers had been treated by other departments for 3 years. The time wasted in this process is very critical for women who intended to get pregnant (44). Notably, the acceptance of ART by the public affects the demand for ART in the public (58). If infertility is included in the coverage of medical insurance reimbursement, it will not only reduce the financial and psychological burden of patients but also effectively guide patients to medical insurance designated units for formal long-term treatment, which may reduce the unscrupulous advertising of criminal elements. In addition, it can encourage the patients to adhere to the same hospital for their continuous treatments and to reduce the duplications of medical resources, which can improve birth outcomes (40).

Affordability of health insurance funds

As shown in the chart (Figure 2), the affordability of the medical insurance fund is limited in China. At the same time, the financing of medical insurance is gradually expanding, and the surplus fund is abundant. The inclusion of infertility treatment in health insurance is debatable, and the burden on the health insurance fund could be potentially reduced by lowering the reimbursement rate or reducing the gross margin of treatment through market competition.

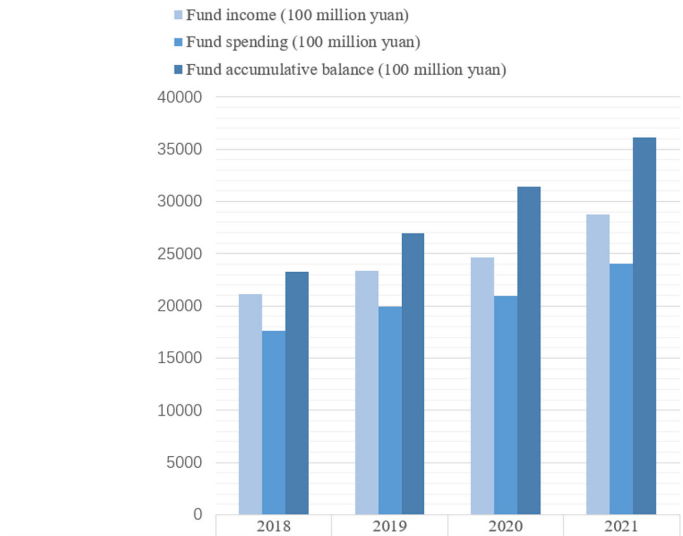
Consideration of the economic burden of indirect diseases

The resources of assisted reproductive services are unevenly distributed, with sufficient resources in eastern provinces and insufficient resources in the central and western regions of China. Considering the large proportion of patients living in remote areas or rural areas with inconvenient transportation and undeveloped medical technology, they have to spend a lot of time and money on transportation and accommodation. Therefore, it is necessary to discuss whether appropriate transportation and accommodation subsidies would be taken into consideration for reimbursement through civil affairs assistance.

Discussion and suggestions

Selective reimbursement and giving preference to vulnerable groups

Belgium provides reimbursement for up to six IVF cycles per patient. In the United States, each state has several IVF cycles or a lifetime maximum reimbursement, and Germany has coverage for an age limit of four cycles. China is entering a depth of aging society dramatically, with an up-trend financial burden continually. According to the forecast, under the precondition of the current healthcare system, the balance of income and expenditure of China's medical insurance fund would be hard to keep steady. In 2026, the annual balance will fall short for the first time, and in 2034, the cumulative balance will fall short for the first time (59). The increasing prevalence of infertility, coupled with the aging trend in China, has increased the need for ART. Affordability is the most essential consideration of the medical insurance system. At the current level of health insurance burden, it is possible to selectively cover some programs most needed by patients. The medical insurance reimbursement level of infertility patients should also be at a magnitude within the affordable range of medical insurance funds. As long as it does not affect the existing Medicare claims,



	2018	2019	2020	2021
Fund income (100 million yuan)	21090.11	23334.87	24638.61	28727.58
Fund spending (100 million yuan)	17607.65	19945.73	20949.26	24043.1
Fund accumulative balance (100 million yuan)	23233.74	26912.11	31373.38	36156.3

Source: national healthcare security administration
Data release date: 2022-06-08

FIGURE 2
Income and expenditure of basic medical insurance fund, 2018–2021.

the examination and treatment of infertility patients should be actively supported to intervene in the fertility rate effectively in the whole nation. For the main function of insurance, it is well recognized and highly recommended that medical insurance should favor vulnerable groups, such as poor and elderly couples who lost their only child. We can learn the advantage of the insurance for severe disease formation, with no minimum payment line and improved reimbursement level, of which corresponding medical assistance could be set up for application aggressively. Under the applicable circumstance, it is suggested that reduction or control of the out-of-pocket costs of poor patients to the acceptable range that would highlight the significant role of medical insurance be tried. Meanwhile, optimization of the structure of fund usage and appropriate consideration of the compensation of indirect costs could also benefit the patients who belong to the priority group with medical insurance support needed.

Some commercial insurance covering infertility and assisted reproduction

The new medical reform plan should continually develop diverse plans of commercial health insurance, as commercial health insurance plays an essential role in the national medical security system. At present, commercial insurance for infertility and assisted reproduction remains limited; it requires more support from national policies to improve the situation by balancing the market related to the supply and demand among the whole society.

Improve the technical skills of infertility treatment in China

The live birth rate per cycle is the highest in the USA, and the multiple pregnancy rate is relatively lower. The development of ART in China is relatively late and immature, and the success rate of ART is not high. Multiple pregnancies and other defects are still common. According to the assessment of ART in Liaoning Province from 2012 to 2016, the clinical pregnancy rate under the therapy of ART was only 45.59–53.63% (60). This means that, even under the circumstance with the assistance from relatively mature ART, nearly half of infertility patients are still unable to achieve their desire to have children (43). By the end of June 2020, there have been 523 medical institutions approved to carry out human-assisted reproduction technology and 27 medical institutions backed to set up human sperm banks (Table 1) in China. Only 396 hospitals were licensed for IVF, and 23.40% of the fertility centers could not meet the requirements of

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TABLE 1 Number of reproductive center gaps by provinces and cities in China in the second half of 2020 (unit: per).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
a	56	33	31	31	31	30	27	23	21	19	18	18	18	17	15	14	13	13	12	12	10	10	9	9	8	8	8	4	2	2	1
b	54	40	33	30	30	30	27	28	20	17	18	16	21	20	19	20	16	13	13	15	12	9	10	8	10	6	4	6	1	2	2
c	-2	7	2	-1	-1	0	0	5	-1	-2	0	-2	3	3	4	6	3	0	1	3	2	-1	1	-1	2	-2	-4	2	-1	0	1

a, number of reproductive centers in the first half of 2020; b, planned number for 2020; c, gap; 1–31, Guangdong, Jiangsu, Shandong, Hebei, Henan, Hubei, Zhejiang, Hunan, Guangxi, Shanghai, Liaoning, Jiangxi, Beijing, Yunnan, Fujian, Anhui, Sichuan, Guizhou, Shanxi, Tianjin, Heilongjiang, Chongqing, Shanxi, Jilin, Neimenggu, Hainan, Xinjiang, Gansu, Qinghai, Ningxia, and Xizang (source: National Health Commission).

IVF technology. Due to the regional factor and lower population economic levels, the number of medical institutions is far less developed in western China compared to those located in eastern China. This imbalance of distribution could make it hard for infertility patients from economically underdeveloped areas to meet their family reproductive aspirations. Therefore, it would be better to encourage the medical institutions in the eastern region to help with the technical implementation and development in the western region by rotations or remote services to alleviate the uneven distribution of health resources and services. Besides this, the reality of assisted reproduction equipment and drugs in China is highly dependent on imports; therefore, it is under high warranty to develop assisted reproductive medical devices and related medication research.

What is worth paying attention to is that, in 2005, Hansen et al. analyzed the results of 25 related studies and reported that the incidence of birth defects in ART offspring increased by 25% or more compared to the rate in a natural pregnancy (61). The increase in birth defects caused by ART should be addressed by improved technology.

Regulate the application of ART

With the continuous development of ART technology, more attention should be paid to the modifications and revisions of relevant regulations. Local governments would help with the implementation of regulations by gradually introducing local regulations and supplementing their policies with special campaigns to encourage or regulate the use of ART in qualified medical institutions. Other issues related to ART might exist in the gray—like egg donation and surrogacy; thus, stricter regulation and legal-level control are also urgently required.

In our modern society with diverse healthcare systems, insurance plays a critical role in ensuring the patients' authority for suitable healthcare and minimizing the related financial burden. Although the examination and treatment of infertility are still far behind the level of full coverage by medical insurance around the whole nation of China at present, there is a huge potential to improve patients' compliance and promote the development and implementation of ART through the selective inclusion of medical insurance reimbursement and policies focusing on the vulnerable groups. In response to Suggestion No. 5581 of the Fourth Session of the 13th National People's Congress, the medical insurance department in China has announced that some eligible fertility support drugs, including bromocriptine, triptorelin, clomiphene, and other ovulation promotion drugs, will be included into medical insurance at the scope of a rational price to improve the Medicare and medication services of infertility patients. In addition, without affecting the existing circumstances of current affordable medical insurance funds, different support measures are further developed—for example, 16 mature, safe, and reliable ART items, such as intrauterine insemination technology, embryo transfer, and sperm selection, which are common in outpatient clinics, will be included in category A of medical insurance reimbursement at 15 designated medical facilities in Beijing this year. With the increase being in public attention, it is believed that the reproductive rights of infertile patients will be under a fast-developing protection pathway in China.

Raise male awareness of infertility

A large proportion of male patients said that they had only discussed their infertility with their wives and felt uncomfortable or even ashamed of talking about it. For a long time, from the bottom of their hearts, most people have believed that women are most responsible for infertility (62), and women have been blamed more and received more useless tests and even treatments. Male factors must be taken into account in the early stages of a fertility assessment, as the minor problems of male infertility can be treated with appropriate drugs to relieve the economic, psychological, and physical stress on both partners. Raising the level of awareness of male infertility is now vital. Through the examination of male infertility, we can achieve timely prediction and prevention of cardiovascular diseases, occurrence or development chronic comorbidities, and mortality and improve the overall male health survival. We can gradually and comprehensively raise people's awareness of male infertility and improve men's awareness of going to a hospital for examination through many ways—classroom education in schools, popularization in the process of routine

physical examination and medical treatment in hospitals, and publicity in communities (7).

Author contributions

LW proposed the central idea and is responsible for the main writing and subsequent revision of the paper. XX and TW also participated in writing the first draft of the paper. YZ analyzed most of the data. QT and JL contributed to the elaboration and additional analysis. YW and WH reviewed and revised the article many times. WW provides financial support. WW is responsible for reviewing the final 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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The potential impacts of circadian rhythm disturbances on male fertility

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A circadian rhythm is an internalized timing system that synchronizes the cellular, behavioral, and physiological processes of organisms to the Earth's rotation. Because all physiological activities occur at a specific time, circadian rhythm disturbances can lead to various pathological disorders and diseases. Growing evidence has shown that the circadian clock is tightly connected to male fertility, and circadian perturbations contribute to infertility. The night shiftwork, insufficient sleep, and poor sleep quality are common causes of circadian disturbances, and many studies have reported that they impair sperm quality and increase the risk of male infertility. However, research on the impacts of light, body temperature, and circadian/circannual rhythms is relatively lacking, although some correlations have been demonstrated. Moreover, as the index of sperm quality was diverse and study designs were non-uniform, the conclusions were temporarily inconsistent and underlying mechanisms remain unclear. A better understanding of whether and how circadian disturbances regulate male fertility will be meaningful, as more scientific work schedules and rational lifestyles might help improve infertility.

KEYWORDS

circadian rhythm, disturbance, male fertility, infertility, sperm quality

1 Background

As the most predictable environmental change in our planet, day/night alteration is accompanied by oscillations in environmental temperature, humidity, and food availability (1). When the sun rises in the morning, diurnal animals awaken from sleep and plants are exposed to the first light of a new day; thus, all organisms have evolved a universally internal rhythmic timing system to adapt to the Earth's rotation (2–4). This endogenous system is called circadian rhythm, which is a precise 24-h oscillating cycle to guarantee optimal performance for biochemical functions and

physiological processes (1, 5), including sleeping habits, body temperature, feeding behavior, hormone secretion, diet (6, 7) and homeostasis (7–9). A recent paper published in *Nature* also showed that time-restricted feeding (TRF) delayed aging and extended lifespan through circadian-regulated autophagy (10).

Before artificial light was invented, humans modified their behavior to match the natural alteration of day/night (3). Although advancements in modern science and technology have greatly changed our daily living habits and improved human health, they are also accompanied by increased diseases (11). Nowadays, elevated social pressures, overloaded work schedules, and personal habits have caused individuals extensively living with artificial lights or luminescent screens (11) that one-fifth terrain in the planet and four-fifth of the world population (99% individuals for Europe and USA) are exposed to light-populated skies or receive misdirected and obtrusive artificial/outdoor light (12). Meanwhile, prolonged night shiftwork, circadian sleep disorders, jet lag, and distance travel across multiple time zones have become more common (11). All these habits inevitably change the daily wake/sleep cycle and gradually lead to circadian misalignment (11), which finally disturb homeostasis, induce oxidative stress, promote an inflammatory response, and accelerate the coagulation process (7–9, 13). These populations are also more vulnerable to hypertension, diabetes mellitus (DM), hyperlipidemia, obesity, atherosclerosis, and other diseases (7, 11, 14, 15).

Fertility is a fundamental process in animal reproduction and is affected by environmental cues such as light, temperature, rainfall, and food availability (5, 16–19); as such, most species reproduce in times with mild weather and optimal food availability (5, 20). For example, many animals evolve a precise seasonal fertility between spring and early summer; others with longer gestations mate in late summer and autumn, which leads to pregnancy in winter and parturition in spring (21–23). Although humans are not seasonal breeders, their sexual activity (24, 25) and reproductive function are still influenced by the peripheral environment (24, 26), although relatively less (27, 28). Infertility is defined as the inability to successfully spontaneously conceive for at least 2 years of unprotected sexual intercourse and is influenced by occupational and lifestyle factors such as smoking, obesity, alcohol consumption, psychological stress, and lack of exercise (29, 30). Currently, infertility affects nearly 15–20% of all couples worldwide (24, 30) and contributes to various psychological, medical, and financial consequences (30).

In fact, numerous studies have shown that elevated night shiftwork, overloaded work schedule, poor sleep quality, and popularization of mobile phones disrupted the circadian rhythm (5, 27, 28, 31, 32) to impair female fertility by affecting the menstrual cycle and altering hormone secretion, which finally increases the incidence of preterm birth, spontaneous abortion, and membrane rupture and reduces breastfeeding success (1, 23). However, males are estimated to be solely responsible for 20–30% cases of infertility (30, 33), while other studies have even indicated a rate of 50% (34). Nevertheless, whether disturbed circadian rhythms contribute to male infertility, like deteriorating female reproduction, is controversial, and whether a normal or healthy circadian rhythm improves male fertility is more interesting. Herein, we review and discuss the potential relationship between the circadian rhythm disturbances and male fertility.

2 Organization and molecular mechanism of the circadian clock system

Circadian rhythm was first described by Konopka in 1971, who cloned the mutant clock gene of *Period* in *Drosophila*; the veil of the circadian clock was subsequently disclosed (1, 35). Recently, the 2017 Nobel Prize in Physiology or Medicine was awarded to Jeffrey C. Hall, Michael W. Young, and Michael Rosbash to reward their huge achievements in exploring and clarifying the regulatory mechanisms in the circadian clock.

The circadian clock system consists of three basic elements: the input pathway, the main oscillator, and the output pathway. The input pathway is a passage that modulates the non-24-h central circadian pacemaker to 24-h and adapts the endogenous timing phase to local environment (1). As the main *Zeitgeber* that synchronizes organisms' circadian clock (9, 36, 37), environmental light is perceived by melanopsin-containing retinal ganglion cells and is transmitted to a central oscillator *via* the retinohypothalamic tract as electrical signal (38) (Figure 1). Other factors, such as environmental temperature, feeding behavior, physical exercise, and social interactions, can also be perceived (38). The master oscillator of the circadian clock for mammals, which is composed of a set of circadian clock genes and coded proteins, is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus which acts by manipulating the daily endogenous rhythms of physiological and behavioral processes (9). The human SCN comprises approximately 50,000 neurons, including diverse cellular components and numerous neurotransmitters and peptides (39, 40). In addition to the main oscillator, the circadian clock system also exists in almost every peripheral tissue and organ (1, 3, 41). For example, when feeding behavior occurs beyond regular eating time, the circadian timing system in SCN is constant, but rhythms in peripheral organs such as stomach, liver, and pancreas are altered and disturbed, which finally contributes to desynchronization between the internal and peripheral circadian clocks (38). Finally, the output pathway transfers central circadian information to peripheral effector organs (1). The output neural pathways are diverse and include the paraventricular nucleus of the thalamus, subparaventricular zone, medial preoptic area, and

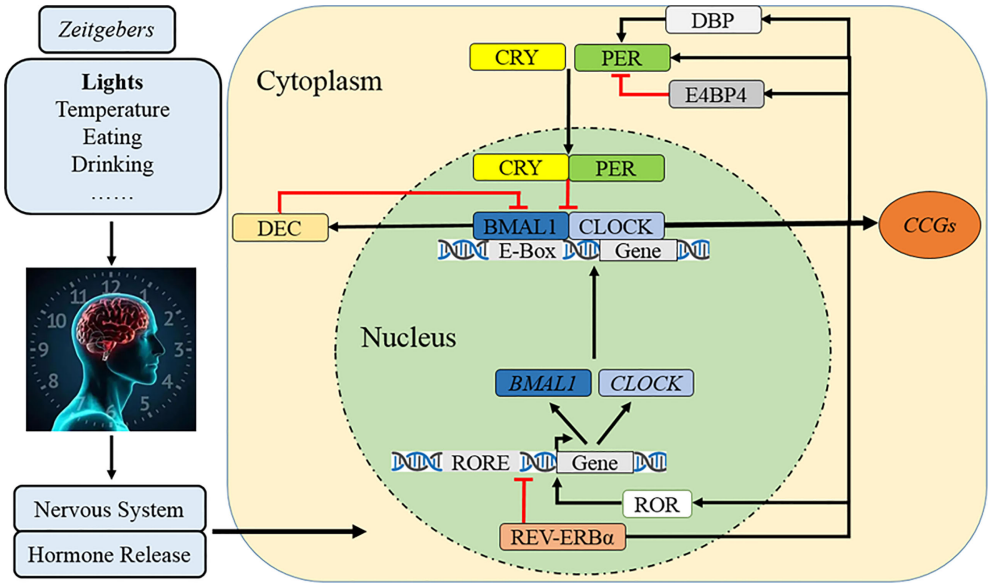

FIGURE 1
Molecular mechanism of the circadian clock. After cues from Zeitgebers are transmitted to SCN as electrical signals, the circadian clock rhythm is orchestrated by a set of genes and proteins which forms interlocked positive and negative feedback loops. Specifically, CLOCK and BMAL1 constitute the core positive heterodimer complex (CLOCK/BMAL1) to activity rhythmic transcription of *PER1/2/3*, *CRY1/2*, and relevant clock controlled genes (*CCGs*). The repressors of PER and CRY accumulate and form a negative feedback loop in cytoplasm during the day and then translocate back to the nucleus to inhibit the level and activity of CLOCK/BMAL1. The complex of PER/CRY is also subsequently disassembled or resolved, while a new circadian cycle is also followed by another 24–h. Meanwhile, REV-ERBα reduces *BAML1* transcription and RORα promotes it to manipulate the CLOCK/BMAL1 complex. The third loop involves that DBP induces *PER* transcription and lengthens the rhythmic period but E4BP4 exerts opposite effects, while DEC inhibits CLOCK/BMAL1-induced *PER1* transcription.

dorsomedial nucleus of the hypothalamus (1, 42–46). Hormones are also regulated by the SCN rhythm and are regularly secreted during the day/night cycle (1, 47, 48).

At the molecular level, the circadian clock system is orchestrated by a set of genes and proteins that form interlocking positive and negative feedback loops of transcription and translation (11, 49) (Figure 1). In the nucleus, the circadian locomotor output cycle kaput (CLOCK) and brain and muscle ARNT-like protein 1 (BMAL1) constitute the core positive transcription factor (CLOCK/BMAL1) (1, 3, 11, 49). This heterodimer complex binds to a specific E-box (5′-CACGTG-3′) sequence on the promoters of *Period* (*PER1*, *PER2*, and *PER3*), *Cryptochrome* (*CRY1* and *CRY2*), and relevant *clock-controlled genes* (*CCGs*), which finally activate their rhythmic transcription (1, 3, 11, 49). The PER and CRY accumulate and dimerize in the cytoplasm during the day and then translocate back to the nucleus with a timed delay to inhibit the activity of CLOCK/BMAL1 and downregulate the transcriptional activation of downstream circadian genes, forming the core negative feedback loop (1, 3, 7). The repressor complex of PER/CRY is also subsequently disassembled or resolved, while a new circadian cycle occurs in the subsequent 24–h (1, 3, 7). The secondary negative regulatory arm involves the nuclear receptors of orphan nuclear receptor (REV-ERBα/β) reducing *BAML1* transcription and retinoic acid-related orphan receptor alpha (RORα/β) promoting it (7, 50, 51). Another CLOCK/BMAL1 negative loop comprises D-site albumin promoter-binding protein (DBP) and E4 promoter-binding protein 4 (E4BP4) which compete binding to the *PER* promoter of the D-box element (52–54); the upregulated DBP cooperatively induces *PER* transcription and lengthens the rhythmic period but E4BP4 exerts opposite effects (52, 54), while elevated differentiated embryo chondrocyte (DEC) inhibits CLOCK/BMAL1-induced *PER1* transcription by direct protein–protein interactions with BMAL1 and/or competition for E-box elements (55, 56).

3 Light exposure and male fertility

Light is a fundamental *Zeitgeber* that synchronizes endogenous circadian rhythms with external environments (57). Nowadays, millions of televisions, computers, and smartphones are operated every day (17), and the time spent on these devices peaks in the evening and shortly prior to sleep onset (17). “2011 Sleep in America” also reported that more than 90% individuals used digital screens within 1–h before sleep (17). Thus, the eyes are increasingly exposed to electronic screens or

artificial light (17), which contributes to various diseases, including male infertility (Figure 2).

A retrospective study recently investigated the impact of digital screen on sperm quality in 116 men (17). Tablet or smartphone usage in the evening or after bedtime was associated with decreased total motile sperm number (−0.173, $p < 0.05$), sperm progressive motility (−0.322, −0.299, $p < 0.05$), sperm concentration (−0.169, $p < 0.05$), and motility (−0.392, −0.369, $p < 0.05$) while increasing the percentage of immotile sperm (0.382, 0.344, $p < 0.05$) (17). The authors hypothesized that artificial light chronically impaired the wake/sleep cycle and disturbed the circadian rhythm, which finally deteriorated male fertility by suppressing melatonin levels (17). However, the exact mechanism has not been explored and other artificial illuminations were not analyzed.

Ogo et al. divided pregnant rats into groups of constant light–light (LL) or light–dark (LD) cycles during the gestation period, but all offspring were housed under a normal LD photoperiod until adulthood (57). Offspring in the constant LL group showed a significantly increased number of abnormal sperm heads and decreased normal sperm number ($p = 0.0001$). The testosterone level, seminiferous tubule diameter, Sertoli cell number, and sperm count in the epididymis were also decreased (57). Moreover, LL reduced glutathione reductase (GR) levels in the epididymis but increased the enzymatic activity of glutathione S-transferase (GST) and glutathione peroxidase (GPx) in the testis (57). These findings suggest that extended light exposure leads to male infertility by decreasing testosterone levels and increasing oxidative stress (57). Moustafa et al. also placed male rats under prolonged light (20-h light and 4-h dark) and darkness (4-h light and 20-h dark) for 12 weeks (57). The prolonged light increased sperm count and motility, while extended darkness reduced the incidence of sperm abnormalities (57). Meanwhile, both extended light and darkness altered hormone secretion and reduced estradiol levels, while increasing FSH, LH, testosterone, and prolactin levels (57). Furthermore, prolonged light exposure increased the expression of *Per1/2* and decreased *Bmal1*, while darkness exposure upregulated *Per1/2*, *Clock*, and *Rer-erab* (57). These results proved that abnormal light exposure disturbed testosterone secretion and the testicular circadian rhythm (57).

Similar to impaired female reproduction, abnormal light exposure may also lead to male infertility (17, 28, 57). However, current results on sperm parameters, hormone levels, and antioxidant indicators are diverse and controversial, while the specific mechanisms of how altered light exposure contributes to male infertility remain to be explored.

4 Shiftwork and male fertility

Sleep is a repetitive behavior that synchronizes with daily circadian rhythms, and a regular wake/sleep cycle is essential for human health (29). Shiftwork refers to working beyond the traditional daytime (08:00 to 16:00), which covers rotating or non-rotating (fixed) shift models (29, 58). The European Working Condition Survey in 2005 estimated that more than 17% of staff in the European Union performed shiftwork (29).

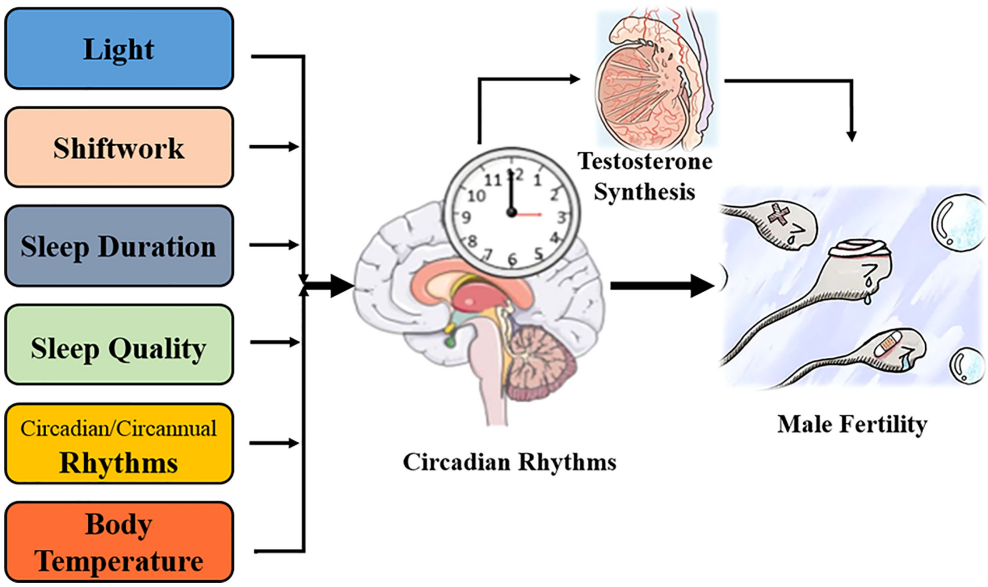

FIGURE 2
Involvement of circadian rhythms with male fertility.

Several studies have shown that shiftwork disturbed the wake/sleep cycle and impaired circadian rhythms, which contributed to cardiovascular disorders, metabolic diseases, and male infertility (29, 59) (Figure 2).

Demirkol et al. found that shift workers (n = 104) had a higher incidence of oligozoospermia (p = 0.006) and lower mean normal morphology (p = 0.036) than non-shift workers (n = 116), while shiftwork (OR = 2.11, 95% CI: 1.03 to 4.34) was independently related to oligozoospermia in multivariate analysis (29). Kohn et al. claimed that shift workers had lower sperm density (p = 0.012), total motile count (p = 0.019), and testosterone levels (p = 0.026) than non-shift workers, whereas no difference was observed in sperm volume and motility or FSH and LH levels (60). El-Helaly et al. explored the impact of occupational exposure on male fertility and found that shiftwork significantly increased the risk of infertility (OR = 3.60, 95% CI: 1.12 to 11.57) (61), which was consistent with findings of Irgens et al. who reported reduced sperm quality among shift workers (OR = 1.46, 95% CI: 0.89 to 2.40) (61). Sheiner et al. also revealed that male infertility was significantly associated with shiftwork (OR = 3.12, 95% CI: 1.19 to 8.13) (62). However, the mechanism by which shiftwork impairs male fertility requires further analysis.

Furthermore, a cross-sectional study with 1,346 men found that rotating shift workers (RSW) had significantly lower total sperm counts (median: 147.3 × 10^6, interquartile range (IQR): 80.7 × 10^6 to 255.3 × 10^6) than day workers (median: 176 × 10^6, IQR: 101.9 × 10^6 to 281.2 × 10^6) (p = 0.034) (63). More RSW (42.4%) revealed lower total sperm count (≤120 × 10^6) than day workers (30.5%) (p = 0.005); this remained true even after controlling for age, income, smoking, alcohol consumption, BMI, educational level, and abstinence period (OR = 1.60, 95% CI: 1.10 to 2.32, p = 0.014) (63). For permanent shift workers (PSW), LH was the only significantly different parameter (p = 0.044) when compared with day workers (63). In consideration of these differences between the two shift working types, the authors suggested that PSW might align their wake/sleep cycle on workdays with their schedule on days off, which finally decreased circadian desynchrony (63). However, this hypothesis remains unexplored. In addition, a study using a mouse model exposed to photoperiod shifting (light on at 20:00 and off at 08:00) for 28 days showed that the mean total sperm count was significantly decreased by inducing apoptosis in seminiferous tubules, while sperm parameters recovered when light shifting was altered to a constant normal photoperiod for another 35 days (63). However, whether such recovery of sperm quality could be observed in shift workers requires more evidence.

In contrast, Tuntiseranee et al. (64) and Bisanti et al. (65) reported that night shiftwork was not related to male infertility, while another study with 456 males also claimed that shiftwork or night work did not alter sperm quality (66). Considering this discrepancy, further studies with large prospective populations are necessary to verify the impact of shiftwork on male fertility before providing suggestions to shiftwork schedules.

5 Sleep duration and male fertility

Similar to proper diet and exercise, normal sleep duration is important for human health (67). The National Sleep Foundation has suggested a sleep period of 7–9 h for people aged 18–64 years and 7–8 h for those over 65 years (68, 69). However, on overloaded work schedule and environmental light/noise pollution, the National Health Interview Survey found that 30% workers (nearly 40.6 million) sleep for <6 h/night in the US (70). Longitudinal studies have claimed that insufficient sleep promoted the risks of mortality, hypertension, T2DM, obesity, and myocardial infraction (69), while others reported a U-shaped association that sleeping for 7–9 h/night had the least risk of deteriorating human health (69, 71). The effect of sleep duration on male fertility has also been explored (69) (Figure 2).

Shi et al. enrolled 328 males and found that the sperm concentration remained constant when sleeping for 4.7–8.0 h/night; however, it remarkably dropped when sleeping for <4.7 h/night and noticeably increased when sleeping for >8 h/night (31). Liu et al. observed that sperm count, motility, and survival rates were significantly decreased in participants sleeping for <6 h/night when compared with average (7–8 h/night) or long (>9 h/night) durations (5, 20). Demirkol et al. also reported a positive correlation between sleep duration and sperm concentration in 104 shift workers (29). These results suggest that insufficient sleep significantly impairs sperm quality.

In contrast, an inverse U-shaped relationship between sleep duration and male fertility was observed (72). The Pregnancy Online Study (PRESTO) with 1,176 couples revealed that the fecundability ratio (FRs) for 8 h/night was significantly higher than that for <6 h/night (FR: 0.62, 95% CI: 0.45–0.87), 7 h/night (FR: 0.97, 95% CI: 0.81–1.17), and ≥9 h/night (FR: 0.73, 95% CI: 0.46–1.15), indicating 8 h/night as the most suitable sleep duration for male fertility (73). The Male Reproductive Health in Chongqing College Students (MARHCS) study, which enrolled 796 individuals in 2013, showed that the highest sperm parameters were found for sleeping for 7.0–7.5 h/night, while both shorter and longer sleep durations were correlated with a declined sperm index (p = 0.001 and 0.002, respectively) in a dose-response manner (72). Sleeping for ≤6.5 h/night was related to a 4.6% (95% CI: -10.5 to 22.3) reduction in sperm volume and a 25.7% (95% CI: -1.2 to 60.1) reduction in total sperm number, while sleeping for >9.0 h/night was correlated with 21.5% (95% CI: 9.2 to 32.2) and 39.4% (95% CI: 23.3 to 52.1) reductions, respectively (72). Moreover, sperm parameters were significantly improved when sleep duration was altered to 7.0–7.5 h/night in the following year, which confirmed the sleep–fertility association again (72). However, this damage to

sperm quality might not be completely reversible or need more time to recover, as Chen et al. revealed that improved sperm quality was only found in a small (but significant) proportion of patients who changed the sleep duration to a "proper" length (72). High DNA stainability (HDS) in the epididymis is an important index of incompletely differentiated sperms (74). Wang et al. reported that HDS was highest when sleeping for 7.0–7.5 h/night, while those with both shorter and longer sleep durations presented lower HDS (71). A preliminary cross-sectional study based on 92 healthy men also demonstrated an inverse U-shaped relationship between sleep duration and testicular volume, with those sleeping for 9.5 h/night showing the largest volume (75).

These epidemiological studies indicate that sleep duration is strongly associated with male fertility and sperm quality, although the specific positive, negative, or inverse U-shaped association remains to be clarified. Moreover, evidence of long sleep duration impairing male fertility is relatively weak and inaccurate.

From the perspective of animal research, sleep restriction (SR) to 6 h/night (sleeping from 10:00 to 16:00) for 21 days significantly increased immotile spermatozoa and impaired sperm motility, whereas total sperm number and transit time through the epididymis did not change (76). Chen et al. observed that SR for 35 days decreased sperm concentration, viability, and motility, while it increased sperm malformation (77). Alvarenga et al. found that SR and paradoxical sleep deprivation (PSD) resulted in 15% and 50% lower sperm viability, respectively; although sperm concentration was similar, spermatozoa with faster movement were significantly decreased compared to the control group (78). Moreover, Rizk et al. showed that PSD for 5 days significantly increased abnormal sperm morphology but decreased sperm count, viability, and motility (30). Choi et al. reported that SD for 7 days (SD7) significantly reduced sperm motility, whereas SD4 and SD7 partially induced seminiferous tubular atrophy and spermatid retention (32). These results suggest that sleep deficiency impairs sperm viability by disrupting sperm cycle maintenance (78); however, the specific mechanism remains to be investigated.

As sperm quality is mainly regulated by hormonal concentration, numerous studies have focused on the relationship between sleep duration and hormonal levels. The testosterone level is the highest in the morning and lowest during evening, and numerous clinical and basic investigations have shown that sleep deficiency/restriction altered the testosterone concentration by disturbing its secreted cycle (23, 69, 79–81). For example, Chen et al. found that sleeping for 6 h/night for 35 days significantly decreased the testosterone concentration (77) and Alvarenga et al. observed that SD for 96 h significantly inhibited testosterone levels by 45% (78). Choi et al. reported that SD4 and SD7 significantly decreased testosterone release but increased corticosterone production (32). Although the mechanism has not been clarified (77), authors inferred that sleep deficiency increased cortisol and corticosterone levels by activating the hypothalamic–pituitary–adrenal (HPA) axis, the feedback of which inhibited the hypothalamus–pituitary–gonadal (HPG) axis to decrease testosterone secretion (30, 32). In contrast, a preliminary cross-sectional study based on 92 healthy men found that insufficient sleep did not alter total/free testosterone levels (75), while the MARHCS trial found that sleep duration had no impact on reproductive hormones (72). Siervo et al. even found that sleep deficiency significantly increased the testosterone concentration in plasma and intratesticle (76, 82). Some authors thus thought that the altered hormones might not be responsible for decreased sperm quality or infertility (72). As sleep deficiency inhibits testosterone secretion during the second half of a biological night (69, 81), others have suggested that different types and definitions of sleep deficiency may contribute to the controversial interaction between sleep duration and male fertility (69).

Sleep deficiency also damages testes by inducing oxidative stress (76, 82). Akindele et al. found that SR for 14 days significantly elevated testicular malondialdehyde and glutathione (GSH) in adult rats (82, 83). Siervo et al. revealed that SR for 21 days sharply increased the tert-butyl hydroperoxide-initiated chemiluminescence (CL) curve of the epididymidis, which suggested an enhanced peroxidative attack by ROS (76, 82). These results infer that sleep deficiency may impair sperm quality by disrupting the balance between oxidative and antioxidant stress (30, 76). Another male Wistar rat model found that SD (sleeping for 4 h/night) impaired functions of the blood–testis and blood–epididymis barriers by increasing its permeability to low/high-molecular-weight tracers and decreasing the expression of tight-junction proteins, androgen, and actin receptors (84). In this model, rat fertility was improved by recovering sleep for 2–3 days, as the percentage of ejaculating males and impregnated females increased (84).

In general, although more studies tend to show that sleep duration plays an essential role in male fertility and sleep deficiency contributes to infertility, some authors still disagree on the aforementioned correlation as the research designs are diverse and the results are inconsistent. Moreover, exploring how sleep duration regulates male fertility is also a huge challenge considering the complex mechanisms of reproductive function. Finally, whether prolonged sleep duration restores sperm quality remains to be proved.

6 Sleep quality and male fertility

Growing evidence has suggested that sleep quality had a significant impact on human health, while poor sleep [defined

as difficulties in falling asleep and lying awake at night (5, 85)] increased the risk of hypertension, T2DM, cardiovascular disease, depression, cancer, and male infertility (85) (Figure 2).

In 2013, Jensen et al. explored the association between sleep disturbance (based on the Karolinska Sleep Questionnaire) and semen quality in 953 young Danish men (16, 85). Males with both lower and higher sleep scores had significantly decreased total sperm count and concentration, percentage of motile spermatozoa and morphologically normal spermatozoa, and testis size than the control group (sleep score: 11 to 20) (16, 85). Those with a sleep score >50 (poor sleep) had a 29% reduction (95% CI: 2 to 48) in sperm concentration, 25% decline (95% CI: -4 to 46) in total sperm count, 0.9% lower (95% CI: -3.1 to 4.9) motile spermatozoa, and 1.6% fewer (95% CI: 0.3 to 3.0) morphologically normal spermatozoa compared to the control group (16, 85). Kohn et al. also found an inverse U-shaped relationship between sleep quality and sperm quality; the total motile count for moderate sleep quality was 15.4 M sperm/ml greater than individuals without sleep difficulty and 4.72 M sperm/ml greater than those with severe sleep difficulty ($p = 0.018$) (60).

Some authors have previously evaluated sleep quality using the Pittsburgh Sleep Quality Index (PSQI) global scores. For instance, Chen et al. recruited 842 healthy males which revealed that poor sleep quality (PSQI >5.0) had a lower total sperm count (8.0%, 95% CI: -15% to -0.046%), motility (3.9%, 95% CI: −6.2% to −1.5%), and progressive motility (4.0%, 95% CI: −6.5% to −1.4%) (86). Du et al. conducted a cross-sectional study among 970 patients and demonstrated that increased PSQI scores indicated lower total sperm number ($r = −0.160008$, $p < 0.001$), sperm concentration ($r = −0.167063$, $p < 0.001$), motility ($r = −0.187979$, $p < 0.001$), progressive motility ($r = −0.192902$, $p < 0.001$), and normal morphology ($r = −0.124511$, $p < 0.001$) (5). Hvidt et al. analyzed the fertility of 140 males and similarly found that sperm quality was reduced when PSQI was increased ($p = 0.04$) (85). Meanwhile, Viganò et al. observed that sperm motility was decreased for patients lying awake most of the night, whereas sperm volume was lower and concentration was higher for those with difficulty in initiating sleep (87).

Studies have suggested that poor sleep quality might decrease the serum testosterone concentration (86, 88) and impair Sertoli cells in the seminiferous tubules (32, 86). However, Jensen et al. (16), Chen et al. (72), Ruge et al. (89), and Du et al. (5) did not observe any correlation between sleep quality and reproductive hormone levels. In general, although some authors speculated that poor sleep quality (86, 90) or inappropriate sleep habits (5, 38, 91) disturbed the circadian rhythms and damaged male fertility, the results were still inconsistent and the evidence was weak. Further epidemiological and fundamental studies are required to clarify this issue.

7 Circadian/circannual rhythms and male fertility

As the two most prominent biological rhythms, day/night alterations (circadian rhythm) and seasonal changes (circannual rhythm) play essential roles in numerous biological functions, including male fertility or sperm quality (24) (Figure 2).

The MARHCS dataset, which analyzed 10,362 community populations, found that the sperm DNA fragmentation index (DFI, the most frequent parameter to assess sperm chromatin integrity) decreased from 08:00 to 11:00 ($p = 0.335$) and then increased after 12:00 ($p < 0.001$), while the DFI was elevated by 4.2% per hour (95% CI: 1.9 to 6.7, $p < 0.001$) after 11:00 (92). The Reproductive Medical Center (RMC) dataset with 630 clinical populations showed that sperm DFI decreased before 11:00 ($p < 0.01$) and each hour (after 07:00) was associated with a 3.8% reduction (92). The rat model proved that sperm DFI varied at different times ($p = 0.05$); it decreased from 03:00 to 07:00 ($p = 0.038$) and then increased until 23:00 ($p = 0.002$) (92). Further cosinor analysis indicated a nadir of DFI at 10:00 (92). Xie et al. analyzed 12,245 sperm samples and found that semen collected before 07:00 had the highest total sperm count and concentration, as well as normal sperm morphology (24). Moreover, Shimomura et al. observed that the total motile sperm count and total sperm count were significantly higher in samples collected in the evening than in the morning (34). This suggests that sperm quality was lower in the morning, while evening-collected semen might be easier to achieve successful intrauterine insemination (34).

The circannual rhythm also significantly influences reproductive behavior, especially in mammals (24). To guarantee the greatest survival chances, many species have adapted to ensure that offspring are born at the most suitable time of the year, when climate and food are the most favorable (24). Although humans are not seasonal breeders, sexual activity (24, 25) and reproductive function still alter with a circannual rhythm (24, 26). Xie et al. analyzed 12,245 semen samples and proved that total sperm count and concentration were higher in spring and lower in summer, while morphologically normal spermatozoids were significantly increased in summer (24). The authors suggested that photoperiod alteration was the most likely cause of circannual variation in sperm quality (24, 93), while temperature variation might be another reason for high hyperpyrexia impaired spermatogenesis (24, 93). Circadian clock genes were also involved, as Akiyama et al. reported that *Bmal1* expression in the hypothalamus and testes was significantly decreased in the transitional season compared to the reproductive and non-reproductive seasons; the *Cry1* level also sharply declined in the transitional season compared to the reproductive season. The testicular morphology and circadian

clock genes (*Bmal1* and *Cry1*) revealed circannual alterations (94). However, whether and how circadian clock genes regulate male fertility in circannual rhythms requires further exploration.

8 Body temperature and male fertility

Similar to light alteration and wake/sleep cycle, daily oscillation of environmental temperature is another typical *Zeitgeber* to synchronize the internal circadian system; thus, body temperature also presents a 24-h oscillation to adapt to the Earth's rotation (95). High ambient temperatures can reduce sperm production by destroying the spermatogenic epithelial structure, inducing testicular oxidative stress, and promoting germ cell apoptosis (95–98). Meanwhile, hyperthermia regulates testicular function by altering the daily secretion of reproductive hormones and changing the expression of circadian and testosterone synthesis-related genes (95) (Figure 2).

Li et al. exposed male mice to hyperthermia (39°C) from 11:00 to 15:00 (4 h/day) for 35 days (95). This hyperthermia disturbed the rhythm of testosterone secretion, which increased at 11:00 h and decreased at 15:00 h (95). Hyperthermia promoted the transcription of testicular *Star* and *Ar* at 11:00 and enhanced the protein level of CYP11A1 at 23:00 (95). Moreover, heat exposure stimulated testicular *Clock* expression but decreased its protein content at 11:00 and increased the BMAL1 concentration at 23:00 (95). This suggests that high external temperature arrests spermatogenesis by disrupting the rhythms of testosterone secretion and clock genes (95). Sabés-Alsina et al. maintained New Zealand White rabbits under environmental temperatures increasing from 22°C to 31°C (maintained for 3 h) and then gradually declined to 22°C until 09:00 on the following day (99). Hyperthermia significantly decreased the percentage of viable spermatozoa (74.21% *vs.* 80.71%) ($p < 0.05$) and increased the ratio of acrosomic abnormalities (36.96% *vs.* 22.57%) and tailless spermatozoa (12.83% *vs.* 7.91%) ($p < 0.01$) (99). However, heat exposure did not alter motility parameters or fertility and prolificacy rates (99). The lack of impairment on fertility and prolificacy may be due to the rapid recovery of reproductive function (99).

In summary, studies on the impact of environmental temperature on sperm quality are diverse and definite; however, whether the oscillation of body temperature influences male fertility and, if so, how it works remains a mystery.

9 Clock genes and testosterone synthesis

Although the diverse reproductive hormones are involved in regulating male fertility and facilitating the spermatogenetic process, only testosterone is essential to maintaining spermatogenesis (100). Mainly released by Leydig cells, the serum testosterone concentration presents rhythmic oscillation in adult male mice and human (101–104) which starts to rise at sleep onset (peaks around 8:00) but falls during the day (trough around 20:00) (104, 105). It is reported that circadian misalignment shifts the summit value of the testosterone's diurnal rhythm to happen soon after waking up (106); however, whether circadian disturbance alters the testosterone concentration is controversial (5, 15, 85, 104, 106–108) and which clock element regulates its secretion is also unknown (103) (Figure 2).

Alvarez et al. have found that BMAL1 protein was rhythmically expressed in mouse Leydig cells (103). Since then, most of the core clock genes like *Bmal1*, *Per1/2/3*, *Cry1/2*, *Rev-erbα/β*, *Rorb*, and *Dbp* in Leydig cells were demonstrated to rhythmically oscillate (101–103, 109), except *Clock*, *Rora*, *Ck1δ*, and *Ck1ε* (102). Meanwhile, the steroidogenic-related genes which are responsible for testosterone production in Leydig cells (including *Star*, *Cyp11a1*, *Cyp17a1*, *Hsd3b2*, *Hsd17b3*, *Sf1*, positive-*Nur77*, and negative-*Arr19*) also exhibited 24-h rhythmic expression patterns (101, 102, 109, 110). Specifically, Baburski et al. found the summits of *Star*, *Cyp11a1*, and *Cyp17a1* oscillation occurred approximately at the middle of the light phase, i.e., a few hours before the testosterone release peak (102). These results indicate a crucial role of the circadian clock in testosterone production (101–103, 110). Furthermore, *Bmal1* knockout or inhibition was reported to decrease testosterone secretion by reducing mRNA levels of steroidogenic genes (*Star*, *Cyp11a1*, *Hsd3b2*, *Hsd17b3*, *3β-Hsd*, *Sf1*, and *Nur77*) (101, 103, 111, 112) and Apo (*Apoa1/2* and *Apoc3*) (112). Moreover, *Bmal1* knockdown inhibited testosterone level by inducing apoptosis of Leydig cells (111), and the circadian clock system was involved to the process of bisphenol A (113) and zearalenone (114) reducing testosterone production. In a word, circadian rhythms can regulate testosterone production by various signaling pathways.

10 Conclusion

The circadian rhythm is strongly correlated with human health, while growing evidence suggests that circadian disorders contribute to male infertility. With the high incidence of night shiftwork, sleep deficiency, and poor sleep quality in modern life, numerous studies have investigated their influences on fertility and found that they impaired sperm quality and increased the risk of male infertility. Evidence for the impacts from light, body temperature, and circadian/circannual rhythms is relatively weak, although some correlations have been uncovered. However, the current conclusions were inconsistent as the abundant indices of sperm quality and male reproduction, while how the circadian clock genes were involved also

remained to be further explored. Nevertheless, a better understanding on the interaction between circadian rhythm disturbance and male fertility will be meaningful, as a more scientific and rational lifestyle and work schedule might help to improve infertility.

Author contributions

All authors researched data, made substantial contributions to discussion content, and edited the manuscript before submission. TL, YB and YJ wrote the article, JG and FS guided and revised it. 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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Hypothesis: Metformin is a potential reproductive toxicant

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Metformin is the first-line oral treatment for type 2 diabetes mellitus and is prescribed to more than 150 million people worldwide. Metformin's effect as a glucose-lowering drug is well documented but the precise mechanism of action is unknown. A recent finding of an association between paternal metformin treatment and increased numbers of genital birth defects in sons and a tendency towards a skewed secondary sex ratio with less male offspring prompted us to focus on other evidence of reproductive side effects of this drug. Metformin in humans is documented to reduce the circulating level of testosterone in both men and women. In experimental animal models, metformin exposure *in utero* induced sex-specific reproductive changes in adult rat male offspring with reduced fertility manifested as a 30% decrease in litter size and metformin exposure to fish, induced intersex documented in testicular tissue. Metformin is excreted unchanged into urine and feces and is present in wastewater and even in the effluent of wastewater treatment plants from where it spreads to rivers, lakes, and drinking water. It is documented to be present in numerous freshwater samples throughout the world – and even in drinking water. We here present the hypothesis that metformin needs to be considered a potential reproductive toxicant for humans, and probably also for wildlife. There is an urgent need for studies exploring the association between metformin exposure and reproductive outcomes in humans, experimental animals, and aquatic wildlife.

KEYWORDS

metformin, reproductive toxicant, testosterone, endocrine disruptor, environment, wildlife, development

Introduction

The oral blood-glucose-lowering drug, metformin, is effective, low-cost and the most commonly used antidiabetic drug in the world (1). Metformin has been used to treat diabetes in European countries since 1958 and is currently recommended as a first-line oral treatment for type 2 diabetes mellitus (T2DM) for both men and women (1, 2). In 2012, metformin was prescribed to more than 150 million people worldwide (3) and was the 4th most prescribed drug in the US in 2019 (4). Unlike insulin, metformin crosses the placenta readily (5) and has the potential to cause negative effects on the developing fetus (6–8). However, metformin is used in pregnant women with T2DM, and benefits on the maternal glycaemic level and neonatal adiposity are demonstrated (9). In addition, there is an increase in experimental studies investigating whether metformin can be used in different diseases and conditions including endometriosis (10). The therapeutic indications for metformin prescription may therefore be expanding, resulting in even more widespread use. However, as stated in a recent review by Triggle et al. (10) metformin may act as an endocrine disrupter through multiple sites of actions and signaling pathways, and this uncertainty may offset the expansion use of metformin.

Recently, we found that offspring of diabetic fathers who were prescribed metformin during the three months before fertilization had an increased risk of malformations, especially in the male sexual organs where malformations were three times more common (11). These findings prompted us to search the literature for indications of other negative reproductive effects of metformin and to present the hypothesis that metformin should be considered a potential reproductive toxicant for humans, and possibly also for wildlife.

Clinical action, metabolism, and excretion to the environment

Evidence suggests that long-term metformin treatment works primarily by inhibiting hepatic gluconeogenesis and secondarily by improving glucose uptake in skeletal muscles and adipocytes, resulting in lowering blood glucose concentrations (12). Metformin presumably affects all tissues within the human body *via* AMP-activated protein kinase (AMPK)-dependent and -independent mechanisms, primarily by inhibition of mitochondrial respiration (13). However, despite more than 60 years of extensive use, metformin's precise mechanism of action is not known (14). Metformin, like many pharmaceuticals, derives from petrochemicals, which again derive from fossil fuels (15). Unlike most pharmaceuticals, metformin is difficult to decompose and not metabolized in the human body and thus enters the environment unchanged, mainly through urine and feces (16). As metformin enters the aquatic compartments, it can be transformed into guanylurea, and several recent reports provide evidence that both metformin and guanylurea are present in the environment (17) (Figure 1). A recent study from 2022, investigating pharmaceutical pollution of the world's rivers, reported metformin as one of the most frequently detected active pharmaceutical ingredients, as it was detected in over half of the monitored sampling sites (18).

Reproductive side-effects of male metformin treatment

Our recent nationwide study by Wensink and colleagues (11) showed an association between preconception paternal metformin treatment and genital birth defects in boys in Denmark. In both, the offspring of the background population and of insulin-treated fathers, the prevalence of major birth defects was 3.3%, while the prevalence in the offspring of the metformin-treated father was 5.6% (adjusted OR 1.40, CI95%, 1.08 to 1.82). In the male sexual organs, the malformations were three times more common. The data also supported an association between metformin exposure and alteration in the secondary sex ratios, as the child exposed to metformin, were less often male (49.4%), compared to those without exposure to diabetes drugs (51.4%) or insulin-exposed offspring (51.3%). These findings align with evidence suggesting men exposed to reproductive toxicants may have altered secondary sex ratios (19, 20). However, it is known that hyperglycemia is a risk factor for fetal malformations (21). In the given study a large proportion (84%) of the insulin group likely had been on insulin for many years, whereas we do not know how many in the metformin group were diagnosed and treated during or shortly before spermatogenesis. As it typically takes weeks or months of metformin treatment to control glycemia, the severity of hyperglycemia during spermatogenesis may have differed between the insulin and metformin groups (22). However, if poor glycemic control did play a major role in relation to the increased prevalence of malformation, we would have expected to see a similar signal with an increased prevalence of malformation in fathers taking insulin (23). There is a need for further studies to support our hypothesis, in particular studies accounting for variables such as obesity and glycemic control.

It is well known that men with T2DM have lower testosterone levels. In a recent randomized controlled study, Hu and colleagues (24) found that in men with T2DM, metformin may cause decreasing testosterone levels independent of blood glucose control. The authors reported that a 1-month treatment resulted in a 27.4% (CI95% -47.2 to -7.6) reduction in testosterone levels among men treated with both insulin and metformin, compared with men only treated with insulin. In addition, the authors did another study with a prolonged duration of 3 months of metformin treatment and their results were consistent with the previous study,

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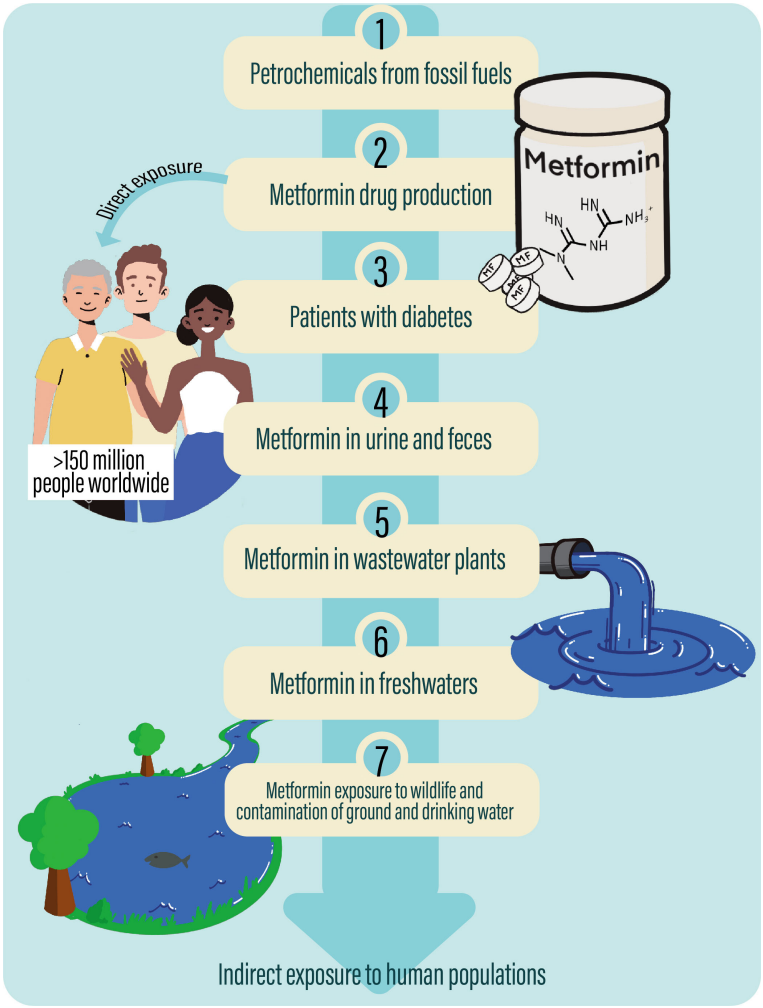

FIGURE 1
Metformin's path from the synthesis of the drug from petrochemicals to contamination of the environment *via* wastewater. 1) Metformin derives from petrochemicals, which again derive from fossil fuels such as oil. 2) From petrochemicals there is a production of the drug, metformin which is 3) prescribed to more than 150 million people, including a large proportion of patients with diabetes 2, who thus are directly exposed through therapeutic treatment. 4) As metformin is not metabolized in the human body, it enters the environment unchanged through urine and feces, and 5) metformin thus reaches wastewater treatment plants and later 6) freshwaters as rivers and lakes and drinking water. 7) Metformin, therefore, contaminates our environment affecting the aquatic wildlife and potentially exists as indirect exposure to human populations all over the world.

as they found that compared with the control group, testosterone levels in the metformin group significantly decreased. Despite these studies being rather small in sample, the data strongly suggest that metformin can reduce the level of testosterone to a clinically significant extent in men (24) and metformin may be another reason for the high prevalence of low testosterone in males with T2DM (25). Furthermore, metformin has been used as an anti-androgen for women with polycystic ovary syndrome (PCOS) (26). Together this shows that the compound is a strong disruptor of steroidogeneses in both sexes. Interestingly, in obese males with metabolic syndrome and reduced fertility, metformin has been shown to improve fertility through increased testosterone production (27). In a study, Morgante and colleagues showed that 6-month metformin treatment resulted in an increase in the serum testosterone in obese males (28). However, this may be related to changes in sex hormone-binding globulin (SHBG) in men with metabolic syndrome, treated with metformin, as obesity and metabolic syndrome are associated with lower SHBG concentrations and thus decreased testosterone production, but normal levels of free testosterone (29, 30). However, when obese males are treated with metformin, their metabolic status improves, resulting in increasing SHBG levels, which can trigger testosterone production, as experimental evidence suggests that an increase in SHBG is associated with an increase in testosterone level (31).

Taken together, these data suggest a scenario where the effects of metformin on reproduction need to be seen in the light of the indication for treatment and the effects of the underlying disease.

Reproductive side-effects of female metformin treatment

In women with PCOS, the effects of metformin treatment are generally described as beneficial, as metformin treatment is associated with a 20% reduction in the testosterone level (26) and improves ovarian cyclicity (27). Furthermore, several randomized trials have shown that metformin treatment in women with PCOS increases clinical pregnancy rates (32).

Metformin is used for the treatment of women with T2DM (9) and currently, there is no evidence that maternal metformin intake is associated with an increased risk of major birth defects. In an international case-control study including 141 malformed offspring, the risk of congenital malformations was regarded as similar in both offspring of women taking metformin for diabetes or PCOS and the background population (33). In addition, a small study on the offspring of women treated with metformin during pregnancy did not find any effects on the testicular size of their sons (34). Thus, in the relatively few and small studies investigating maternal metformin intake during pregnancy, there is found no association with birth defects or other adverse reproductive outcomes. However, studies of large populations of pregnant women using metformin, with a focus on genital malformations, are lacking. There is thus a need for additional studies evaluating the risk of congenital malformations in offspring of mothers treated with metformin. However, it remains clear that metformin in the clinic on one hand is used as an antiandrogenic compound to decrease testosterone in women with PCOS, while it on the other hand is frequently used in women with gestational diabetes or T2DM during pregnancy without considering the potential effects on hormonal regulation of the developing fetus.

Experimental evidence of reproductive effects of metformin from animal models

Disruption of e.g., hormonal regulation during fetal life can have adverse health outcomes later in life (35). Hence, exposure to drugs during sensitive periods of sexual differentiation can induce alterations in cell numbers, leading to irreversible reductions in sperm and oocyte production that ultimately can influence fecundity later in life (36, 37). Evidence from experimental studies suggests that metformin can interfere with fetal life through at least four essential processes: (*i*) steroidogenesis (38), (*ii*) epigenetics (39), (*iii*) metabolism (40), and (*iv*) gamete development and maturation (27) (Figure 2). The exact mechanism of metformin's actions remains to be completely understood, however, it is clear that metformin inhibits Complex I of the mitochondrial respiratory chain at relatively high concentrations (mM) (27). This inhibition results in a decline in ATP production by mitochondria and activation of the AMPK pathway. It has been suggested that the effects on epigenetics, metabolism, and gamete development might be through AMPK, as the signaling pathway is a crucial cellular energy sensor that maintains cellular energy homeostasis. However, not all effects can be explained by activation of the AMPK pathway and the effects on steroidogenesis have been suggested to be AMPK-independent (38).

The potential effects on fertility have especially received much attention and it is clear that maternal exposure to metformin can interfere with reproductive parameters in male offspring. In a study investigating the effect of *in utero* and lactational exposure to metformin in male rat offspring (41), the authors reported a significant decrease in the number of spermatids and spermatids per organ, as well as daily sperm production in male rat offspring exposed to metformin during gestation and lactation, compared to controls. Interestingly, the decrease in sperm count was only observed in the offspring of mothers exposed to metformin during both gestation and lactation, suggesting that the exposure is needed to cover the entire critical period of male sexual differentiation in the rat.

In another study, Tartarin et al. (42) reported that metformin can reduce testosterone production *in vitro*, and probably also *in vivo*. The authors reported that *in vitro*, metformin decreased the secretion of testosterone by human fetal testicular tissue at a therapeutic dose (50 µM) by 45% and found a reduced testicular size and Sertoli cell population *in vivo*. The authors thus suggest that metformin can alter the masculinization of human offspring when mothers are exposed during pregnancy. Furthermore, the authors reported that the testosterone secretion by mouse fetal testes was reduced by 20% at a concentration of 500µM. Furthermore, the authors reported that metformin decreased mRNA expression of the main factors involved in steroidogenesis by 60-90% in mouse fetal testis. *In vivo* exposure of metformin to mice during pregnancy reduced the size of both fetal and neonatal testes of offspring. The number of Sertoli, but not germ cells, was slightly increased in both the fetal and neonatal period and the Leydig cell population was reduced in the fetal period.

Taken together, this evidence suggests that metformin may have anti-androgenic effects and that it might influence the development of the male reproductive tract and thus alter male fecundity later in life (43).

The direct effect on fertility is supported by a study from 2021, where Faure et al. (44) reported that metformin exposure *in utero* induced sex-specific metabolic and reproductive changes in adult rat offspring. Adult males exhibited reduced fertility, manifested as a 30% decrease in litter size compared to controls whereas adult females presented no clear reduction in

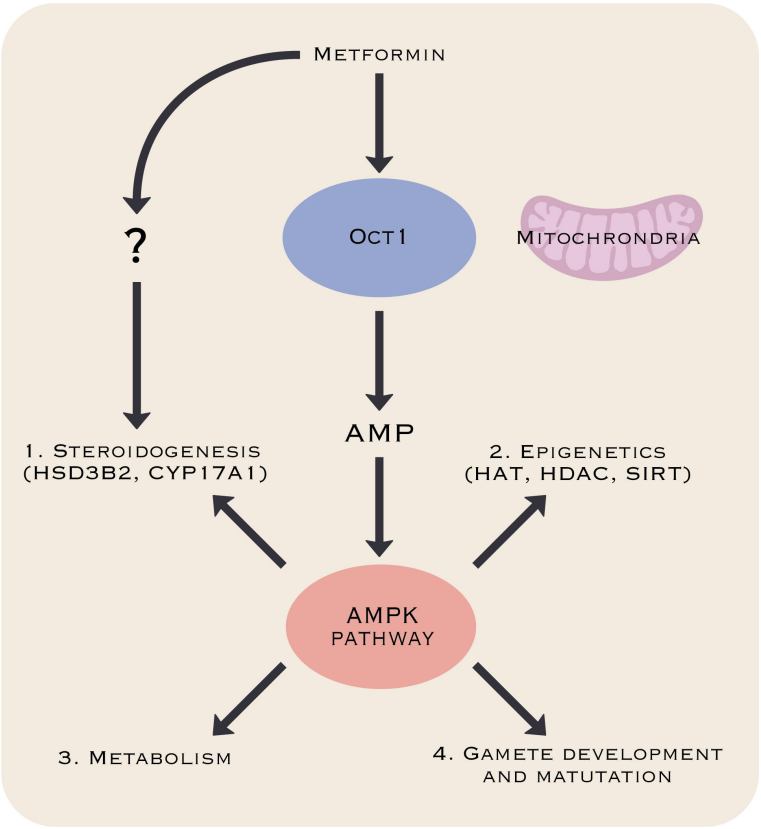

FIGURE 2
Metformin's processes of potential interference with fetal development. Metformin may interfere with fetal development through effects on steroidogenesis (38), epigenetics (39), metabolism (40), and gamete development and maturation (27). The mechanism behind these effects remains poorly understood, but data suggest that the AMP-induced protein kinase (AMPK) pathway might play a central role except for effects on steroidogenesis that might be AMPK-independent (38).

fertility. In this study the lower fertility in male rats was not due to changes in sperm production or motility, but rather to lower sperm head quality, including significantly increased spermatozoa head abnormality with greater DNA damage. The authors suggested that metabolic modification by metformin may alter the expression of epigenetic regulators, which could contribute to reduced fertility. It has been suggested that the predominant mechanisms behind the effects leading to decreased fertility are likely through the AMPK pathway, while the effects on androgen production regulating key steroidogenic enzymes HSD3B2 and CYP17A1are likely through an AMPK independent pathway (38).

Metformin in the environment

The expanded metformin use and its resistance to decompose may have an influence on the environment. Several studies have investigated the presence of metformin and guanylurea in our environment, and the results are unambiguous: Metformin is ubiquitous in the aquatic environment, all over the world. In a systematic review, Ambrosio-Albuquerque, and colleagues (45) reported measurable concentrations of metformin in several different aquatic sources, including influent, sludge, and effluent from wastewater treatment plants, sewage, different surface waters, e.g., rivers, lakes, and oceans, drinking water and sediment. The percentage of detection of metformin varied across the different sources, ranging from e.g., 8% for drinking water, 28% for surface waters, and 51% for urban wastewater. In addition, a study from 2022, investigating active pharmaceutical ingredients in 258 of the world's rivers, from 1052 locations, in 104 countries, detected metformin at over 50% of the sampling sites. The authors reported a similar frequency of metformin across continents, and metformin was one of the pharmaceuticals analyzed, which was present in the highest concentrations (18). In fact, metformin has been recognized as the most frequently detected anthropogenic-organic contaminant in the aquatic environment among several analyzed pharmaceuticals in

different studies and is considered an emerging pollutant of concern (45).

As active pharmaceutical ingredients, such as metformin, are biologically active molecules, specially designed to interact with several biochemical pathways within the human body (18), the widespread presence of metformin in the aquatic environment should be of concern. Notably, metformin is also found in several sources of drinking water around the world, meaning that numerous people worldwide potentially are exposed. Despite the removal of metformin in wastewater treatment plants having a high efficacy rate, ranging from 84% to 99% (46), metformin, and guanylurea are still widely detected in both surface- and drinking water. Another concern is metformin's chlorination byproducts. Chlorine is used for the disinfection of drinking water all over the world, and there is evidence that chlorine can oxidize metformin into two byproducts: Y; $C_4H_6C_1N_5$ and C; $C_4H_6C_1N_3$ (47). Evidence suggests that disinfection byproducts formed during chlorine disinfection have a larger negative effect on human health compared to their parent compounds (48). The byproduct C has been detected in 68.40% of tap water in 32 cities in China (48). In addition, in a recent study (49) byproduct C was detected in urban drinking water from multiple countries, including China and the US, and it was demonstrated that the production of both byproduct C and Y could be increased with increasing metformin concentration exhibiting marked toxicities of a potential health concern and thereby being a hidden threat to the global water supply. So, although the current levels of metformin present in drinking water are not regarded as a direct health concern to humans, the potential threats of metformin's chlorination byproducts should be explored further (49).

Effects on aquatic wildlife

As metformin is detected widely in the aquatic environment, it is important to consider whether this constitutes a threat to aquatic wildlife. Several studies report that metformin exposure in environmentally relevant concentrations can cause potential endocrine disruption in fish. Niemuth and Klaper (50) reported that exposure to metformin in a concentration relevant to wastewater effluent levels (40 μg L^{-1}), caused the development of several alterations in male fathead minnows, including a significantly higher occurrence of intersexuality, compared to control males. Furthermore, they reported a significant reduction in overall size in metformin-treated males as well as significantly fewer cumulative clutches laid per mating pair over time and mean clutch size for metformin-treated males, compared with controls. In contrast, in a fish model using Oryzias latipes, Lee et al. (51) reported no intersex in male gonads, but the occurrence of intersex in F0 generation female gonads in a dose-dependent manner was found.

Additionally, they found that among F0 generation male fish, metformin significantly increased gene expression of both CYP19a and estrogen receptor α. Among F0 generation female fish metformin significantly decreased the expression of ERβ1 and VTG2. Among the F1 generation, metformin significantly increased the expression level of estrogen receptor α in female fish, and significantly decreased the expression of VTG1 in male fish. These sex-specific effects indicate that metformin exposure may cause feminization in male fish and deactivate the reproductive system in female fish (51). Several other studies have investigated the association between metformin and alterations in the expression of specific genes related to reproduction in fish models (52, 53). Niemuth and Klaper (52) have provided evidence that metformin may be an endocrine disrupter, as they showed that among fathead minnows exposed to 40 μg L^{-1} metformin for a year, there was an upregulation of the expression of five endocrine-related genes (AR, 3β-HSD, 17β-HSD, CYP19A1, and SULT2A1) in male gonads tissue. Furthermore, they reported a significant correlation between the expression of three endocrine-related genes (3β-HSD, 17β-HSD aCYP19A1) in the testis and the occurrence of intersex in the gonads. In addition, a significant upregulation of mRNA encoding for VTG in metformin-treated male fish, compared with controls has been reported (53).

Taken together, the experimental findings suggest that metformin can interfere with not only fecundity in mammals but also can act as a disruptor of sexual development in fish at environmentally relevant concentrations. However, the bioaccumulation of metformin in surface water worldwide results in human exposure and is of emerging concern. However, the effects of chronic exposure are poorly understood and need future attention for evaluating the consequences of the increasing amount of metformin found in the environment (45, 54).

Discussion and conclusion

Evidence is accumulating that metformin, besides its well-documented glucose-lowering effects, may act as a reproductive toxicant in humans, experimental rodents, and fish. We recommend that the adverse reproductive effects of metformin should be examined further. Particularly, there is an urgent need for studies exploring the association between metformin exposure and reproductive outcomes in humans and experimental animals concerning the safety of the offspring following parental metformin treatment. The study by Wensink and colleagues (11) on paternal metformin intake should be repeated in another cohort. Clinical studies in normal and diabetic men investigating the impact of metformin on sex hormones are urgently needed. Furthermore, the effect of maternal exposure to metformin in early pregnancy on the development of congenital malformations and its impact on offspring should be investigated. In experimental animals, a randomized study on

intrauterine metformin exposure and e.g., the anogenital distance in male offspring can help shed light on metformin's antiandrogenic effects and reproductive toxicity. In addition, to gain insight into the underlying mechanisms of metformin on steroidogenesis and whether these in fact are AMPK-independent, experimental studies using AMPK-knockout mouse models can be executed. If our hypothesis that metformin is a reproductive toxicant is supported, alternative drugs for the management of T2DM must be considered. Furthermore, evidence of a widespread presence of metformin in the aquatic environment raises concern. Ubiquitous exposure to metformin may not only be considered a potential threat to aquatic wildlife, but also to humans and wildlife in general through continuous exposure from drinking water. As metformin has been used to treat diabetes since 1958 and is difficult to decompose, we speculate that metformin might be accumulated in sedimentary deposits over the latest 60 years, especially in coastal environments associated with river outlets. This is a potential huge reservoir for metformin pollution close to densely populated urban areas in the world.

Data availability statement

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

Author contributions

MT, RL-J, and NS have made substantial contributions to the conception and design of the work. MT, RL-J, NS, DK, and EM have made substantial contributions to the acquisition, analysis, and interpretation of data/literature. MT, RL-J, NS, EM, DK, A-MA, and KK have been drafting the work or revising it critically for important intellectual content. All authors have approved the final version of the paper to be published and have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Joint analysis of m^{6}A and mRNA expression profiles in the testes of idiopathic nonobstructive azoospermia patients

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Background: Growing evidence has indicated that epigenetic factors might be associated with the pathophysiology of idiopathic nonobstructive azoospermia (iNOA). As the most common RNA modification, N6-methyladenosine (m^{6}A) methylation has recently attracted more attention in the regulation of spermatogenesis; however, its role in the mechanisms of iNOA is still unclear.

Objective: To determine the differential expression of mRNA and m^{6}A methylation status in the testes of iNOA patients.

Methods: Testes tissues from diagnosed iNOA and controlled obstructive azoospermia (OA) patients were collected and grouped according to the histological examinations. Total RNA was isolated and quantified by an m^{6}A RNA Methylation Quantification Kit. The expression level of mRNAs was detected by qRT-PCR analysis. Differentially expressed m^{6}A genes were analyzed using the human ArrayStar m^{6}A epitranscriptomic microarray, and bioinformatics analyses were applied.

Results: A total of 36 iNOA and 8 controlled patients were included. The global expression of m^{6}A in the iNOA group was significantly decreased. A dosage relationship was observed between the m^{6}A decline and the degree of impaired spermatogenesis, with the successive process of normal spermatogeneis, hypospermatogenesis (HP), maturation arrest (MA), and Sertoli cell-only syndrome (SO). Four down-expressed genes (*BDNF,*

TMEM38B, RPL3L, and C22orf42) displayed significantly lower expression of m^6A methylation. Additionally, they also showed a gradually down-expressed tendency in the three groups (OA, HP, SO/MA groups). Moreover, m^6A reader EIF3A was approved to have differential expression through microarrays analysis, which was consistent with the result from the qRT-PCR test.

Conclusions: The m^6A expression was gradually downregulated in the testes tissue from iNOA patients in accordance with the degree of spermatogenic dysfunction. The determined differential expression of mRNA and m^6A methylation status may represent potentially novel molecular targets for the mechanism study of iNOA in the epigenetic level, which could benefit the understanding of the pathophysiology of iNOA.

KEYWORDS

m^6A, methylation, azoospermia, spermatogenesis, male infertility

Introduction

Infertility affects approximately 15% of couples of reproductive age worldwide, where male factors account for up to 50% of cases (1). As the most severe condition, nonobstructive azoospermia (NOA) contributes to 10-15% of male infertility, with an incidence rate of 1% in the adult male population (2). There are multiple identified factors that could lead to NOA, including chromosomal abnormalities (Klinefelter syndrome, Y-chromosome microdeletions, etc.), cryptorchidism, and chemotherapy or radiotherapy history. However, collectively, these reasons may account for just 30% of NOA instances (2, 3). With ineffective medical therapy and unpredictable sperm extraction rates, the remaining idiopathic NOA (iNOA) cases whose pathological results include Sertoli cell-only syndrome (SO), maturation arrest (MA), and hypospermatogenesis (HP), are the most challenging and frustrating ones in the clinical practice.

With the advancement of epigenomics, it is becoming increasingly apparent that the mechanisms of iNOA might be accompanied by epigenetic variables (4). Generally, the epigenetic modification includes DNA methylation, histone modifications, and non-coding RNAs (5, 6). Nowadays, RNA modifications have shown a critical role in the epigenetic programming of spermatogenesis, with N^6-methyladenosine (m^6A) being the most common (7, 8). With three well-established regulation patterns, namely m^6A writers, erasers, and readers, m^6A has shown its extensive function in mRNA splicing and stability, microRNA processing, and so on. In spermatogenesis research, the deletion of m^6A writers, Mettl3 and Mettl14, could disrupt spermiogenesis through the experiments *in vitro* (9), and the ablation of Mettl3 could severely inhibit meiosis in the germ cells (10).

Furthermore, the m^6A eraser ALKBH5 had an essential role in correct splicing in the round spermatids phase (11). In addition, the elevated expression of Mettl3 and Mettl14 was identified in the semen of asthenozoospermia patients, indicating the correlation between m^6A and sperm quality (12). However, it is still unclear how m^6A affects the spermiogenesis process; to our knowledge, there have been only a few reports so far on the subjects.

In this case-control study, we collected the testes tissues from iNOA patients and controlled obstructive azoospermia (OA) patients. Additionally, the testes tissues from iNOA patients were divided into SO/MA or HP groups, indicating the extent of spermatogenesis damage according to the pathological findings. m^6A and mRNA expression profiles were then tested among all the groups by applying ArrayStar m^6A microarrays, and verified by qRT-PCR. Bioinformatic analysis was also employed to predict the differentially expressed genes involved in the regulation of m^6A methylation for spermatogenesis in iNOA.

Materials and methods

Testes tissue collection

Approved by the Ethics Committee of Women's Hospital of Nanjing Medical University, Nanjing Maternity and Child Health Care Hospital (2019KY-027), this study was conducted in the reproductive medical center of Nanjing Maternity and Child Health Care Hospital from November 2019 to February 2021. For the case group, patients with male infertility who had been diagnosed with azoospermia were enrolled. The

semen analysis, with a complete lack of sperm in the ejaculate at least twice, combined with hormonal evaluation, was used to determine the diagnosis of iNOA. The exclusion criteria include 1) obstructive azoospermia (OA), which diagnosis was based on the physical examination, sex hormone testing, and transrectal ultrasound performed by a skilled urologist; 2) NOA with genetic causes (abnormal karyotype, pathogenic Y-chromosomal microdeletions, etc.); 3) NOA with a detectable disorder affecting hypothalamic-pituitary-gonadal axis based on hormonal evaluation and imaging diagnoses (hypogonadotropic hypogonadism, hyperprolactinemia); 4) NOA caused by secondary testicular failures, such as cryptorchidism and iatrogenic factors (chemotherapy, radiotherapy). Besides, the OA patients who chose ICSI with testicular sperm extraction (TESE) were included in the control group, and who chose to undertake anastomosis were excluded in this study. All the participants' testes tissues were collected using the residual tissues after the TESE operation with written informed consent.

Histological analysis

All collected testes tissues were fixed in 4% Paraformaldehyde (PFA) for 24 hours and then embedded with paraffin after dehydration. Next, samples were cut into 4-micron sections and stained with hematoxylin and eosin (H&E) using a fully automatic H&E staining machine and Dako CoverStainer (Agilent Technologies, Inc., USA). The improved Mayer hematoxylin (CS700, Dako) was used in the process. Finally, the stained slides were evaluated, and the images were analyzed under a light microscope (Eclipse 80i, Nikon, Japan) at 100× to 400× magnifications.

RNA extraction and m^{6}A quantification of the overall m^{6}A levels

Total RNA was isolated from each testicular sample using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The concentration and purity of total RNA samples were determined using NanoDrop 2000 (Thermo Fisher Scientific, Inc.). The total RNA samples were stored at -80°C for subsequent experiments. The m^{6}A quantification was conducted using the m^{6}A RNA Methylation Quantification Kit (Epigentek) following the manufacturer's protocol. Briefly, 200 ng total RNA sample was added to each test well, followed by the addition of capture antibody and test antibody in turn, and then the reaction was stopped with stop solution. Finally, the absorbance at 450 nm of the test wells was detected by a microplate reader to calculate the percentage of m^{6}A in total RNA.

Analysis of related mRNA using qRT-PCR

The reverse transcription step to cDNA was performed using the PrimeScript RT reagent Kit (Takara, Tokyo, Japan), and qRT-PCR analysis was conducted by SYBR® Premix Ex Taq™ on the LightCycler 480 II real-time fluorescent quantitative PCR system (Roche Diagnostics). The $2^{-\Delta Ct}$ method was used to calculate the relative expression level of related mRNAs. GAPDH was used as an internal control for mRNA quantification. All the reactions were run in duplicate. The primers used for qRT-PCR in this study were verified by previous published papers which sequences were listed in Table 1.

ArrayStar m^{6}A epitranscriptomic microarray analysis

The sample preparation and microarray hybridization were performed according to Arraystar's standard protocols. Briefly, the total RNAs were immunoprecipitated with anti-m^{6}A antibody. Next, the modified RNAs "IP" and the unmodified RNAs "Sup" were labeled with Cy5 and Cy3 using Arraystar Super RNA Labeling Kit. The synthesized cRNAs were then hybridized onto Arraystar Human mRNA&lncRNA Epitranscriptomic Microarray (8×60K, Arraystar). Then, the arrays were scanned using the Agilent Scanner G2505C system.

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze microarray data. First, raw intensities of IP and Sup were normalized and log2-transformed. After Spike-in normalization, the probe signals having Present (P) or Marginal (M) QC flags in at least 3 out of 9 samples were retained as "All Targets Value" for further analyses. Then it was followed by the following procedures: 1) the "m^{6}A methylation level" was calculated for the percentage of modification based on the IP and Sup normalized intensities; 2) the "m^{6}A quantity" was calculated for the m^{6}A methylation amount based on the IP normalized intensities; 3) the "expression level" was calculated based on the total of IP and Sup normalized intensities of RNA. Differentially m^{6}A-methylated RNAs or RNAs expression among the three groups were compared by calculating the fold change (FC) with a cutoff of 2-fold ($P < 0.05$). Finally, the clustering analysis heatmaps and the Venn diagram were performed to show the distinguishable m^{6}A methylation, m^{6}A quantity, and mRNA expression among the three groups.

Bioinformatic analysis

Gene Ontology (GO) terms, which encompass molecular function, cellular structure, and cellular processes, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analyses were performed using DAVID (https://

10.3389/fendo.2022.1063929

TABLE 1 Sequences of primers for mRNAs qRT-PCR analyses.

Gene symbol	Primers	Sequences (5′-3′)
METTL3	Forward	AGCTGCACTTCAGACGAAT
	Reverse	GGAATCACCTCCGACACTC
METTL14	Forward	AGAAACTTGCAGGGCTTCCT
	Reverse	TCTTCTTCATATGGCAAATTTTCTT
WTAP	Forward	GGCGAAGTGTCGAATGCT
	Reverse	CCAACTGCTGGCGTGTCT
VIRMA	Forward	CGATAACTTGATGACCCCAGAA
	Reverse	ATAACGGCAAGATTCCATTTC
RBM15	Forward	TCCCACCTTGTGAGTTCTCC
	Reverse	GTCAGCGCCAAGTTTTCTCT
RBM15B	Forward	CGAGAGTTTGACCGCTTTGGG
	Reverse	CCGAGTCTCCTCTGCTTTGGC
ZC3H13	Forward	AGAAAAGAGAGACAAGCCAAGGT
	Reverse	GAGAGGCAGAGCGTCGTAAAG
METTL16	Forward	TTCCTCGCAACAGAAGTGGA
	Reverse	GTCTTCTGTGGCACTTTCACC
FTO	Forward	GGGTTCATCCTACAACGG
	Reverse	CTCTTCAGGGCCTTCAC
ALKBH5	Forward	CCGAGGGCTTCGTCAACA
	Reverse	CGACACCCGAATAGGCTTGA
YTHDF1	Forward	CTCAGCATGGGGGACAAGTG
	Reverse	GAGGAGCTGACGTCCCCAAT
YTHDF2	Forward	GGCAGCACTGAAGTTGGG
	Reverse	CTATTGGAAGCCACGATGTTA
YTHDF3	Forward	CTACTTTCAAGCATACCACCTCAA
	Reverse	GCATTTCCAGAGTCTACATCGTTA
YTHDC1	Forward	CCACACCATCCTTACTATCAGCA
	Reverse	CTCTTTCACGGGGTCTACTTCTC
YTHDC2	Forward	GGAGCCAATGTCCATAGTAAAGC
	Reverse	ACTTCCATTTGTTTGAACCAGAG
IGF2BP1	Forward	GTCCCTCTCCCGTGTAGGTTTC
	Reverse	AGTTAGCGTCCCCTTCCCAGTG
IGF2BP2	Forward	GTGGCAAGACCGTGAACGAACT
	Reverse	TGCTCCTGCTGCTTCACCTGTT
IGF2BP3	Forward	AGTTGTTGTCCCTCGTGACC
	Reverse	GTCCACTTTGCAGAGCCTTC
FMR1	Forward	AAAAGAGCCTTGCTGTTGGTGGT

(Continued)

TABLE 1 Continued

Gene symbol	Primers	Sequences (5′-3′)
	Reverse	TTGTGGCAGGTTTGTTGGGATTA
EIF3A	Forward	TCAAGTCGCCGGACGATA
	Reverse	CCTGTCATCAGCACGTCTCCA
GAPDH	Forward	GGATTTGGTCGTATTGGG
	Reverse	CTGGAAGATGGTGATGGGATT

david.ncifcrf.gov/). To assess the importance of genes whose m^{6}A methylation level was significantly downregulated in the iNOA group, we established an evaluating system using five pieces of evidence that has hierarchical significance (i to v), based on the tendency and cooperativity in the scales of m^{6}A methylation, m^{6}A quantity, and mRNA expression for differentially expressed genes.

Statistical analysis

Data were expressed as mean ± SEM. Mann-Whitney U test was used for continuous variables and the Chi-square test for categorical variables (GraphPad software, San Diego, California). One-way ANOVA was used for multiple group comparisons. A *P*-value <0.05 was considered statistically significant (*$P < 0.05$).

Results

The testes tissues from 36 iNOA and 8 OA patients were collected in this study. And their demographic characteristics are listed in Table 2. The histological results included 7 SO, 17 MA, and 12 HP in iNOA patients. Among them, testes tissues from 30 iNOA patients with 6 SO, 15 MA, and 9 HP cases, and 5 randomly selected OA patients were used for the m^{6}A quantification experiment. The tissue samples from the remaining 6 iNOA containing 1 SO, 2 MA, and 3 HP, as well as 3 OA patients were used to perform the ArrayStar m^{6}A microarrays. No significant differences could be seen in the comparison of age and BMI in different groups, while the smoking and alcohol drinking status of OA groups were significantly lower ($P < 0.05$). In addition, the LH and FSH values were lower in OA and HP groups when compared with that in the SO or MA groups ($P < 0.05$). Also, the volumes of testes in OA groups were significantly more prominent than in other groups ($P < 0.05$).

The expression of m^{6}A was downregulated in iNOA patients' testes

The global expression of m^{6}A in testes from iNOA patients was significantly decreased compared to that in the

TABLE 2 Baseline characteristics, gonadal hormone concentrations, testes volumes and the success testicular sperm extraction rates of all the patients.

	SO	MA	HP	OA
Age (years)	27.7 ± 2.7	29.5 ± 3.3	30.5 ± 5.0	29.0 ± 2.4
BMI (kg/m^2)	25.2 ± 2.6	26.7 ± 9.1	23.9 ± 3.5	26.8 ± 9.3
Alcohol drinking, n (%)	2 (28.6)	5 (29.4)	4 (33.3)	1 (12.5)*
Passive smoking, n (%)	4 (57.1)	9 (52.9)	8 (66.7)	3 (37.5)*
LH (mIU/mL)	8.7 ± 2.1	6.1 ± 3.5	4.8 ± 4.5$^{\#}$	4.0 ± 1.7$^{\#}$
FSH (mIU/mL)	22.0 ± 8.7	16.8 ± 7.5	7.2 ± 4.3$^{\#}$	5.7 ± 4.3$^{\#}$
E2 (pg/mL)	36.4 ± 1.5	36.5 ± 1.6	32.9 ± 1.6	34.1 ± 1.5
T (ng/mL)	3.6 ± 0.9	3.2 ± 0.8	3.5 ± 1.3	4.1 ± 1.4
Testis volume (mL)	11.9 ± 0.4	11.4 ± 2.6	12.4 ± 2.4	15.0 ± 2.5*
Success testicular sperm extraction, n (%)	0 (0)	6 (35.3)	9 (75)	8 (100)

SO, Sertoli cell-only syndrome; MA, maturation arrest; HP, hypospermatiogenesis; OA, obstructive azoospermia; BMI, Body Mass Index; LH, Luteinizing hormone; FSH, Follicle-stimulating hormone; E2, Estradiol; T, Testosterone. Mann-Whitney U test was used for continuous variables and Chi-square test for categorical variables. *Compared with other three NOA groups, P<0.05. $^{\#}$Compared with SO or MA groups, P<0.05.

OA group. After dividing the iNOA group into three subgroups according to the pathological types, it was discovered that the m^{6}A expression gradually declined from OA to HP, MA, and SO groups, with a dosage relationship observed between the decline and the degree of spermatogenic dysfunction (Figure 1).

iNOA is associated with the alterations of mRNA expression and their m^{6}A methylation status

The expression profiles of mRNA and their m^{6}A methylation status in the SO/MA, HP, and OA groups were identified by the

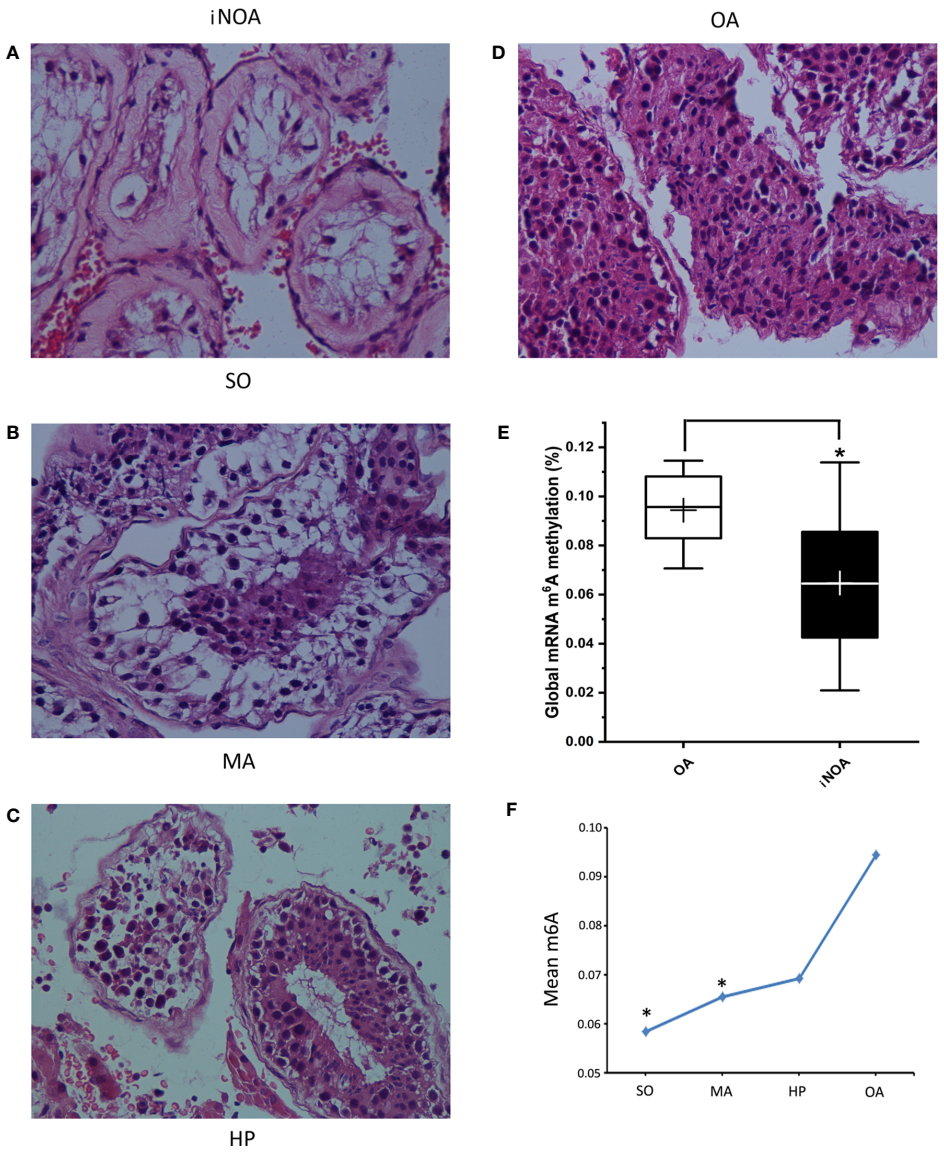

FIGURE 1
The iNOA group was divided into three groups according to the pathological types, namely the SO group **(A)**, the MA group **(B)**, and the HP group **(C)**. **(D)** shows the histology of OA. All the four representative images are 400× magnifications using H&E staining. **(E)** shows the significantly decreased global expression of m^{6}A in testes tissues from iNOA groups. **(F)** shows the a dosage relationship between the m^{6}A expression decline and the degree of impaired spermatogenesis. SO: Sertoli cell-only syndrome, MA: maturation arrest, HP: hypospermatogenesis, OA: obstructive azoospermia. *P < 0.05 compared with OA group.

ArrayStar m^{6}A microarrays. The three heatmaps showed the clustering analysis of m^{6}A methylation, m^{6}A quantity, and mRNA expression in the three groups (Figure 2).

As a result, there are 160 significantly up-expressed genes in the SO/MA group and 649 in the HP group compared to the OA group. The intersection of the two results found 31 up-expressed genes (Figure 3A). Meanwhile, 2469 significantly down-expressed genes from the SO/MA group and 230 ones from the HP group were identified compared to the OA group. Moreover, 287 significantly down-expressed genes were placed in the SO/MA group compared to the HP group. The intersection of the three results highlighted 4 down-expressed genes, which are listed in Figure 3B.

Furthermore, the Gene Ontology (GO) analysis was performed to investigate the potential functions of the 31 up-expressed and 4 down-expressed genes. As Figure 3C showed, the most significant GO terms related to molecular function and cellular components were protein binding, plasma membrane, and extracellular exosome. We mainly focused on the hypomethylated genes when comparing the difference in gene m^{6}A methylation level or m^{6}A quantity in three groups. As a result, 9 genes with significantly lower m^{6}A methylation levels in the SO/MA group and 7 in the HP group were found compared to the OA group. In addition, there were 1937 genes with significantly lower m^{6}A methylation quantity in the SO/MA group and 309 in the HP group compared to the OA group. The intersection of these two results included 141 genes, and their KEGG pathway analysis result was shown in Figure 3D. The most enriched KEGG pathway was the cell cycle.

To predict the genes with m^{6}A methylation status alteration potentially involved in the mechanisms of iNOA, we further compared the genes with significantly decreased m^{6}A methylation levels from the SO/MA and HP groups to the OA group. From Table 3, four genes (*BDNF, TMEM38B, RPL3L, and C22orf42*) displayed significantly lower m^{6}A methylation status associated with decreased gene expression, which was accompanied by the gradual down-expressed tendency in the three groups, reflecting the degree of spermatogenic dysfunction.

An m^{6}A reader was upregulated in the testes of iNOA patients

Corresponding to the global decline of m^{6}A expression in the iNOA group, the majority of m^{6}A-related writers' and readers' genes were down-expressed in the iNOA groups, especially in the SO/MA group (Figure 4A). However, one m^{6}A reader, eukaryotic translation initiation factor 3 subunit A (EIF3A), was significantly upregulated in the iNOA group, which was also verified by the qRT-PCR test. As for the significantly declined m^{6}A writers (METTL3, WTAP, and RBM15), their ArrayStar m^{6}A microarrays results were not testified by the qRT-PCR (Figure 4B).

Discussion

m^{6}A plays diverse functions in stem cell pluripotency regulation, posttranscriptional regulation, and mRNA splicing and stability (13, 14). Notably, there is growing evidence in support of its significance in human spermatogenesis (12, 15, 16), which is a highly organized process. A previous clinical study showed that the m^{6}A expression in sperm RNA was upregulated in asthenozoospermia (12), in which the researchers illustrated that the abnormal expression of m^{6}A might regulate the mRNAs of certain genes related to sperm motility. Being different from the study subject, our study

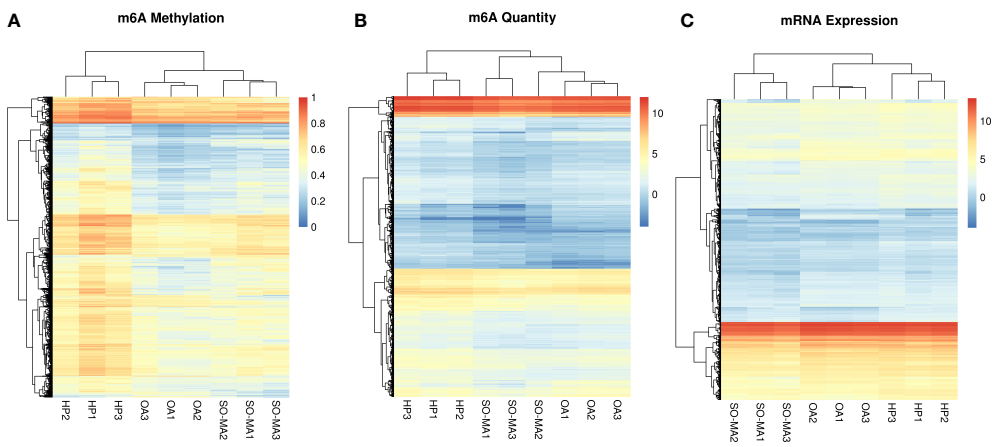

FIGURE 2
The clustering analysis heatmaps of m^{6}A methylation **(A)**, m^{6}A quantity **(B)**, and mRNA expression **(C)** in the SO/MA, HP, and OA groups.

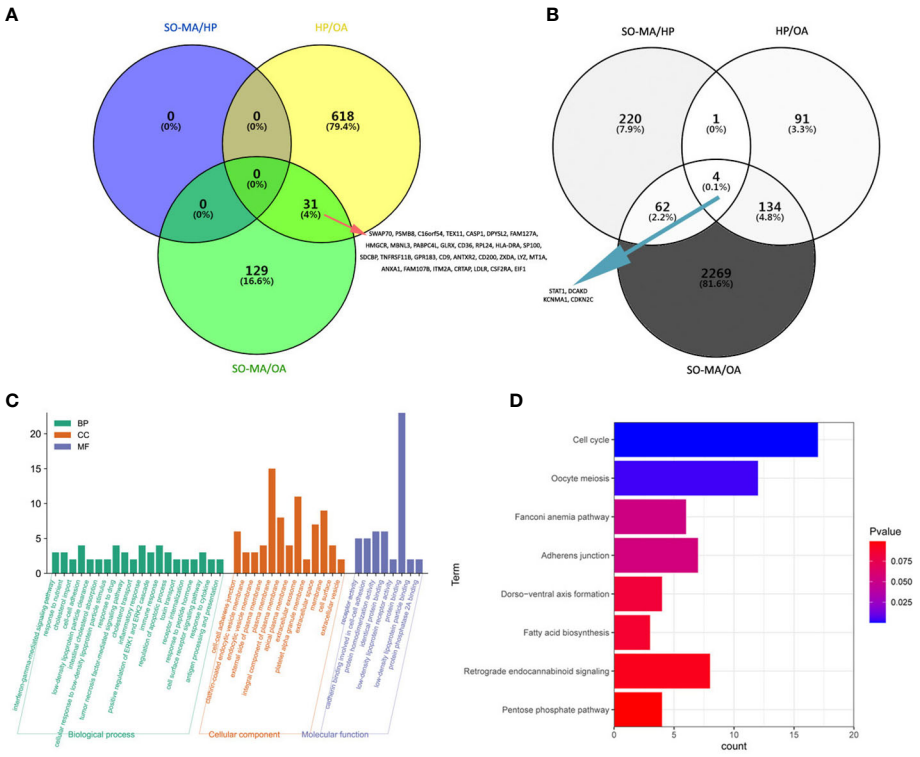

FIGURE 3
Bioinformatics analysis of differentially expressed genes and m^{6}A methylation status in the three groups (the SO/MA, HP, and OA groups). **(A)** The Venn diagram of up-regulated mRNA gene in three groups, and the intersection area included 31 listed genes. **(B)** The Venn diagram of down-expressed mRNA gene in three groups, and the intersection area included four listed genes. **(C)** GO analysis of the 31 up-expressed and 4 down-expressed genes. **(D)** KEGG pathway analysis of 141 genes with significantly lower m^{6}A methylation quantity both in the SO/MA and HP groups when compared to that in the OA group.

explored the m^{6}A expression in the testes tissues of iNOA patients. As is well-known, there is a distinction between the spermatids in testes and the ejected spermatozoa, which experience the capacitation in the epididymis, and have a potential contamination by the somatic cell. Overall, it could be illustrated that dynamic and varied mechanisms participate in the m^{6}A regulation in different spermatogenesis processes for male fertility.

It is well known that, spermatogenic and even spermatogonial cells are absent in SO patients. In contrast, the MA patients include the complete MA characterized by the absence of haploid spermatids, and the incomplete MA, in which a few round or later stage spermatids could be found potentially (17). Studies have shown that m^{6}A is dynamically present in all mRNAs from different stages of mouse spermatogenic cells, with the peak expression in pachytene spermatocytes and round spermatids (11, 18); thus, the lack of these stages of meiosis with or without spermiogenesis might lead to the decline of m^{6}A expression. Consistently, our results also identified the dosage relationship between the m^{6}A and different spermatogenesis conditions. The m^{6}A expression decreased gradually within OA, HP, MA, and SO gourps, and a significant decline was presented in the MA and SO groups.

It is reported that the m^{6}A writers, methyltransferase like 3 (METTL3) and METTL14, likely act critical roles in the spermatogonial stem cells differentiation/proliferation and the spermatids differentiation in the late stages of spermiogenesis (9, 10). METTL14 can form a component with METTL3, as well as strengthen their activities as m^{6}A writers (9). Moreover, WTAP, a mammalian splicing factor, was reported to interact with the complex of METTL3- METTL14 and affect m^{6}A methylation (19). In our study, METTL14 and WTAP were tested down-expressed based on the ArrayStar m^{6}A microarrays' results, which were not verified by the qRT-PCR. More investigations are warranted in this aspect for further studies.

Based on the findings from Kasowitz SD et al., the YTHDC1 KO mice lacked germ cells and exhibited a SO phenotype, manifesting its necessary regulations in spermatogonial differentiation (20). The YTHDC2 could affect the spermatocyte stage *via* interacting with the meiosis-specific

10.3389/fendo.2022.1063929

TABLE 3 To predict the importance of differentially expressed genes with significantly downregulated m^6A Methylation levels in iNOA group using a 5-degree evidence evaluating system.

Gene Symbol	m^6A Methylation Level (iNOA)	m^6A Methylation Level (OA)	m^6A Methylation Quantity (iNOA/OA)	Gene Expression Fold Change (HP/OA)	Gene Expression Fold Change (SO-MA/OA)	Significant Mechanism of Gene m^6A Methylation in iNOA (Evidence)
MITF	6.80%	19.40%	Up	↑↑*	↑	No (i)
PUM1	6.80%	18.40%	Up	↑↑*	↑	No (i)
NPPB	35.10%	78.50%	Up*	↑	↑↑*	No (ii)
CLEC7A	32.30%	66.00%	Down	↓	↑	No (iii)
GRB10	20.10%	40.40%	Down	↑	↓	No (iii)
VSTM5	20.60%	55.70%	Down	↑	↓	No (iii)
ARF1	19.60%	41.20%	Down	↑	↓	No (iii)
CCDC61	15.70%	34.30%	Down	↑*	↑	No (iii)
CTXN2	21.80%	50.80%	Down*	↓*	↓↓	Yes (iv)
ANKRD60	28.50%	64.10%	Down*	↓↓*	↓↓	Yes (iv)
BDNF	32.70%	67.50%	Down*	↓	↓*	Yes (v)
TMEM38B	14.40%	36.20%	Down*	↓	↓↓*	Yes (v)
RPL3L	27.20%	55.00%	Down*	↓	↓*	Yes (v)
C22orf42	35.60%	72.60%	Down*	↓↓	↓↓*	Yes (v)

*With statistical significance ($P < 0.05$).
Evidence i: Lower percentage of m^6A methylation level of this gene with increased m^6A methylation quantity in all groups manifesting that erased m^6A methylation accompanied with enhanced gene transcription; ii: This gene may be important in NOA for its strengthening transcription, but the m^6A function is attenuated; iii: The gene expression change is unordered in three groups, and with no statistical significance; iv: Significantly declined m^6A methylation together with decreased gene transcription, but no statistical significance can be found when the gene expression was compared between the SO-MA group and the OA group; v: Significantly lower m^6A methylation associated with decreased gene expression, and the tendency is gradually in three groups which reflect the degressive spermatogenesis. ↑-expressed and ↓-expressed.

protein MEIOC (21). And YTHDF2 might influence spermatogonia proliferation by affecting the stability of m^6A-containing transcripts (22). However, these 'readers' did not show the significantly differential expressions in iNOA patients' testes based on our analysis. It might be partially explained by the differences in the spermatogonial stem cells and spermatogenesis stages between humans and rodents (23). By applying the bioinformatic methodology, two newly-emerging fields of study from the same study group revealed that m^6A methylation-related ALKBH5 and METTL3 were significantly downregulated, and YTHDF3 was upregulated considerably in the iNOA group comparing to the normal group (15, 16). However, their original data were based on Affymetrix Human Gene microarrays' results, and further verification could provide more evidence on the functions of YTHDF3.

Our study reported that EIF3A was upregulated in the iNOA group, which was not a classic m^6A reader and seldom studied in spermatogenesis. As the largest subunit of eIF3, eIF3a is a 170-kDa protein consisting of 1382 amino acids, and is a major initiation factor in mRNA translation progress (24). Moreover, eIF3a involves in cell cycle, DNA synthesis and repair regulation, and serves as a negative regulator of cell differentiation in some tissues (24–26). In carcinogenesis studies, eIF3a has been recognized as a proto-oncogene, and may be a potential anticancer drug target in the eIF family (24). Recently, researchers found that eIF3a could involve in fibrosis through regulation of the TGF-β1 signaling pathway (27); the m^6A reader YTHDF3 could recruit eIF3a to facilitate FOXO3 translation, subsequently initiating autophagy (28). These findings may provide directions for subsequent studies of eIF3a in the mechanisms of iNOA. In the present study, considering that the majority of iNOA spermatogenesis states entire translational repression, we hypothesized that the compensated mechanism might be responsible for the upregulation of EIF3A in the testes of iNOA patients.

Furthermore, four genes (*BDNF, TMEM38B, RPL3L, and C22orf42*) were predicted to be involved in the dysregulation of m^6A methylation in iNOA mechanisms in the present study. First, Brain-derived neurotrophic factor (BDNF) was detected in human spermatozoa which might influence sperm mitochondrial activity and apoptosis (29, 30). Second, Transmembrane protein 38B (TMEM38B) encodes trimeric intracellular cation channel type B, expressed in most mammalian tissues' endoplasmic reticulum (31). Finally,

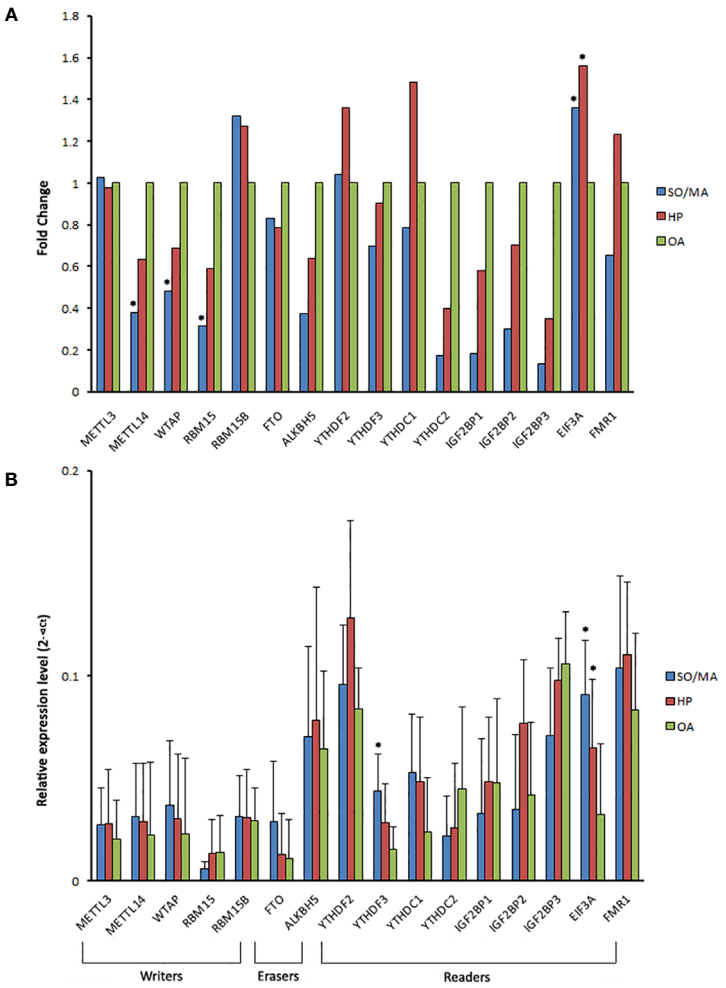

FIGURE 4
The gene expression of m^{6}A related writers, erasers, and readers in the different groups. **(A)** The gene expression fold change of the m^{6}A related writers, erasers, and readers in the SO/MA or HP group compared to the OA group *via* the detection of ArrayStar m^{6}A microarrays. **(B)** The comparison of m^{6}A related regulators' mRNA expression between the three groups using qRT-PCR. *$P < 0.05$ compared with the OA group.

Ribosomal protein L3-like gene (RPL3L) is a novel gene located near the PKD1 and TSC2 genes on chromosome 16p13.3, expressed mainly in skeletal muscle and heart tissue (32). It may be involved in the translation initiation. All these genes' potential functions in iNOA still need further research.

There are some limitations and flaws in this study. First, the testis tissue contains other types of cells besides the spermatogenic cells, such as Leydig cells, Sertoli cells, myoid cells, macrophages, etc. Therefore, the findings might be impacted by the contamination to some extent. Second, the fixation of PFA and HE staining applied in the pathological examination might cause some limitation because the results always displayed shrinkage artifacts between seminiferous tubules and germ cells. Third, the patients' number was relatively small for that the residual testicular tissue after sperm retrieval in clinic was always not enough for the following RNA experiments. And just because of this, the SO and MA patients were combined into one subgroup in this study. Besides, we considered that the pathology of SO and MA were homologous to some extent, and clinically their sperm retrieval rate were both very low. Nevertheless, more studies with a large population size of iNOA patients are highly desired in the future.

Conclusions

This study, to our knowledge, is a first exploration in the joint expression profiles of m^{6}A and mRNA in the testes of iNOA patients. As shown in Figure 5, the m^{6}A expression was gradually downregulated in the testes tissue from iNOA patients

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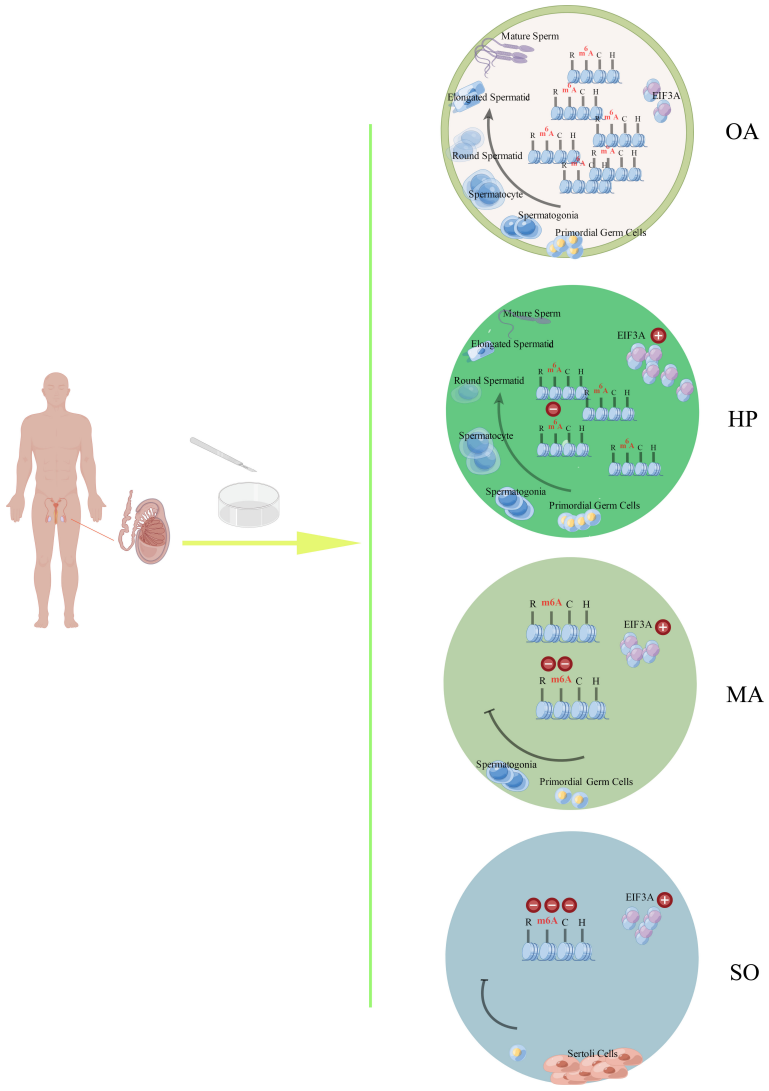

FIGURE 5
The schematic drawing of the main findings from this study by Figdraw. The m^{6}A expression was gradually downregulated in the testes tissues from different groups (OA, HP, MA, and SO groups) in accordance with the degree of spermatogenic dysfunction. As m^{6}A reader, EIF3A was testified to be upregulated in the testes from iNOA patients, which include the patients with HP, MA, or SO. OA: obstructive azoospermia (with the successive process of normal spermatogeneis), HP, hypospermatogenesis; MA, maturation arrest; SO, Sertoli cell-only syndrome.

in accordance with the degree of spermatogenic dysfunction. As m^{6}A reader, EIF3A was testified to be upregulated in iNOA patients' testes. iNOA is associated with the alterations of mRNA expression and their m^{6}A methylation status. Four genes displayed significantly lower m^{6}A methylation status associated with decreased gene expression were hypothesized to be involved in the dysregulation of m^{6}A methylation in the iNOA mechanisms. Overall, the determined differential expression of mRNA and m^{6}A methylation status may represent potentially novel molecular targets for the mechanism study of iNOA in the epigenetic level.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by The Ethics Committee of Women's Hospital of Nanjing Medical University, Nanjing Maternity and Child

Health Care Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conception and design: QT, WW, and FP Acquisition of data: QT, YL, and YZ. Analysis and interpretation of data: WW, LP, XL, and FP Drafting the manuscript: QT and FP Laboratory testing: YL, YZ, and WFW. Critical revision of the manuscript: WW, LP, XL, FP, and JL. Final approval to be published: all authors. Agreement to be accountable for all aspects: all authors. 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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Impact of body composition analysis on male sexual function: A metabolic age study

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Introduction: Metabolic Age (MetAge) and body composition analysis may reflect an individual's metabolic status, which is believed to influence male sexual and gonadal functions. Although erectile dysfunction (ED) and hypogonadism are increasingly prevalent with age, they are also detected among younger men. This study aims to assess the impact of MetAge and body composition on male sexual and gonadal status overall, and particularly in men younger than 40 years of age.

Methods: This was a cross-sectional study of 90 male healthcare workers, between the ages of 18-55, randomly selected based on their corporation numbers. In addition to Bioelectric Impedance Analysis, subjects were requested to fill the International Index of Erectile Function questionnaire (IIEF-5) and to provide an early morning serum testosterone (T) sample.

Results: The mean participants' age was 39.4 $\pm$ 9.4 years, MetAge was 45.54 $\pm$ 10.35 years, serum T level was 13.68 $\pm$ 4.49 nmol/L and BMI was 28.8 $\pm$ 4.7 kg/m^2. Significant negative correlations were obtained between serum T, MetAge, body weight and fat composition. Significant negative correlations between the IIEF-5 score, MetAge, and fat composition, were only reported in subjects <40 years of age. Significantly lower T levels (p=0.002), significantly older MetAge (p=0.034), and higher BMI (p=0.044) and degree of obesity (p=0.042) were observed in participants <40 years with erectile dysfunction (ED) compared to their counterparts without ED.

Discussion: MetAge and body composition parameters significantly impact the androgenic state. ED in men <40 years is associated with lower T levels, older MetAge and higher BMI and degree of obesity.

KEYWORDS

body mass index, basal metabolic rate, erectile dysfunction, hypogonadism, metabolic age

Introduction

The human body is composed of balanced percentages of fat, muscle, bone, water and connective tissue (1). This balance is a potential indicator of the state of health of each person, in relation to his or her origin, chronological age, sex, diet, physical activities as well as their lifestyle (2). These variables are known to influence body weight and body fat composition and consequently body mass index (BMI) and basal metabolic rate (BMR) (1, 2).

An individual's BMR, defined by the number of calories burned during the awake resting state determines the metabolic age (MetAge). The latter is obtained by comparing the BMR of an individual to the BMR average of his/her chronologic age group in the general population (3). Body fat is essential for maintaining optimal body temperature, as well as protecting joints and internal organs (4, 5). However, it is well known that an increase in fat mass, more precisely in the visceral region (6), is associated with a disturbance of internal homeostasis, endocrine and metabolic imbalance and a cascade of inflammations causing functional impairments of different organs and systems (7). Indeed, many diseases are diagnosed in overweight and obese subjects such as cardiovascular disease, type-2 diabetes, dyslipidemia, metabolic syndrome, as well as other pathologies whose complications can result in different types of cancer or even mortality in some cases (8). Body composition and fat mass can also interfere with reproductive function (9). Metabolic disturbances caused by excess weight such as metabolic syndrome, increased adipocyte secretions, hyperinsulinemia and dysregulation of steroid metabolism, have repercussions at central and peripheral levels leading to reproductive disorders of varying intensity (10, 11). Hypogonadism and erectile dysfunction (ED), defined by the inability to obtain and maintain an erection allowing satisfactory sexual intercourse (12), are examples of reproductive conditions that have been strongly correlated with obesity (13). While these sexual disorders are generally more prevalent among aging men (14), several studies have shown them to be not uncommon in younger generations holding poor metabolic status and obesity as the responsible risk factors for such an observation (15, 16).

Evaluation of the body composition is integral to weight reduction programs and might help in understanding the risks to sexual disorders that are linked to excessive fat deposition and weight gain. Several methods have been utilized for body composition analysis including dual-energy X-ray absorptiometry, waist circumference, visceral adiposity index, anthropometric measurements and bioelectric impedance analysis (17, 18). These methods provide a detailed understanding of the body composition ratios and hence are appealing means that can be used to investigate disorders associated with impaired metabolism (17, 18). While several studies investigated the impact of obesity on reproductive function, very few have assessed the relationship between individual body composition parameters and male sexual and gonadal health. Therefore, the objectives of this study were to (1) investigate the overall relationship between body composition parameters, serum testosterone levels and erectile function; and (2) compare testosterone levels, MetAge and body composition analysis results in men younger than 40 of age with and without ED.

Material and methods

Study design, participants and setting

This cross-sectional study was conducted at tertiary medical center between March, 2016 and March, 2017. A total of 120 male healthcare providers, randomly selected based on their corporation numbers through computer-generated randomization tables, were electronically invited to participate in this study.

The Institutional Review Board approved the study protocol and informed consents were signed by participants before enrollment in the study.

Adult, sexually active men >18 years of age were included in this study. The exclusion criteria were: subjects [1] receiving treatment with phosphodiesterase inhibitors or any other erectogenic supplements; [2] taking testosterone replacement therapy or other medications to increase endogenous testosterone levels including estrogen receptor modulators, aromatase inhibitors and gonadotrophins; [3] known to have endocrine abnormalities that may impact sexual function including hyperprolactinemia, pan-hypopituitarism and hypothyroidism; [4] with debilitating medical diseases including end-stage renal disease, liver failure and congestive heart failure; [5] who were receiving recreational drugs or alcoholic; [6] who had a history of infertility due to a male factor including cryptorchidism, orchitis, and testicular torsion or trauma.

Study procedures

All participants reported in the morning following an overnight fast of 12 hours and abstinence from for at least 24 hours, to provide an early morning serum testosterone sample, and to undergo Bioelectric Impedance Analysis according to the protocol validated by Lukaski et al., 1986 to assess body composition (19). Serum testosterone levels (reference range 10.4 – 34 nmol/L) were analyzed using the immunoassay chemiluminescence method, Architect i1000SR® (Abbott systems, Illinois, USA). Hypogonadism is defined in subjects who had a serum testosterone level <10.4nmol/L. Subjects were also requested to fill in the English five-item version of the International Index of Erectile Function questionnaire-5 (IIEF-5) a brief, reliable, and valid self-administered questionnaire containing five questions that have been widely used in many

countries to detect the presence and severity of ED (score range: 5-25, severe ED: 5-7, moderate ED: 8-11, mild-moderate ED: 12-16, mild ED: 17-21, no ED: 22-25) (20). All participants had a higher level of education and good command of the English language.

The metabolic status of participants was assessed using the TANITA body analyzer (TBF-410GS) (Arlington Heights, Illinois, USA) which is a reliable method for assessing body composition (21). Measurements were performed with subjects fasting for a minimum of 8 hours, wearing light clothing and no shoes or socks. Quality control for all measurements was monitored regularly. Results of the body composition analysis include weight, body fat %, body mass index (BMI), degree of obesity, MetAge and visceral fat. The degree of obesity is an estimate of overweight in relation to the norm in percentage and is calculated using the following formula: [(weight – ideal weight)/ideal weight] x 100.

Data, variables

Risk factors for sexual dysfunction such as diabetes mellitus, hypertension, coronary artery disease, dyslipidemia and smoking were assessed through interview.

Demographic data (age), clinical (testosterone, results of IIEF-5) and body composition results (noted above) were also collected. Participants were divided according to their chronologic age into two groups: (i) group 1 with participants whose age was under 40 years (18-39), and (ii) group 2 with participants whose age was equal or above 40 years (40-65) (22–24).

Statistical analysis

The distribution of the data was assessed using the Shapiro-Wilk test. All numerical data were presented as means ± Standard Deviation (SD), while categorical data were presented with frequencies (%).

The relationship between participants' sexual and androgenic state and their body composition parameters were assessed using Pearson's correlations. Student T-test was used to compare testosterone levels and results of body composition analysis between men <40 years of age with or without sexual dysfunction. Clustered box plots were used to picture differences in chronologic and MetAge in men with serum testosterone (<10.4 or ≥ 10.4) and with or without ED. A p-value <0.05 was considered statistically significant. Statistical analysis of collected data was performed using SPSS version 25 (IBM, Armonk, NY, USA).

Results

Among the 120 participants, only 90 subjects met the inclusion and the exclusion criteria for this study. None of the participants had undergone any bariatric surgery. The average chronologic age of the population was 39.41 ± 9.39 years [30-68 years], of which 53 individuals were under the age of 40 and 37 individuals were over the age of 40. A low testosterone level was detected in 18 subjects (20%) and risk factors were detected in 41 (45.6%) subjects. Risk factors were more prevalent among men ≥40 compared with men <40 years of age (54.1% vs. 39.6%). The characteristics of the study population including demographic, clinical and body analysis data are shown in Table 1.

ED was reported by 48 (53%) subjects, with an average IIEF-5 score of 20.04 ± 3.0.

Table 2 reports the correlations between the different continuous study variables.

Overall, a significant negative correlation was detected between testosterone and metabolic age, weight and BMI as well as various fat body composition elements (Fat%, fat mass, visceral fat and degree of obesity). On subgroup analysis these correlations were stronger in men ≥40 years of age.

With regards to the IIEF-5 score, no significant correlations were observed with other variables among participants, overall. However, significant negative correlations were observed with testosterone, MetAge, BMI, fat mass, visceral fat and degree of obesity only in men < 40 years of age.

Comparison of demographic, clinical and body composition analysis data in subjects < 40 years of age with/without ED was established (Table 3). ED was reported by 24 subjects who were < 40 years old, while the remaining 29 subjects had no ED with the IIEF-5 score.

Participants younger than 40 with ED had significantly lower T levels (10.88 ± 4.05 vs. 14.52 ± 4.14, p=0.002) compared to their counterparts without ED. Older MetAge was observed in ED vs no ED participants as well (44.63 ± 6.9 vs 39.9 ± 8.59, p=0.034) (Table 3).

Clustered box plots for chronologic age (Figure 1) and MetAge (Figure 2) by sexual function and testosterone levels stratified according to the study groups were drawn.

In subjects < 40 years of age, the mean MetAge was significantly higher in those with hypogonadism or with ED compared to their counterparts with normal testosterone levels or normal sexual function (Figure 2). No significant differences were noted for MetAge in men ≥40 years of age. Furthermore, no significant differences were noted in the chronologic age of men with low/normal testosterone or with/without ED among the two study groups (Figure 1).

Discussion

This study revealed that body composition parameters including MetAge can significantly influence the sexual and gonadal health of men. More importantly, the increase in body mass index and MetAge may be associated with sexual dysfunction in men younger than 40 years of age.

10.3389/fendo.2022.1050441

TABLE 1 Characteristics of the study population: whole population, participants < and ≥ 40 years of age.

Demographic and clinical data	Whole population (n=90)	Participants <40 years of age (n=53)	Participants ≥40 years of age (n=37)
Age (years)	39.41 ± 9.39	32.77 ± 2.74	48.92 ± 7.02
Testosterone (nmol/L)	13.68 ± 4.49	12.87 ± 4.45	14.84 ± 4.34
IIEF-5 score	20.91 ± 2.86	22.38 ± 2.34	20.43 ± 3.12
Mild -Moderate ED	7 (7.7)	3 (5.6)	4 (10.8)
Mild ED	41 (45.5)	21 (39.6)	20 (54.1)
No ED	42 (46.6)	29 (75.5)	13 (35.1)
Risk Factors	41 (45.6)	21 (39.6)	20 (54.1)
Diabetes Mellitus	7 (7.8)	1 (1.9)	6 (16.2)
Hypertension	12 (13.3)	4 (7.5)	8 (21.6)
Smoking	29 (32.2)	17 (32.1)	12 (32.4)
Coronary artery disease	1 (1.1)	0 (0)	1 (2.7)
Body composition analysis			
Metabolic Age (Years)	45.54 ± 10.35	42.04 ± 8.14	50.57 ± 11.67
Weight (kg)	88.28 ± 17.8	89.49 ± 16.87	86.54 ± 19.14
BMI (kg/m^2)	28.8 ± 4.77	29.02 ± 4.49	28.49 ± 5.18
Fat (%)	26.03 ± 6.82	25.87 ± 5.84	26.27 ± 8.09
Fat mass (kg)	23.74 ± 9.88	24.27 ± 9.97	22.97 ± 9.8
Visceral fat (rate)	10.52 ± 4.1	9.57 ±3.91	11.89 ± 4.01
Degree of obesity (%)	31.12 ± 21.51	32.06 ± 20.33	29.78 ± 23.31
Muscle mass (kg)	61.42 ± 10.05	62.53 ± 8.45	59.84 ± 11.92
Bone mass (kg)	3.22 ± 0.51	3.25 ± 0.43	3.19 ± 0.61
BMR (Kj)	8050.77 ± 1280.03	8199.34 ± 1203.01	7837.95 ± 1371.54

IIEF-5, International Index of Erectile Function-5 item version; ED, Erectile Dysfunction; BMI, Body Mass Index; BMR, Basal Metabolic Rate.

Significant negative correlations were obtained between serum testosterone level and metabolic age, body weight and degree of fat deposition. Many studies have confirmed the presence of a close association between testosterone deficiency and obesity (25). The excessive secretion of leptin and pro-inflammatory cytokines by adipose tissue as well as the prevailing state of insulin resistance in obese men exert a negative impact on the hypothalamic-pituitary-gonadal axis ultimately reducing the secretion of gonadotropins (26). At the peripheral level, the increased production of leptin may further reduce the receptivity of Leydig cells to LH (27). While insulin resistance decreases hepatic sex hormone-binding globulin production and increases the availability of free testosterone (26, 28, 29), it thereby renders free testosterone a substrate for excessive aromatization to estradiol in adipose tissues (26). Estradiol consequently exhibits a negative effect on the hypothalamic-pituitary function and further aggravates the process (26). The European Male Aging Study showed that a decline in testosterone was observed in 73% of overweight or obese men; the serum testosterone in men with a BMI> 30 kg/m^2 was on average 5 nmol/l lower to those of normal weight (30). In a cross-sectional study of young non-diabetic obese men, hypoandrogenemia was directly associated with adiposity (31).

Our results showed that in men < 40 years of age, the IIEF-5 score was significantly negatively correlated with serum testosterone levels, MetAge, BMI and degree of fat deposition. On the other hand, this finding was not obtained in men ≥ 40 years of age. This may be explained by the higher incidence of risk factors among men ≥ 40 years old, suggesting that the presence of risk factors in this population may undermine the effect of body composition parameters on sexual function which was observed in the younger age group. Age has been confirmed to be an independent risk factor for ED (32). Older men, are prone to systemic diseases such as hypertension, cardiovascular disease, type-2 diabetes or even psychoneurological diseases and are hence more likely to develop ED (33). However, some studies

10.3389/fendo.2022.1050441

TABLE 2 Pearson correlation between variables in the whole study population and the subjects < or ≥ 40 years old.

r	Testosterone			IIEF score		
	Whole population (n=90)	<40 years (n=53)	≥ 40 years (n=37)	Whole population (n=90)	<40 years (n=53)	≥ 40 years (n=37)
Testosterone (nmol/L)	1	1	1	0.135	-0.296*	0.049
Age (years)	0.186	-0.145	0.086	-0.203	-0.057	-0.084
Metabolic age (years)	-0.267*	-0.347*	-0.467**	-0.090	-0.304*	0.253
IIEF score	0.135	-0.296*	0.049	1	1	1
Weight (Kg)	-0.391**	-0.372**	-0.402*	0.034	-0.190	0.245
BMI (Kg/m^2)	-0.430**	-0.389**	-0.483**	-0.065	-0.283*	0.142
Fat %	-0.474**	-0.472**	-0.530**	-0.080	-0.259	0.087
Fat mass (Kg)	-0.477**	-0.447**	-0.518**	-0.070	-0.291*	0.174
Visceral fat (rate)	-0.377**	-0.441**	-0.505**	-0.151	-0.276*	0.106
Degree of obesity (%)	-0.439**	-0.394**	-0.499**	-0.067	-0.288*	0.145
Muscle mass (Kg)	-0.192	-0.210	-0.132	0.108	-0.115	0.259
Bone mass (Kg)	-0.178	-0.033	-0.320	0.098	-0.083	0.239
BMR (Kj)	-0.296**	-0.237	-0.326*	0.082	-0.136	0.265

▪: Negative correlation, ▪: Positive correlation, (*): Significant correlation P< 0.05, (**): highly significant correlation P<0.001, IIEF, International Index of Erectile Function; BMI, Body Mass Index; BMR, Basal Metabolic Rate; r, Pearson correlation coefficient.

have suggested that ED in young men may be more frequent than we thought and could be attributed to life style exposures including smoking, alcohol or drug intake (34) as well as alterations in metabolic profiles of these individuals. Several studies have explored the relationship between various body composition parameters and sexual dysfunction in men. Visceral fat, represented by the visceral adiposity index which is a mathematical parameter obtained using waist circumference,

TABLE 3 Comparison of demographic, clinical and body composition analysis data in subjects < 40 years of age with/without erectile dysfunction.

	No ED (n=29)	ED (n=24)	P value
Testosterone (nmol/L)	14.52 ± 4.14	10.88 ± 4.05	<0.01*
Weight (Kg)	86.42 ± 15.56	93.2 ± 17.97	0.14
Fat (%)	24.52 ± 5.95	27.5 ± 5.38	0.06
Fat mass (Kg)	21.88 ± 8.85	27.17 ± 10.66	0.07
Muscle mass (Kg)	61.41 ± 8.12	63.88 ± 8.82	0.29
Bone mass (Kg)	3.21 ± 0.41	3.29 ± 0.46	0.48
BMR (Kj)	8022.03 ± 1128.98	8413.58 ± 1277.82	0.24
Metabolic age (Years)	39.9 ± 8.59	44.63 ± 6.9	0.03*
Visceral fat (rate)	8.69 ± 3.82	10.63 ± 3.83	0.07
BMI (Kg/m^2)	27.9 ± 4.42	30.38 ± 4.28	0.04*
Degree of obesity (%)	26.93 ± 20.12	38.25 ± 19.2	0.04*
Risk factors, n (%)	14 (48.27)	7 (29.17)	0.13

Independent t- test and Chi squared test, *: significant result p<0.05.
BMI, body mass index; BMR, basal metabolic rate.

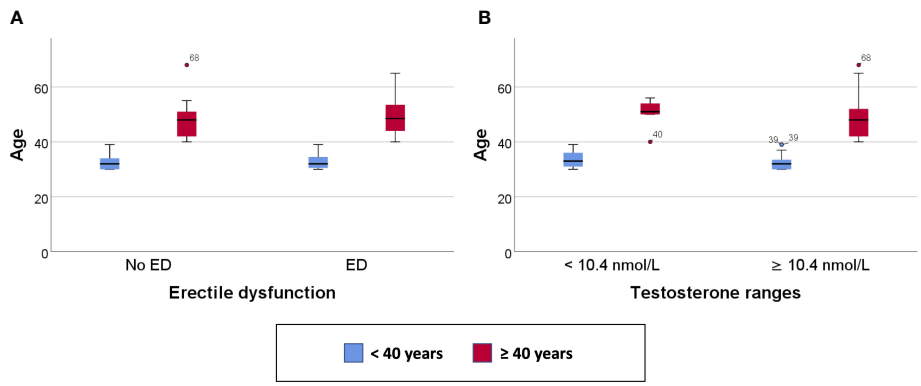

FIGURE 1
Clustered Box plot for: Age by sexual function **(A)** and serum testosterone level **(B)** in participants < or ≥ 40 years of age.

BMI, serum triglyceride and high-density lipoprotein levels, was found to be significantly higher in men with ED in comparison to those without ED (35). Body fat mass, on the other hand, had a U-shaped relationship with erectile function in a study of Korean men indicating that worse sexual performance was reported by men with either too little or too much fat mass (36). A recent observational study by Molina-Vega et al. (2020) established an association between ED and obesity in a group of young non-diabetic obese men between the ages of 18-49 years (37). The authors revealed that the severity of ED was directly related to an increase in BMI, metabolic syndrome components, fat mass and lipid balance. While these results do corroborate with our findings, their study may have a selection bias as it only included obese patients who may be predisposed to other risk factors of ED and did not include a non-obese control group to confirm the association. In our, randomly selected study participants, we compared serum testosterone levels and body composition parameters between 2 equally sized groups <40 years of age with/without ED. We reported significantly lower serum testosterone, older MetAge and higher BMI and degree of obesity in men with ED compared to those without ED.

The impact of hypogonadism on the vascular tone has been investigated and reports have established a link between endothelial dysfunction and testosterone deficiency predisposing patients for ED (38). Hypogonadism may manifest with decreased production of nitric oxide (NO) synthetase thereby reducing the NO levels in the vascular endothelium (10, 39). This in addition to an upregulation in vasoconstrictor levels including endothelin-1 and pro-inflammatory factors (IL-6, CRP) result in impairment of cavernous smooth muscle hemodynamic properties leading to altered relaxation, or in other words, ED (11, 40).

This study was not without limitations. The study participants were healthcare workers who are not necessarily representative of the general population. Furthermore, ED was subjectively assessed by the participants who might under- or

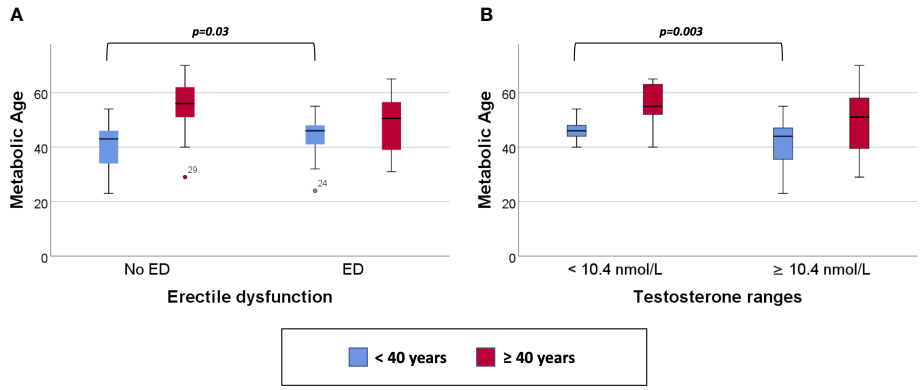

FIGURE 2
Clustered Box plot for: Metabolic Age by sexual function **(A)** and serum testosterone level **(B)** in participants < or ≥ 40 years of age.

overestimate their sexual performance. HBA1c was not assessed and therefore it is unknown whether some of the participants might be pre-diabetic or with insulin resistance. The body composition analysis results were as valid as the accuracy of the machine utilized. Despite the fact that men with infertility were excluded from the study, we did not assess serum LH and FSH levels in our study population, nor did we measure their testicular volumes. As such we cannot exclude with certainty the presence of primary testiculopathies and therefore we cannot rule out preexisting hypogonadism that may be unrelated to metabolic age alteration. Moreover, the diagnosis of ED was based only on the IIEF-5 results and as such some patients with psychogenic ED may have been included. Finally, lifestyle factors that may interfere with sexual function such as cigarette smoking were not evaluated.

Conclusion

MetAge is significantly inversely correlated with serum testosterone levels overall. In men < 40 years of age, higher MetAge seems to have a negative impact on sexual function. This association may serve as an additional motive towards adopting a healthy lifestyle among the general population. Further studies of larger sample size are required to confirm or dispute these results.

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 Medical research center, Hamad Medical Corporation, protocol number 13453/13. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors contributed to the article and approved the submitted version.

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Conflict of interest

RH is employed by LogixX Pharma.

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.

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Nickel oxide nanoparticles exposure as a risk factor for male infertility: "*In vitro*" effects on porcine pre-pubertal Sertoli cells

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Lately, nickel oxide nanoparticles (NiO NPs) have been employed in different industrial and biomedical fields. Several studies have reported that NiO NPs may affect the development of reproductive organs inducing oxidative stress and, resulting in male infertility. We investigated the *in vitro* effects of NiO NPs on porcine pre-pubertal Sertoli cells (SCs) which undergone acute (24 h) and chronic (from 1 up to 3 weeks) exposure at two subtoxic doses of NiO NPs of 1 μg/ml and 5 μg/ml. After NiO NPs exposure we performed the following analysis: (a) SCs morphological analysis (Light Microscopy); (b) ROS production and oxidative DNA damage, gene expression of antioxidant enzymes (c) SCs functionality (AMH, inhibin B Real-time PCR analysis and ELISA test); (d) apoptosis (WB analysis); (e) pro-inflammatory cytokines (Real-time PCR analysis), and (f) MAPK kinase signaling pathway (WB analysis). We found that the SCs exposed to both subtoxic doses of NiO NPs didn't sustain substantial morphological changes. NiO NPs exposure, at each concentration, reported a marked increase of intracellular ROS at the third week of treatment and DNA damage at all exposure times. We demonstrated, un up-regulation of SOD and HO-1 gene expression, at both concentrations tested. The both subtoxic doses of NiO NPs detected a down-regulation of AMH and inhibin B gene expression and secreted proteins. Only the 5 μg/ml dose induced the activation of caspase-3 at the third week. At the two subtoxic doses of NiO NPs a clear pro-inflammatory response was resulted in an up-regulation of TNF-α and IL-6 in terms of mRNA.

Finally, an increased phosphorylation ratio of p-ERK1/2, p-38 and p-AKT was observed up to the third week, at both concentrations. Our results show the negative impact of subtoxic doses NiO NPs chronic exposure on porcine SCs functionality and viability.

KEYWORDS

Sertoli cells, nickel oxide nanoparticles, ROS, comet, MAPK pathways

Introduction

Recent advancements in the discipline of nanotechnology have introduced the employment of engineered nanoparticles (NPs) in the production systems of numerous consumer products, as well as in various industrial applications and some innovative medical practices.

NPs are also widespread in the environment and they can proceed to enter the human body *via* inhalation, ingestion, skin adsorption, and *via* intravenous injection when used for medical applications (1).

One of the most common metal nanomaterial, nickel oxide nanoparticles (NiO NPs) have found broad application prospects in many fields, such as magnetism, energy technology and biomedicine and have therefore attracted great interest. In the biomedical field, NiO NPs can be used in many ways, like creation of biological probes, isolation of DNA from total proteins, targeted drug delivery, treatment of malignant tumor cells with hyperheat, contrast-enhanced magnetic resonance imaging (2).

In the last decade, various studies have reported on the dangers of exposure to NiO NPs that have been found to induce pulmonary toxicity (3–5), liver and spleen toxicity (6, 7) cardiovascular toxicity (8), genotoxicity (9, 10) and spermiotoxicity (11) and, even to induce cancer (6, 7, 9, 12).

Notably, the lung toxicity of NiO NPs was greatly investigated, demonstrating relevant inflammatory, cytotoxicity and apoptotic effects on the alveolar cells (13, 14). Other studies highlight that NiO NPs can reach the gastrointestinal tract through the unintended ingestion of food and water contaminated with NiO (15).

Studies by Saquib et al. estabilished that NiO NPs may interest many different tissues of male wistar rats, resulting in genotoxicity and imbalanced enzymes activity (16). The NiO NPs were described as an hepatotoxic agent due to their ability to induce oxidative stress and apoptosis in the human liver cells (17).

Singh et al. reported that NiO NPs may also induce a state of oxidative imbalance in the testes of male rats after oral exposure, leading to DNA damage and subsequent infertility (18).

Recently, assessing male reproductive health and infertility, one of the most crucial problem caused by environmental pollution, has attracted increased attention from many scientists.

It has been hypothesized that exposure to NiO NPs through industrial use or occupational environment may compromise the reproductive system; in fact several studies reported that NiO NPs has reproductive and developmental toxicity (11, 19–23). In an adult albino rat model, it has been estabilished that nanomaterials can easily cross the blood-testis barrier and can cause germ cell damage due to their properties related to their size (23). To date, however, the precise mechanism that correlates Nickel NPs exposure with testicular damage is not entirely clear, still it is relevant that mitochondrial damage has been shown to be an important player in altered sperm parameters and damage to Leydig cells and testis (24).

In the present study, we focused our attention on the *in vitro* effects of NiO NPs on primary cultures of Sertoli cells (SC).

SC are a key element in spermatogenesis through their ability to support stem cells, providing both nourishment and physical support. They are also able to protect them from the host immune system, either through the formation of an SC- based blood- testis barrier (BTB) or through the release of numerous immunomodulatory factors.

Their main contribution to the unfolding of spermatogenesis is characterized by the production of critical factors necessary for the successful development of spermatogonia, throughout the stage of mature spermatozoa (25, 26).

Our experimental studies conducted in the last years have enabled us to develop a system, using *in vitro* pre-pubertal porcine bioengineered cell culture system as a new model for experimental studies on male infertility. We have successfully isolated pure and functional porcine pre-pubertal SC preparations (SCs), preferred to adult cells since the latter are very difficult, if not impossible, to isolate.

The ability to obtain functional SCs was demonstrated by their secretion of Anti-Mullerian hormone (AMH) and inhibin B, as key functional parameters of superior mammalian SCs, after follicle stimulating hormone (FSH) stimulation (27).

Our primary SCs cultures show many similarities to human SC and hold great potential as an experimental model to study the "*in vitro*" effects of toxic substances and heavy metals, as demonstrated in our previous work that confirmed the negative impact of Titanium dioxide NPs on SCs, and our works on toxicity of cadmium and lead (28–30).

The goal of the present study was to evaluate the influence of acute (24 h) and chronic (from 1 up to 3 weeks) exposure to subtoxic doses NiO NPs of 1 µg/ml and 5 µg/ml on our "*in vitro*" model of SCs.

Materials and methods

Preparation and characterization of NiO NPs

Chemicals: $Ni(CH_3COO)_{2}{\cdot}_2H_2O$, and NH_4HCO_3 were purchased from Sigma-Aldrich.

For the synthesis of NiO NPs 50 mL of a NH_4HCO_3 1 M solution were dropped, under ultrasound irradiation for 15 minutes at 50% of amplitude at 25°C, to a 100 mL of a $Ni(CH_3COO)_{2}{\cdot}_2H_2O$ $4x10^{-3}$ M solution. The pH was maintained at 9. The high power ultrasound irradiation was carried out by an Ultrasonic processors VC750 Sonics and Materials, 20 kHz with a diameter tip of 13 mm. The precipitate was dried, calcined at 350°C for 1 h and characterized by ICP-OES, FE-SEM-EDX and XRD (31).

SCs culture and NiO NPs exposure

Animal studies were conducted in agreement with the guidelines adopted by the Italian Approved Animal Welfare Assurance (A-3143-01) and the European Communities Council Directive of November 24, 1986 (86/609/EEC). The experimental protocols were approved by the University of Perugia. Number 3 Danish Duroc pre-pubertal pigs (15 to 20 days old) underwent bilateral orchidectomy after general anaesthesia with ketamine (Ketavet 100; Intervet, Milan, Italy), at a dose of 40 mg/kg, and dexmedetomidine (Dexdomitor, Orion Corporation, Finland), at a dose of 40 g/kg, and were used as SCs donors. Specifically, pure porcine pre-pubertal SCs were isolated, and characterized according to previously established methods (27).

In detail, SCs have undergone acute (24 h) and chronic (from 1 up to 3 weeks) exposures at two subtoxic doses of NiO NPs of 1 µg/ml and 5 µg/ml according to 3-(4, 5-Dimethyl-thiazol-2-yl)22,5-diphenyl-tetrazolium bromide (MTT) assay. The control group consisted of unexposed SCs (0 NiO NPs µg/ml).

ICP-OES

NiO NPs -treated SCs were detached by trypsin/ethylenediaminetetraacetic acid (EDTA) (Lonza, Verviers, Belgium) at 37°C for 8 min, to promote the enzymatic reaction. After washing with 1 ml Hank's balanced salt solution (HBSS) (Sigma-Aldrich Co., St. Louis, MO, USA), samples were centrifuged at 150×g for 6 min, the supernatant was removed, the pellets were freeze-dried, and accurately weighed. Samples were dissolved by treatment with 10 ml of a mixture of sulfuric acid (H_2SO_4), (97% Sigma-Aldrich Co., St. Louis, MO, USA)/nitric acid (HNO_3 70%), (Sigma-Aldrich Co.,St. Louis, MO, USA) (2:1). After solubilization, the obtained solutions were diluted with the EDTA solution (1:10) prior Ni2+ content determination using a Varian 700-Es series spectrometer (Agilent, Milan, Italy) in triplicate. Calibration was performed diluting a Nickel nitric acid stock solution for ICP (Sigma Aldrich, Milan, Italy) to obtain Nickel standard solutions in the 1–15-mg/ml range. The Ni2+ uptake in SCs was calculated per unit weight of freeze-dried NiO NP-treated SCs and % of the total amount added and the error expressed as SEM.

MTT assay and cell viability

NiO NPs cytotoxicity was evaluated by the MTT (Sigma-Aldrich Co., St. Louis, MO, USA) test on unexposed and exposed SCs. Briefly NiO NPs at the concentrations of 2.5, 5, 15, 30, 45, 60 and 120 µg/ml were added to each well and cultured for additional 24 or 48 h. Then, the experiment was performed, as previously reported (28). Unexposed (0 NiO NPs µg/ml) SCs served as controls. Viability was expressed as a percentage with respect to unexposed SCs (NPs-exposed SCs ×100/unexposed SCs). The sub-toxic doses of 1 and 5 µg/ml were chosen for all subsequent experiments at 24 hours (acute exposure) and 1, 2, 3 weeks (chronic exposure) and MTT assay was performed at each experimental time-point.

ROS determination

Intracellular ROS were measured by treating unexposed and exposed SCs with 50 mM dichlorofluorescein diacetate (DCFHDA) (Sigma-Aldrich Co., St. Louis, MO, USA) solution in Dulbecco's phosphate-buffered saline (D-PBS) (Sigma-Aldrich Co., St. Louis, MO, USA) at 37°C for 30 min. Fluorescence was read by using a plate reader (DTX 880 Multimode Detector, Beckman Coulter). Data were normalized for cell viability (MTT assay) and expressed as the percentage of unexposed SCs. The sensitivity of the test was confirmed by adding 30 µM hydrogen peroxide (H2O2) (30 min) on unexposed SCs as positive control.

Oxidative DNA damage quantification

To evaluate the oxidative DNA damage, unexposed and exposed SCs were processed in the comet assay under alkaline conditions (alkaline unwinding/alkaline electrophoresis, pH >13), basically following the original procedure (32). Briefly, SCs treated with 1 mM 4-nitroquinoline N-oxide (4NQO) (Sigma-Aldrich, Milan, Italy) for 1 h at 37°C (33) were used as positive control. At the end of treatments, the cells were detached with trypsin (Invitrogen, Milan, Italy) and collected by centrifugation (70×g, 8 min, 4°C). Then, cell pellets were gently resuspended in low-melting point agarose (Sigma-Aldrich, St. Louis, MO, USA) at 37°C, layered onto a conventional microscope slide precoated with 1% normal melting point agarose and covered with a coverslip (Knittel-Glaser, Braunschweig, Germany). Then, electrophoresis runs were then performed as previously reported (28).

The comets in each microgel were analysed (blind), at ×500 magnification with an epi-fluorescent microscope (BX41, Olympus, Tokyo, Japan), equipped with a high sensitivity black and white charge-coupled device (CCD) camera (PE2020, Pulnix, UK), under a 100-W high-pressure mercury lamp (HSH-1030-L, Ushio, Japan), using appropriate optical filters (excitation filter 510–550 nm and emission filter 590 nm). Images were elaborated by Comet Assay III software (Perceptive Instruments, UK). A total of 100 randomly selected comets (50 cells/replicate slides) were evaluated for each experimental point.

AMH and inhibin B secretion assays

Aliquots of culture media from all the experimental groups were collected and stored at -20°C for subsequent assessment of AMH (AMH Gen II ELISA, Beckman Coulter, Webster, TX, USA) and inhibin B (inhibin B Gen II ELISA, Beckman Coulter) secretion levels as previously described (34).

Reverse transcriptase-polymerase chain reaction analysis

AMH, inhibin B, TNF-α,IL-6, SOD1, HO-1, GHSPx and NRF2 were analysis by reverse transcriptase-polymerase chain reaction (RT-PCR) as previously described in Arato et al. (35) employing the primers listed in Table 1.Total RNA was extracted using the TRIzol reagent (Sigma-Aldrich), and quantified by reading the optical density at 260 nm. In detail, 2.5 µg of total RNA was subjected to reverse transcription (RT, Thermo Scientific) to a final volume of 20 µl. We performed the qPCR with the use of 25 ng of the cDNA obtained by RT and an SYBR Green Master Mix (Stratagene). This procedure was performed in an Mx3000P cycler (Stratagene), using FAM for detection and ROX as the reference dye. We normalized the mRNA level of each sample against β-actin mRNA and expressed it as fold changes versus the levels in the control group.

Immunoblot

Total protein extracts were prepared for immunoblot analysis as described in Mancuso et al. (36). Briefly, the cell extracts were separated by 4–12% SDS-PAGE, and then blotted on nitrocellulose membranes (BioRad, Hercules, CA, USA). The membranes were incubated overnight in a buffer containing 10 mM TRIS (Sigma-Aldrich Co., St. Louis, MO, USA), 0.5 M NaCl (Sigma-Aldrich Co., St. Louis, MO, USA), 1% (v/v) Tween 20 (Sigma-Aldrich Co., St. Louis, MO, USA), rabbit anti-ERK1/2 (Millipore, MA, USA, 1:2000), mouse anti-phospho- ERK1/2 (Millipore; MA, USA, 1:100), rabbit anti-JNK (Millipore; MA, USA, 1:1000), rabbit anti-phospho-JNK (Millipore; MA, USA, 1:500), rabbit anti-posphop38 (Millipore, MA, USA, 1:2000), mouse anti p38 (Millipore, MA, USA, 1:2000), anti-Akt (Cell Signalling, 1:100), rabbit anti-phospho-Akt (Cell Signalling, 1:1000), rabbit anti-phospho-NF-kB p65 antibody (AbCam, Cambridge, UK, 1:1000), rabbit anti-NF-kB p65 antibody (AbCam, Cambridge, UK, 1:1000), and mouse anti-β-actin (Sigma-Aldrich Co., St. Louis, MO, USA, 1:100) primary antibodies.

Primary antibody binding was then detected by incubating membranes for an additional 60 min in a buffer containing horseradish peroxidase conjugated anti-rabbit (Sigma-Aldrich Co., St. Louis, MO, USA,1:5000) and/or anti-mouse (Santa Cruz Biotechnology Inc., 1:5000) IgG secondary antibodies. The bands were detected by enhanced chemiluminescence and acquired by ChemiDoc imaging System (Bio-Rad, Hercules, CA, USA).

Data analysis

Normality analysis was performed by Shapiro–Wilk test, and statistical comparisons were analyzed using one-way ANOVA followed by Tukey's HSD *post hoc* test (SigmaStat 4.0 software, Systat Software Inc., CA, USA). Values were reported as the means ± SEM of three independent experiments, each performed in triplicate. Differences were considered statistically significant at *$p < 0.05$, and **$p < 0.001$ compared to unexposed SCs (0 NiO NPs).

Results

Characterization of NiO NPs

The synthesis of NiO NPs was performed by XRD analysis, the resulting diffractogram was characteristic of nichel oxide crystals in anatase form (JCPDS 00-001-0562) (Figure 1A).

TABLE 1 Primer sequences for PCR analyses.

Gene	Forward sequences (5′–3′)	Reverse sequences (5′–3′)
AMH	GCGAACTTAGCGTGGACCTG	CTTGGCAGTTGTTGGCTTGATATG
Inhibin B	CCGTGTGGAAGGATGAGG	TGGCTGGAGTGACTGGAT
SOD1	TCGGGAGACCATTCCATCAT	ACCTCTGCCCAAGTCATCT
HO-1	CTGGTGATGGCGTCCTTGTA	TTGTTGTGCTCAATCTCCTCCT
GHSPx	CGAGAAGTGTGAGGTGAATGG	GCGGAGGAAGGCGAAGAG
NRF2	TTCACTAAACCCAAGTCCCAGCAT	AAGCCAAGCAGTGTGTCTCCATA
IL-6	AATGCTCTTCACCTCTCC	TCACACTTCTCATACTTCTCA
TNF-α	CTCTTCTCCTTCCTCCTG	GCTTTGACATTGGCTACA
β-actin	ATGGTGGGTATGGGTCAGAA	CTTCTCCATGTCGTCCCAGT

AMH, anti-Müllerian hormone; SOD1, superoxide dismutase 1; HO-1, heme-oxygenase 1; GHSPx, gluthatione peroxidase; NRF2, Nuclear factor erythroid 2-related factor 2; IL-6, interleukin-6; TNF-α, Tumor necrosis factor-alpha.

A representative SEM image of NiO NPs in dry form is showed in Figure 1B, and the mean size distribution reports values of 20 ± 5 nm diameter, calculated by measuring over 100 particles in random fields of view. Results showed that NiO NPs tended to form aggregates of submicrometric dimensions. DLS analysis, confirmed some aggregation of NiO NPs in suspension. The mean hydrodynamic diameter of NiO NPs mainly distributed in a range of 100–800 nm (Figure 1C).

Uptake of NiO NPs by SCs

ICP-OES was used to quantify the uptake of NPs expressed as the percentage of internalized NPs and the amount of metal adsorbed per cell number (expressed as ng/10^5), at each concentration, after 5 hours of treatment (Figure 1S). In the treatment after 5 hours, the percentage of internalized NPs showed a range between 1 and 3% (Figure 1S), where the lower treatment dosage (1µg/ml) exhibited a higher percentage of uptake than the higher dosage (5µg/ml). On the contrary, the amount absorbed and expressed as ng/10^5 was much higher at the higher dosages (1.57 for NiO).

We could speculate that this difference between percentage and net amount absorbed was probably due to the gradual saturation of SCs, leading to a progressive slowing down in uptake as the concentration of NPs increases, further confirming literature data (37).

Other factors that could have negatively influenced the absorption of NiO particles are their marked hydrophobicity and their tendency to stick to surfaces, phenomena due to the high surface energy of these particles that causes a reduced availability for absorption by part of the SCs (38).

NiO NPs cytotoxicity evaluation

For the preliminary study, at 24 hours, as shown in Figure 2S, panel A, NiO NPs at low concentrations appeared to induce proliferation of SCs, probably due to an adaptive response to the increasing NPs concentration. Proliferative effects due to metal NPs have been found many times before and this has been attributed to the activation of specific pathways, such as MAP kinases (28), but many aspects remain to be elucidated. The percentage of metabolically active cells began to decline at doses of 40 µg/ml (*p< 0.05 vs. unexposed SCs), showing a LD50 of 80 µg/ml (**p<0.001 vs. unexposed SCs). Finally, a collapse in SCs viability was also identified at 100 and 120 µg/ml (**p<0.001 vs. unexposed SCs). At 48 hours, a statistically significant reduction in metabolic activity was identified, in SCs exposed to a concentration of 40 µg/ml compared to controls (*p< 0.05 vs. unexposed SCs), with a LD50 dropping to 70 µg/ml (**p<0.001 vs. unexposed SCs), followed by a drastic reduction in viability at higher concentrations (Figure 2S). Due to the obvious toxicity of NiO NPs, for the 3-week treatment the sub-toxic concentrations of 1 µg/ml and 5 µg/ml, were chosen, the former to simulate a mild exposure to seemingly harmless concentrations, to which humans could easily be exposed in everyday life, while the latter represents a higher dosage to allow an evaluation of possible mechanisms of toxicity.

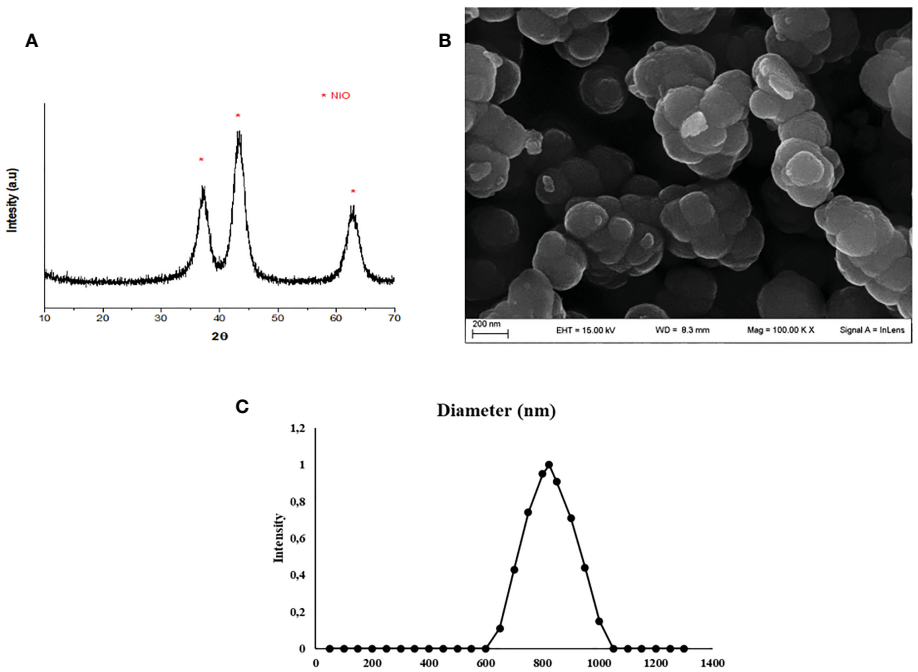

FIGURE 1
NiO NPs characterization. **(A)** XRD analysis: diffractogram, * represents the peaks of NiO NPs in anatase form (JCPDS 00-001-0562). **(B)** SEM analysis: the mean size distribution of TiO2 NPs in dry form was 20 ± 5 nm. **(C)** DLS analysis: mean hydrodynamic diameter of TiO2 NPs at 10 µg/ml in culture medium.

MTT assays performed during the 3 weeks of treatment with NiO NPs showed an increase in metabolically active cells at the dosage of 5 µg/ml at 1 week (Figure 2S panel B *p<0.05 vs. unexposed SCs). This effect could be due to a defensive mechanism put in place by the cell in response to a noxious stimulus, however it does not seem to be sufficient to preserve the cell from the statistically significant reduction in the percentage of metabolically active cells of 10% observed at the third week compared to the unexposed SCs (Figure 2S panel B, *p<0.05 vs. unexposed SCs).

In contrast, at the concentration of 1 µg/ml the only significant effect was observed at the second week with a 10% loss of viability (*p<0.05 vs. unexposed SCs) which then recovered at the third week, demonstrating the low level of toxicity potential at this concentration, which allowed the cell to recover from the stress suffered (Figure 2S panel B).

SCs light microscopy

Morphological analysis revealed that SCs exposed to both subtoxic doses of NiO NPs did not undergo substantial changes compared with the untreated monolayer at all times of exposure, shown in the Figures 2A, D, G, J. In fact, cells exposed to NiO NPs maintained the typical squamous shape of epithelial cells with vacuoles containing lipid hormones, likely testosterone coniugated with androgen binding protein (ABP) and estradiol, well evident and abundantly distributed in the cell surface (Figures 2B, C, E, F, H, I, K, L).

Impact of NiO NPs on the liveness of ROS and oxidative DNA damage

As shown in Figure 3A, the dose of 1 µg/ml NiO NPs did not affect ROS intracellular level up to 2 weeks post exposure. On the contrary, at 3 weeks post treatment, ROS level significantly increased compared to unexposed SCs. Conversely, the dose of 5 µg/ml NiO NPs induced a significant increase of intracellular ROS amounts over time and until the end of treatment compared to unexposed SCs (Figure 3A, **p < 0.05 **p < 0.001 vs. 0 NiO NPs). As expected, H_2O_2 (positive control) induced a significant increase in ROS intracellular levels (Figure 3A, **p < 0.05 **p < 0.001).

The levels of oxidative DNA damage induced by NiO NPs were measured as the % of DNA in tail by the alkaline comet assay over time, after acute (24 h) and chronic exposures (from 1 to 3 weeks) to 1 and 5 µg/ml of NiO NPs. Both doses of exposure, induced a significant increase in the oxidative DNA damage over time and until the end of treatment with respect to the unexposed SCs (Figure 3B, **p < 0.05 vs. 0 NiO NPs).

Antioxidant response

The gene expression of SOD1 significant increased at the dose of 1 µg/ml only at the third week of treatment, meanwhile at the dose of 5 µg/ml it showed a significant increase from second up to third week (Figure 4A, *p < 0.05 and **p < 0.001 vs. 0 NiO NPs).

The gene expression of HO-1 increased at both concentrations only at the third week of NiO NPs-exposure (Figure 4B, **p < 0.001 vs. 0 NiO NPs).

We observed a significant increase in GHSPx gene expression only at the second week at the dose of 1 µg/ml and, at first week at the dose of 5 µg/ml; with a significant reduction at 24h at the dose of 1 µg/ml (Figure 4C, *p < 0.05 vs. 0 NiO NPs).

The gene expression of NRF2 showed a significant increase at both dose from second up to third week of treatment (Figure 4D, *p < 0.05 and **p < 0.001 vs. 0 NiO NPs).

NiO NPs effects on SCs functionality

Exposure of SCs to both concentrations of NiO NPs induced a significant increase in AMH and inhibin B gene expression at 24 h, followed by a significant decrease after 2 week up to the third week, compared to unexposed SCs (Figures 5A, B *p < 0.05 and **p < 0.001 vs. 0 NiO NPs).

At both concentrations, AMH and inhibin B secretion was significantly increased at 24h of treatment. The secretion of AMH was significantly decreased after dose of 1 µg/ml NiO NPs only the third week, whereas at 5 µg/ml NiO NPs, we observed a significant reduction from the second, up to the third week of treatment respect to unexposed SCs (Figure 5C **p < 0.001 vs. 0 NiO NPs).

In contrast, inhibin B secretion was significantly decreased after exposure to both dose of treatment only at third week with respect to unexposed SCs (Figure 5D **p < 0.001 vs. 0 NiO NPs).

Caspase-3 evaluation

We observed that NiO NPs exposure, at each concentration, induced the activation of caspase-3 at third week, with the cleavage of p19 and p17 fragments p19 kDa active fragment.

Only at the dose of 5 µg/ml NiO NPs, we demonstrated a statistical increase of both active p19 and p17 with respect to the inactive p35 fragments, expression of a more prominent apoptotic process (Figures 6A–D **p < 0.001 vs. 0 NiO NPs).

Pro-inflammatory response

At both concentration, the gene expression of TNF-α showed a significant increase at 24 h and during third week, (Figures 7A, **p < 0.001 vs. 0 NiO NPs).

Moreover, IL-6 gene expression showed a significant increase at 24 h only at dose of 5 µg/ml NiO NPs, meanwhile at both concentrations, the increase resulted after week 2 up to the third week, with respect to unexposed SCs (Figures 7B, *p < 0.05 and **p < 0.001 vs. 0 NiO NPs).

MAPK kinase signaling pathway activation

We performed Western blotting analysis to investigate the involvement of different MAPK family members (ERK1/2, JNK,

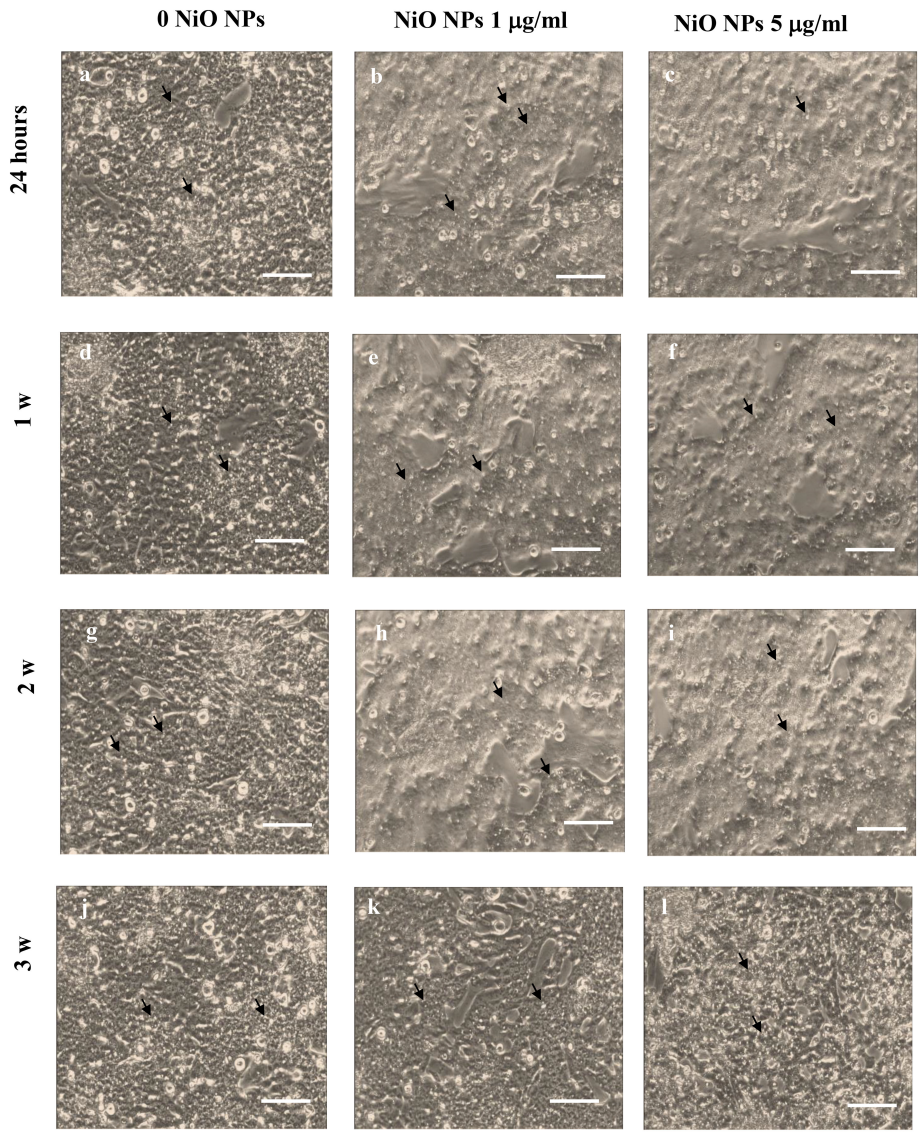

FIGURE 2
SCs Morphological characterization. Light microscope of unexposed (0 NiO NPs) SCs **(A, D, G, J)**, NiO NPs exposed SCs at 1 **(B, E, H, K)**, and 5 µg/ml **(C, F, I, L)** at 24 h **(A–C)** and 1 **(D–F)**, 2 **(G–I)**, and 3 weeks **(J–L)**. Black arrows point to some of the abundant vacuoles containing lipid hormones, likely testosterone coniugated with androgen binding protein (ABP) and estradiol. The scale bar corresponds to 200 µm for **(A–L)**. The images are representative of three separate experiments.

p38, AKT) and NF-kB signaling pathway after NiO NPs exposure (Figure 8A). The phosphorylation ratio of ERK1/2 showed a significant increase at both concentrations, from the second up to third week (Figure 8B, **p < 0.001 vs. 0 NiO NPs).

The phosphorylation ratio of JNK increased at 24 h in both concentrations, with a significant increase at the second week only at the 1 µg/ml NiO NPs dose and a significant reduction at third week at the 5 µg/ml NiO NPs dose (Figure 8C, **p < 0.001 vs. 0 NiO NPs).

The phosphorylation ratio of p38 showed a significant increase from second up to third week, at both NiO NPs doses (Figure 8D, **p < 0.001 vs. 0 NiO NPs).

The phosphorylation ratio of AKT showed a significant increase at 24 h, second and third week, with a significant reduction only at first week at both concentrations of treatment (Figure 8E, **p < 0.001 vs. 0 NiO NPs).

Finally, the phosphorylation ratio of p-NF-kB showed a significant increase only at first week at dose of 1 µg/ml NiO NPs, meanwhile at dose of 5 µg/ml NiO NPs, the increase resulted after 24 h and at third week of treatment compared to unexposed SCs (Figures 8F, **p < 0.001 vs. 0 NiO NPs).

Discussion

According to World Health Organization estimation, infertility, defined as 'the inability of a sexually active, non-contracepting couple to achieve spontaneous pregnancy in one year', affects about

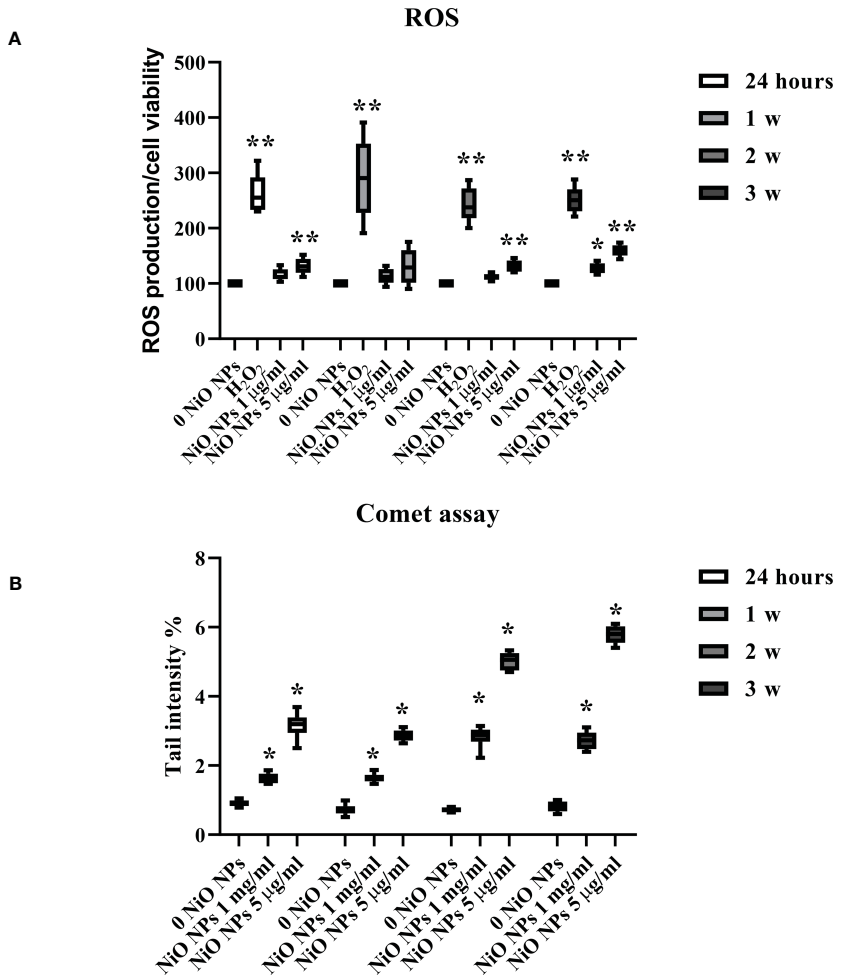

FIGURE 3
ROS production and DNA damage after NiO NPs treatment in SCs. **(A)** Total intracellular ROS production in SCs exposed to NiO NPs 1 and 5 µg/ml for 24 h and 1, 2, and 3 weeks. Data represent the mean ± SEM (**p < 0.001 with respect to 0 NiO NPs of three independent experiments). **(B)** DNA damage expressed as tail intensity % in unexposed SCs and exposed to NiO NPs and 5 µg/ml for 24 h and 1, 2, and 3 weeks. Data represent the mean ± SEM (*p < 0.05 vs. 0 NiO NPs of three independent experiments).

15% of couples of childbearing age in industrialized countries (39). Since a male factor is responsible in about 30% of the cases, and in 20% of cases both male and female factors are involved, about 50% of cases of infertility are related to the male partner (40). Unfortunately, despite progress, the etiology of male infertility is still unknown in 30 to 40% of the cases, thus defined as idiopathic infertility (40, 41). Patients with idiopathic infertility do not have a history compatible with fertility-altering diseases, and their physical, laboratory, genetic, and instrumental examinations are unremarkable; however, their semen frequently shows significant alterations in sperm parameters. Such idiopathic sperm anomalies (such as azoospermia, oligozoospermia, teratozoospermia, and/or asthenospermia) are presumed to be caused by several factors, including reactive oxygen species (ROS), unknown genetic and epigenetic abnormalities, and endocrine disruption due to environmental pollution (42).

As a nanomaterial, NiO NPs are widely used in various fields (43). Humans can be exposed to NiO NPs through environmental and occupational settings. Currently, NiO NPs have been shown to impair the development of reproductive organs, resulting in male infertility.

Notably, in previous studies, it has been demonstrated that NiO NPs cause reproductive toxicity in healthy adult rats, increasing the ratio of epididymis weight to body weight, changing sperm motility parameters in rats, disturbing spermatogenic tubule cells, inducing apoptosis and necrosis (44).

Employing animal models, it was verified that various types of nanoparticles, including NiO NPs, have a negative impact on male germ cells; their damage potential differing in regard to nanoparticle modification, composition, concentration, route of administration, and the species of the animal (2).

During present investigation, an attempt was made to study the influence of acute (24 h) and chronic (from 1 up to 3 weeks) exposure to subtoxic NiO NPs doses of 1 µg/ml and 5 µg/ml on our *"in vitro"* model of SCs.

The NiO NPs doses were chosen according to MTT cytotoxicity assay and, the viability was expressed as a percentage of cells compared to unexposed SCs (NPs-exposed SCs ×100/unexposed SCs).

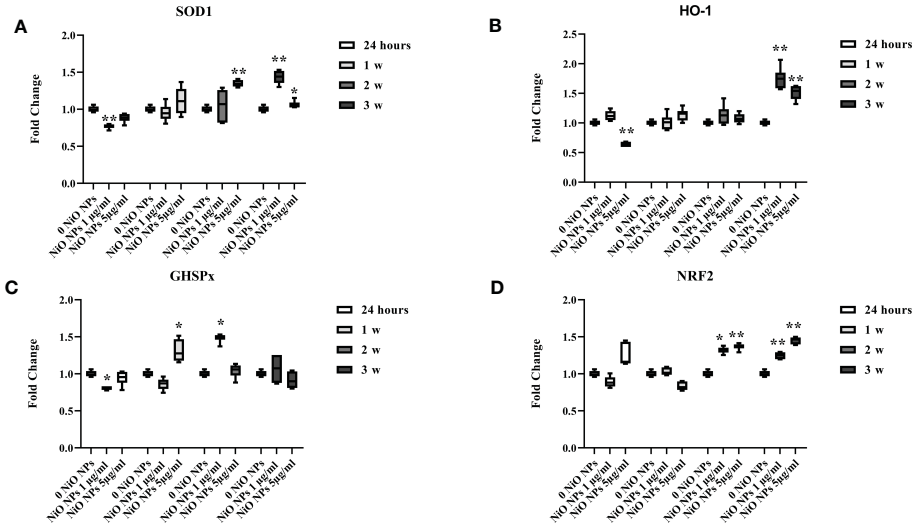

FIGURE 4
Real-time PCR analysis of antioxidant and metabolic enzymes. Gene expression of SOD1 **(A)**, HO-1 **(B)**, GHSPx **(C)**, and NRF2 **(D)** in SCs at 24 h and 1, 2, and 3 weeks of incubation with NiO NPs 1 and 5 µg/ml. Data represent the mean ± SEM (*p < 0.05, **p < 0.001 vs. 0 NiO NPs of three independent experiments, each performed in triplicate).

Due to the evident toxicity of NiO NPs at the 3-week treatment, the sub-toxic concentrations of 1 µg/ml and 5 µg/ml were chosen. Although *in vitro* studies with NPs enable the identification of conceptual models of mechanistic interaction with cells, they do not represent a full realistic model of how NPs will interact with the specific organ of the body *in vivo*. Unfortunately, nowadays no consistent epidemiologic studies exist on the association between reproductive health and the risk of NiO NPs exposure in humans.

In the first analysis, our data demonstrated that the SCs exposed to both subtoxic doses (1 µg/ml and 5 µg/ml) of NiO NPs didn't show substantial morphological changes.

Oxidative stress is a key contributor to the reproductive toxicity caused by NPs (45). Reactive oxygen species (ROS) are a major factor in inducing 30–80% of infertility issues in men (46), since the increased production of ROS leads to cell apoptosis and impaired spermatogenesis (47).

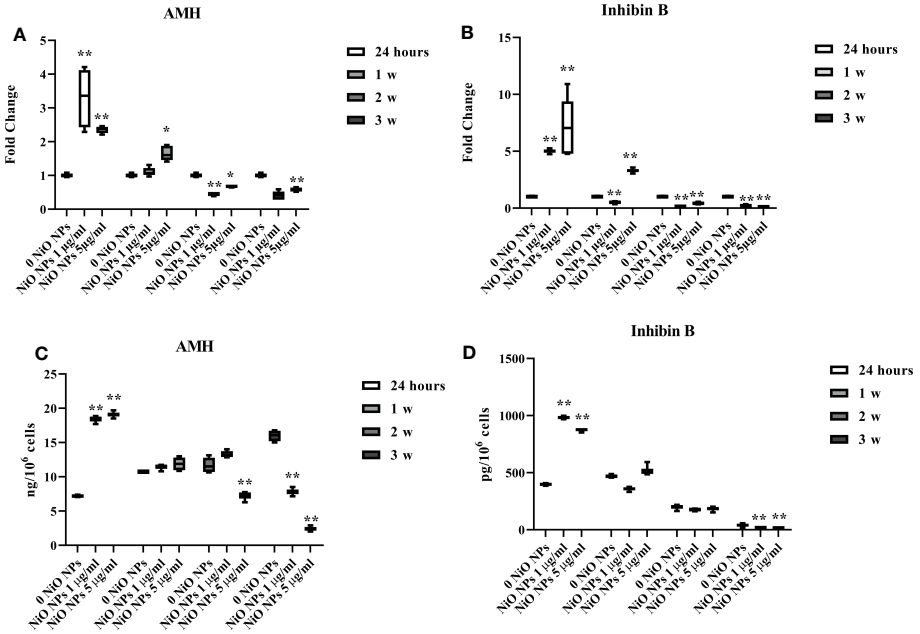

FIGURE 5
Effect of NiO NPs on SCs functionality parameters AMH and inhibin Gene expression of AMH **(A)**, and inhibin B **(B)** in SCs at 24 h and 1, 2, and 3 weeks of incubation with NiO NPs 1 and 5 µg/ml. Data represent the mean ± SEM (*p < 0.05 and **p < 0.001 vs. 0 NiO NPs of three independent experiments, each performed in triplicate). ELISA assay of **(C)** AMH and **(D)** inhibin B secretion in SCs at 24 h and 1, 2, and 3 weeks of incubation with NiO NPs 1 and 5 µg/ml. Data represent the mean ± SEM (**p < 0.001 vs. 0 NiO NPs of three independent experiments, each performed in triplicate).

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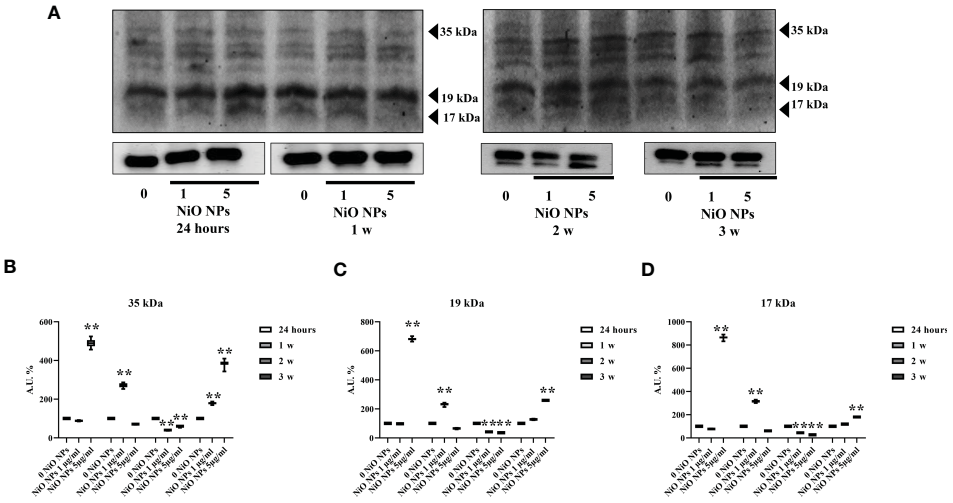

FIGURE 6
Caspase-3 Evaluation by WB analysis. **(A)** Immunoblots of caspase-3 p35, p19, and p17 in SCs at 24 h and 1, 2, and 3 weeks of incubation with NiO NPs at 1 and 5 µg/ml. Densitometric analysis of the protein bands of caspase-3 p35 **(B)**, p19 **(C)**, and p17 **(D)** in SCs at 24 h and 1, 2, and 3 weeks of incubation with NiO NPs 1 and 5 µg/ml. Data represent the mean ± SEM (**p < 0.001 vs. 0 NiO NPs of three independent experiments, each performed in triplicate).

NiO NPs induced oxidative damage has been demonstrated in different organs of rats (16, 48, 49) and mice (50).

Several studies have investigated the relationship between the depletion of cellular antioxidants in male reproductive organs and infertility (51, 52).

The increase in ROS may induce lipid peroxidation, leading to loss of cell membrane integrity and axonemic damage, reduced sperm viability, and later increased sperm abnormalities (53). It has also been reported that lipid peroxidation alters the germ cell membrane, leading to inhibition of spermatogenesis and cell death, resulting in decreased sperm count (54). Therefore, oxidative stress damage induced by NiO NPs may be the main mechanism of their toxicity, which may be related to the binding of nickel to amino acids, polypeptides and proteins to promote the production of ROS (55).

Our results would agree with these data. In fact, in our model, NiO NPs exposure, at each concentration, induced a marked increase of intracellular ROS at the third week of treatment and DNA damage at all exposure times.

Studies by Kong et al. discovered that NiO NPs reduce the activity of superoxide dismutase (SOD) and catalase (CAT) in rats. Following exposure to NiO NPs, the cell concentration of the antioxidant enzymes SOD and CAT increases in an attempt to counteract the injury caused by ROS. When the antioxidant effect is inadequate to resist the action of ROS, the balance between the production of ROS and the antioxidant system response breaks down, which subsequently leads to reduced levels of antioxidant enzymes, increased ROS content, oxidative stress, and eventually cell death (56).

We measured gene expression of antioxidative enzymes (ROS removal agents) including SOD, HO-1, and GHSPx as downstream molecules of Nrf2/ARE pathway. The SCs exposed to both concentration of NiO NPs showed the upregulation of SOD1 and HO-1, while, the increase of GHSPx was evident only at second week (at 1 µg/ml NiO NPs) and first week (at 5 µg/ml NiO NPs).

We might hypothesize that the Nrf2/ARE pathway activation was enough to cope with ROS production only during the acute exposure to subtoxic doses of NiO NPs; meanwhile, its activation

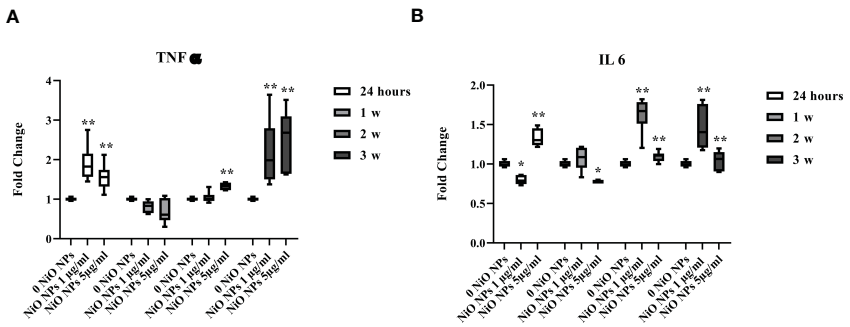

FIGURE 7
Real-time PCR analysis of SCs proinflammatory response. Gene expression of TNF-α **(A)**, IL-6 **(B)** in SCs at 24 h and 1, 2, and 3 weeks of incubation with NiO NPs 1 and 5 µg/ml. Data represent the mean ± SEM (*p < 0.05, **p < 0.001 vs. 0 NiO NPs of three independent experiments, each performed in triplicate).

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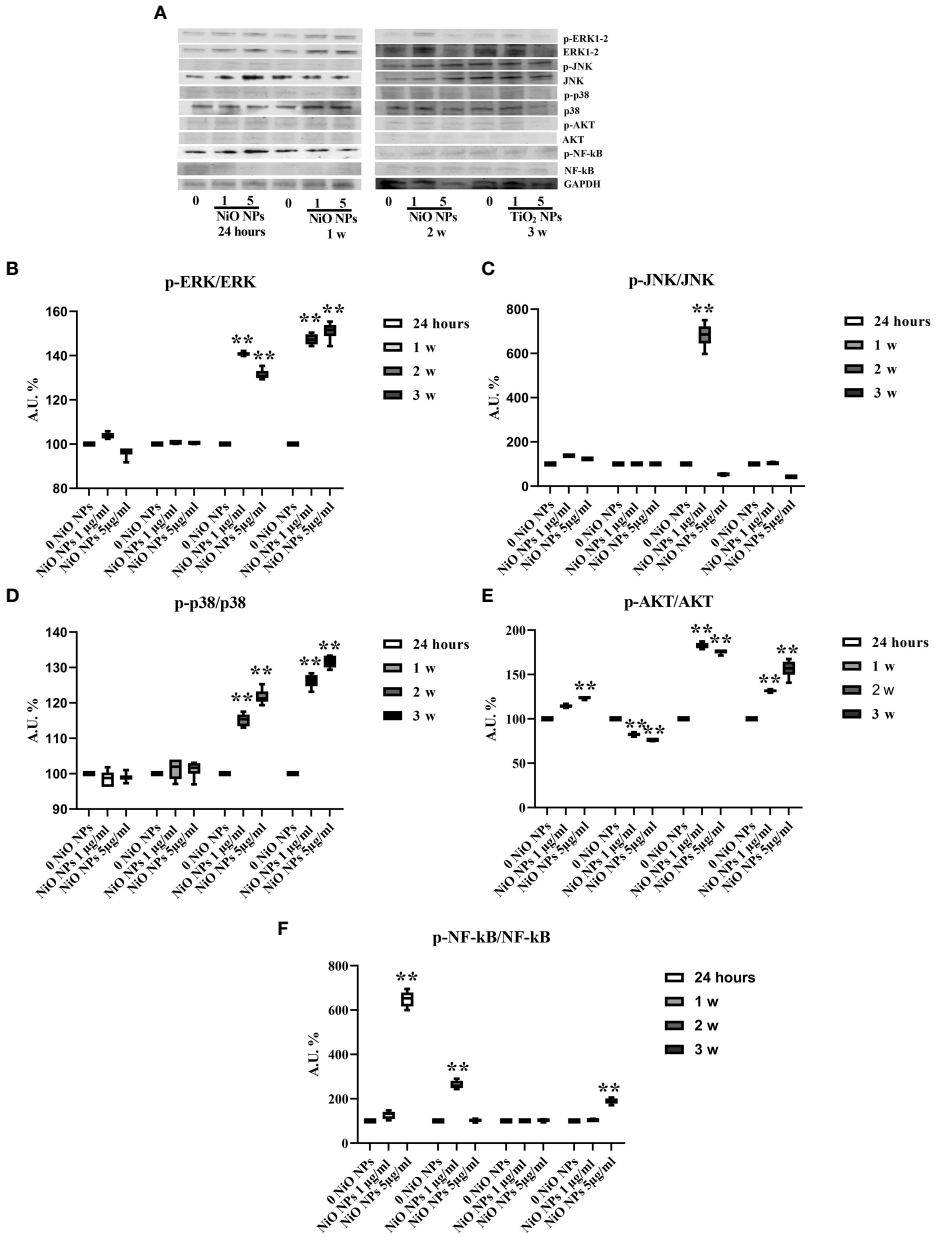

FIGURE 8
MAPK Kinase Signaling Pathway analysis in WB. **(A)** Immunoblots of phosphoERK1-2/ERK1-2, phosphoJNK/JNK, phosphop38/p38, phosphoAKT/AKT, posphoNF-kB p65/NF-kB, and GAPDH in SCs 24 h and 1, 2, and 3 weeks of incubation with NiO NPs 1 and 5 µg/ml. **(B)** Densitometric analysis of the protein bands of phosphoERK1-2/ERK1-2, **(C)** phosphoJNK/JNK, **(D)** phosphop38/p38, **(E)** phosphoAKT/AKT, and **(F)** phosphoNF-kB p65/NF-kB in SCs 24 h and 1, 2, and 3 weeks of incubation with NiO NPs 1 and 5 µg/ml. Data represent the mean ± SEM (**p < 0.001 vs. 0 NiO NPs of three independent experiments, each performed in triplicate).

was not able to counteract the oxidative stress generated throughout the chronic exposure at the subtoxic toxic dose.

The effects on SCs exposed to NiO NPs toxicity were evaluated using functional biomarkers of these cells, such as the gene expression and secretion of AMH and inhibin B.

AMH is a dimeric glycoprotein that belongs to the transforming growth factor-β (TGF-β) superfamily, which includes inhibin B, activins, and others (57). It is exclusively secreted by SCs, thus representing a useful markers of testis functionality during the pre-pubertal period (58). Inhibin B is a heterodimeric glycoprotein, which plays a role in the negative feedback control of FSH secretion in men (59). Inhibin B is a marker used in clinical practice to evaluate the presence and function of SCs during childhood (60). We observed that AMH and inhibin B gene expression and secretion significantly decreased up to the third week at both concentrations of NiO NPs-exposure. This result is an expression of the reduced Sertolian functionality caused by subtoxic doses of NiO NPs chronic exposure on our SCs model.

We then evaluated the activation of apoptosis assessing the caspase-3 protein expression.

Magaye and Zhao discovered that NiO NPs is able to induce genotoxicity by switching on apoptosis-related genes. Apoptosis

induced by NiO NPs mainly engages the death receptor-mediated pathway and the mitochondria-mediated pathway (9). Kong et al. found that NiO NPs increased the levels of pro-apoptotic factors, such as caspase-3, caspase-8, caspase-9 and reduced the levels of anti-apoptotic factor Bcl-2 protein. The apoptosis process mediated by caspase-3 can be triggered by p53 activated by NiO NPs (61). Our results showed the activation of caspase-3 during third week of treatment with 5 μg/ml NiO NPs, with increase of both active p19 and p17 compared to the inactive p35 fragments, expression of a more prominent apoptotic process.

Regarding inflammation, administration of NiO NPs in mice alters the balance between pro-inflammatory and anti-inflammatory response (62), during which monocyte-differentiated macrophages produce pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α (63). After intratracheal instillation of the same concentration of NiO NPs, various cytokines were found to work as proliferation and/or survival drivers, and TNF-α and IL-6 were also significantly increased in all experimental groups compared with the control group. Accordingly, it is possible to suppose that up-regulation of pro-inflammatory effects of IL-1β, IL-6, and TNF-α may deepen the inflammatory reaction and microstructure damage of testicular tissues.

We were able to observe, at both subtoxic doses of NiO NPs a clear pro-inflammatory stress with the steady increase in the gene expression of TNF-α and IL-6.

The MAPK signal transduction pathway, also known as the mitogen-activated protein kinase pathway, includes three parallel pathways, namely the ERK pathway, the JNK/SAPKK pathway and the P38MARK pathway (64). Being some of the most widely used transcription factors in cells, they are implicated in many significant cellular activity processes, including proliferation, differentiation, and apoptosis (65).

Magaye et al. reported that NiO NPs at concentrations of 0, 2.5, 5, 7.5, and 10 μg/cm2 significantly up-regulated the protein expression of phosphorylated ERK1/2 (p-ERK1/2), phosphorylated JNK (p-JNK) and phosphorylated P38 (p-P38) (6, 7).

SCs treated with both concentrations of NiO NPs markedly increased the phosphorylation ratio of p-ERK1/2, p-38 and p-AKT from the second up to the third week of treatment, as a response to a state of inflammation and apoptosis.

This study points out the importance of deepening the effects of the chronic exposure to subtoxic doses of NiO NPs on *"in vitro"* model of SCs, underlining that to identify damages in the Sertolian pre-puberal phase is crucial to predict future irreversible alterations of spermatogenesis in adulthood.

The *limitation* of this study is represented by the difficulty of to isolate (if not impossible) adult SC because of very tight intercellular junctions.

Disruption of such junctions, during testis digestion, severely damages SC viability. In fact, adult Sertolian cell lines are commonly used (66), that are very far from simulating physiological characteristics of SC when investigated as primary cultures (67).

Obvioulsly, pre-pubertal human SC are quite difficult to find and harvest and, above all, raise unsolved ethical problems (in fact, many Countries, including ours, prohibits retrieval of reproductive organs from cadavers).

The use of SCs does not impact on results.

Conclusions

The present study has concluded that the chronic exposure to subtoxic doses of NiO NPs induces adverse effects on SCs functionality and viability. Our *in vitro* pilot study could help to adopt future containment strategies and active surveillance programs, as preventive measures before irreversible damage to SCs may occur and consequently affects spermatogenesis.

Data availability statement

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

Ethics statement

Animal studies were conducted in agreement with the guidelines adopted by the Italian Approved Animal Welfare Assurance (A-3143-01) and the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Author contributions

IA designed and drafted the manuscript. The experimental procedures and data analysis were performed by AM, CB, CL, MA, DB, MC, EE, FG and TB. SG, AG, and GM gave experimental guidance. FM and GL supervised and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1063916/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Uptake of NiO NPs by inductively coupled plasma-optical emission spectrometry (ICP-OES). Percentage of internalized nanoparticles (% uptake) and amount of metal adsorbed per cell number (expressed as ng/105) in SCs 5 h of incubation with NiO NPs 1 and 5µg/ml. Data represented as mean ± SEM of three independent experiments, each are performed in triplicate.

SUPPLEMENTARY FIGURE 2
NiO NPs Cytotoxicity Evaluation by MTT test. **(A)** Evaluation of NiO NPs toxicity in SCs at 24 (grey line) and 48 hours (black line) of incubation with NiO NPs 2.5, 5, 15, 30, 45, 60 and 120 µg/ml. **(B)** Evaluation of NiO NPs toxicity in SCs at 24 hours, 1, 2 and 3 weeks of incubation with NiO NPs 1 (grey line) and 5 µg g/ml (black line). Data represented as mean ± SEM. (*p<0.05 and **p<0.001 *vs* unexposed SCs of three independent experiments, each performed in triplicate).

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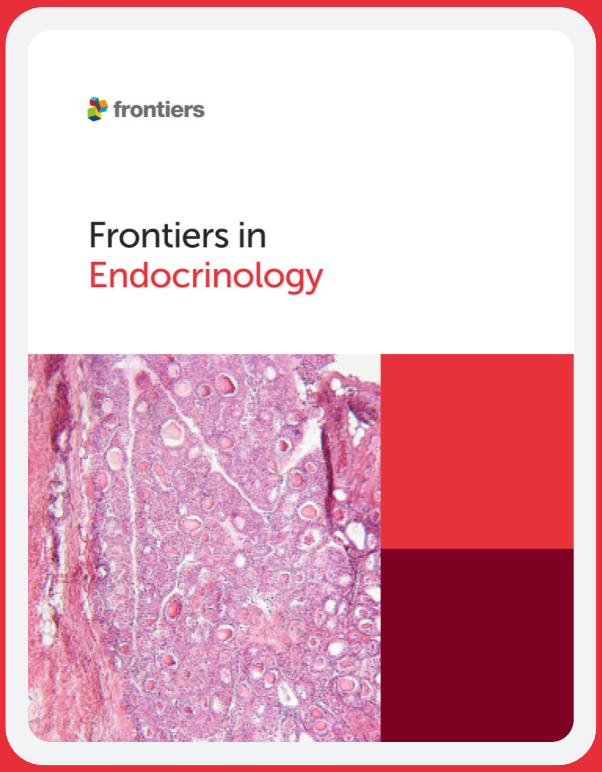

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