

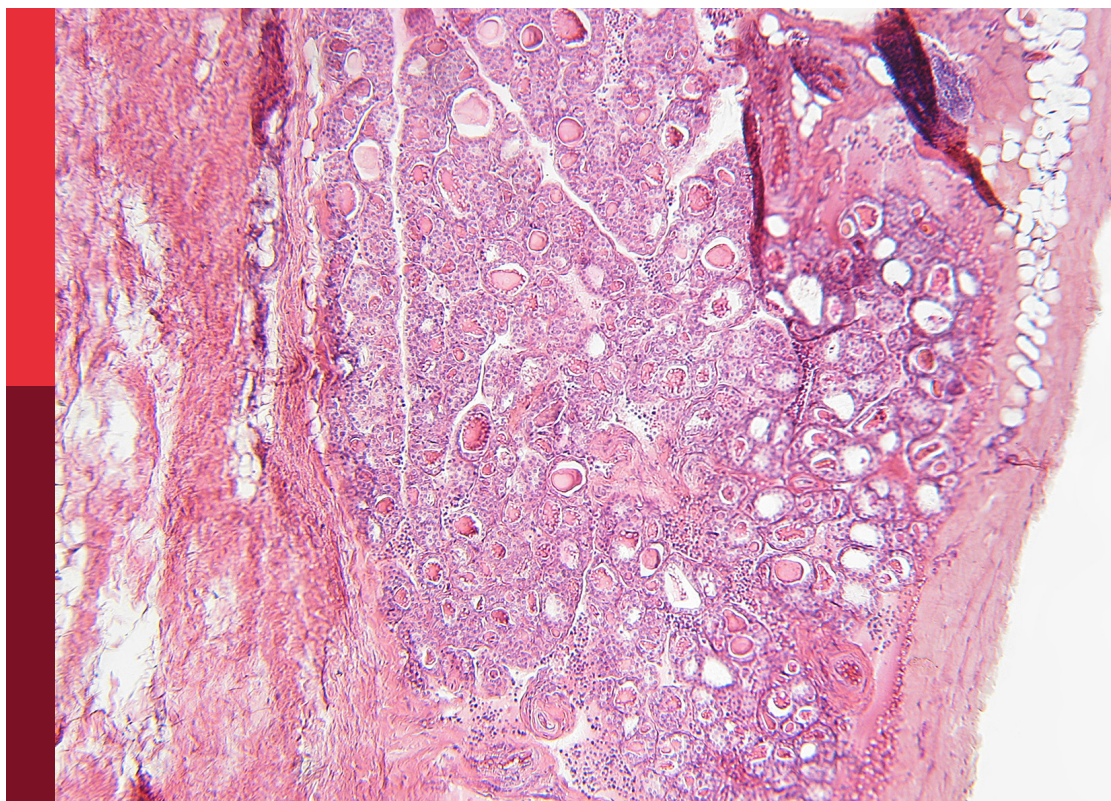
Male reproduction and oxidative stress, volume II

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Published in

Frontiers in Endocrinology



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ISSN 1664-8714
ISBN 978-2-8325-6997-9
DOI 10.3389/978-2-8325-6997-9

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Male reproduction and oxidative stress, volume II

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Citation

Bai, S., Ali, A., Bai, W., eds. (2025). *Male reproduction and oxidative stress, volume II*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-6997-9

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OPEN ACCESS

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RECEIVED 06 March 2024

ACCEPTED 19 April 2024

PUBLISHED 14 May 2024

CITATION

Fan S, Zhang Z, Wang H, Luo L and Xu B
(2024) Associations between tobacco
inhalation and semen parameters in
men with primary and secondary infertility:
a cross-sectional study.
Front. Endocrinol. 15:1396793.
doi: 10.3389/fendo.2024.1396793

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Associations between tobacco inhalation and semen parameters in men with primary and secondary infertility: a cross-sectional study

ShiWei Fan, Zeling Zhang, HuiRu Wang, Lei Luo* and Bo Xu*

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Objective: To examine the impact of tobacco smoking on seminal parameters in men with both primary and secondary infertility.

Methods: This cross-sectional study analyzed 1938 infertile men from China who were categorized as nonsmokers (n=1,067) and smokers (n=871), with the latter group further divided into moderate smokers (1-10 cigarettes per day) (n=568) and heavy smokers (>10 cigarettes per day) (n=303). We assessed semen volume, concentration, total sperm count, progressive motility, and normal morphology following World Health Organization (WHO 2010) guidelines. A logistic regression model was used to analyze the relationships between smoking and seminal parameters while also controlling for lifestyle factors.

Results: The analysis demonstrated a statistically significant correlation between smoking and adverse seminal parameters in both primary and secondary infertility patients. Specifically, primary infertile men who smoked had a lower semen concentration, with heavy smokers showing a median sperm concentration of $59.2 \times 10^6/\text{ml}$ compared to $68.6 \times 10^6/\text{ml}$ in nonsmokers ($P=0.01$). The secondary infertile men who smoked exhibited reduced forward sperm motility, with heavy smokers demonstrating a median progressive motility of 44.7%, which was significantly lower than the 48.1% observed in nonsmokers ($P=0.04$).

Conclusion: Smoking is significantly associated with detrimental effects on seminal parameters in infertile men, thus highlighting the need for cessation programs as part of fertility treatment protocols. Encouraging smoking cessation could substantially improve semen quality and fertility outcomes in this population.

KEYWORDS

primary infertility, progressive sperm motility, secondary infertility, semen quality, smoking, sperm concentration

1 Introduction

Infertility is characterized as the inability of couples within the reproductive age spectrum to conceive despite engaging in regular, unprotected coitus for a span exceeding one year. A thorough examination of 25 population-based studies demonstrated that approximately 9% of individuals of reproductive age worldwide, across both advanced and developing nations, are impacted by infertility. Significantly, male factors play a role in half of these instances (1). Infertility exerts a substantial global influence, whereby it affects an estimated 70 million individuals throughout the world. The World Health Organization (WHO) has recognized infertility as a paramount public health challenge, thus highlighting the need for enhanced clinical focus. Research suggests that a worldwide decrease in sperm quality, including decreased sperm counts among men of reproductive age, is linked to environmental pollutants, occupational stress, and suboptimal dietary habits (2). This Introduction section emphasizes the importance of exploring infertility, acknowledges the current insights into its prevalence and causative factors, and positions this study as a significant step forward in deepening our understanding of the factors contributing to infertility and possible interventions. By refining the description of infertility to avoid repetitiveness, the narrative can become more clear, thus focusing on the core criteria for defining infertility.

Routine semen analysis is essential before assisted reproductive medicine treatment. Evidence-based recommendations highlight the importance of a comprehensive medical history, detailed physical examination, and assessment of factors affecting semen quality as initial evaluative measures for male patients. Abnormalities in semen analysis can provide valuable insights into various aspects of male infertility, which often results from a complex interplay of genetic and socioenvironmental factors. A significant body of research, including a 2021 meta-analysis on molecular genetics, has identified genetic underpinnings for approximately half of all infertility cases in men, thus manifesting in reduced sperm count, sperm viability, or increased morphologically abnormal sperm (3). Despite these advancements, the specific mechanisms of male genetic infertility require further exploration (4). In addition to genetic factors, lifestyle factors significantly impact male fertility. In particular, smoking has been extensively shown to detrimentally affect male reproductive health, with smokers often requiring more *in vitro* fertilization (IVF) attempts to achieve conception (5). Lead and cadmium, which are prevalent in tobacco smoke, contribute to decreased male fertility through oxidative stress pathways, thus damaging sperm DNA and reducing sperm production (6). This finding has been corroborated by population-based epidemiological studies and animal experiments (7). Therefore, lifestyle modifications, such as maintaining a balanced diet, weight control, moderate exercise, and minimizing exposure to environmental pollutants, are advocated to enhance men's sexual health and reproductive outcomes (8).

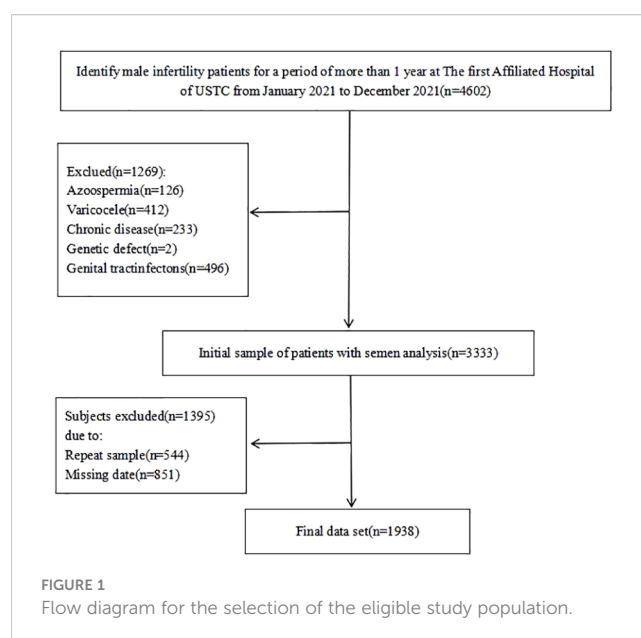
Male infertility is typically categorized into primary infertility, wherein individuals have been infertile both previously and currently, and secondary infertility, wherein individuals were fertile in the past but are currently experiencing infertility issues (1, 9). Although earlier research, including studies by Al-Turki in

2014 and 2016 (1), have targeted specific demographics, such as infertile men in Saudi Arabia, these investigations were limited by small sample sizes (258 and 425 cases, respectively) and data that may no longer reflect current trends (spanning the years of 2008–2013). This scenario suggests that the information collected during this time period may not accurately represent the latest developments or changes in infertility patterns, thus necessitating more recent and broader studies to understand the current state of male infertility. Therefore, we conducted the present study to offer valuable and superior research data in this field. Reports indicate that the causes of secondary infertility in men may include surgical procedures, radiotherapy, varicocele and aging, which are also significant factors in the development of secondary infertility (10). Given the prevalence of smoking as a lifestyle habit among adult men throughout the world, with more than one-third of them using tobacco (Tobacco-WHO), an understanding of its impact on male infertility has become particularly pertinent.

2 Methods

2.1 Patients

To assess the link between cigarette smoking and semen quality among infertile men, a cross-sectional analysis was performed from January to December 2021 in Anhui Province, China (refer to [Figure 1](#) for details). The eligibility criteria required male participants to be diagnosed with infertility, with an absence of concurrent reproductive abnormalities (e.g., varicocele, syringomyelia, cryptorchidism, and inguinal hernia) or hormonal abnormalities. The female partners were expected to exhibit regular fertility cycles indicative of ovulatory function and normal uterine structures. Initially, the study aimed to include a cohort of 4,602 men. However, during data verification, 1,269 participants were excluded for various reasons, including azoospermia ($n=126$),



varicocele ($n=412$), reproductive tract infections ($n=496$), chronic severe debilitating conditions ($n=233$), or specific genetic abnormalities ($n=2$). Notably, some participants were disqualified for multiple reasons ($n=544$), and a significant number ($n=851$) were excluded for not providing complete information on their smoking consumption habits. Ultimately, the study included 1,938 men, all of whom presented with abnormal semen parameters in conjunction with the 5th edition of the WHO criteria. Ethical approval for this research was obtained from the Ethics Committee of the First Affiliated Hospital of USTC, China (Ethics Approval Number: 2021-RE-072).

The study delineated two primary groups based on smoking behavior: smokers (who were further divided into moderate smokers, consuming 1-10 cigarettes per day (11), and heavy smokers, consuming more than 10 cigarettes per day (12)) and nonsmokers (nonsmokers included quitters and never-smokers). This classification was pivotal in examining the potential gradations in semen quality across different levels of tobacco exposure among the infertile male population.

2.2 Semen parameters

Prior to the collection of semen samples, participants were instructed to urinate to ensure a clean urethra and advised to abstain from ejaculation for a period of two to seven days. To further ensure sample purity, an increased intake of water was recommended on the day preceding the collection. The WHO Laboratory provided sterile plastic containers for the collection of semen samples. Semen volume was measured by weight (milliliters), and samples were allowed to liquefy at 37°C for 30 minutes before analysis, thus adhering to the WHO 2010 standards. The analysis included evaluations of sperm volume, concentration, progressive motility, and nonprogressive motility. Cytological staining was conducted by using the Diff-Quick staining kit supplied by Anke Biotechnology Co., Ltd., thus facilitating the assessment of sperm morphology through microscopic examination of more than 200 spermatozoa. The identification of anti-sperm antibodies (AsA) employed the mixed antiglobulin reaction (MAR) method, which was also provided by Anke Biotechnology (Hefei, China). Leukocytospermia was determined when leukocyte counts exceeded $1 \times 10^6 \text{ ml}^{-1}$.

2.3 Clinical characteristics

Body mass index (BMI), which is a universally recognized metric for assessing obesity levels, was calculated for each participant by using the formula $\text{BMI} = \text{weight (kg)} / \text{height}^2 (\text{m}^2)$, based on the WHO China BMI thresholds from the 2004 WHO Expert Consultation.

2.4 Research design

Participants completed a detailed questionnaire regarding their smoking habits to facilitate data collection. Smoking frequency was

recorded as the number of cigarettes smoked per day, dividing participants into quitters (nonsmokers), moderate smokers (1-10 cigarettes per day), and heavy smokers (more than 10 cigarettes per day). The classification also considered the participants' reproductive history, distinguishing those with primary infertility (those who were unable to conceive for more than a year without contraception) from those with secondary infertility (those who previously had children but were unable to conceive). Additional clinical characteristics, such as age, BMI, ethnicity, education level, duration of infertility, alcohol consumption, late-night snacking habits, dietary patterns, working hours, and sleep duration, were also collected. Semen analysis followed the protocols outlined in the 2010 WHO manual, with abnormal semen parameters defined as a semen volume less than 1.5 ml, a sperm concentration less than $15 \times 10^6 \text{ ml}$, and a motility pattern less than 4%.

2.5 Statistical analysis

The statistical analysis was conducted by using Prism 9.0 software (San Diego, CA), with a significance threshold set at $P < 0.05$. Quantitative variables are presented as the mean $\pm$ standard deviation (SD) for normally distributed data, whereas nonnormally distributed data are presented as the median and interquartile range (min–max or ideally with 25th–75th percentiles). The Kolmogorov-Smirnov test was employed to determine the distribution of the variables. For normally distributed data, one-way ANOVA or t tests were used, whereas Kruskal-Wallis or Mann-Whitney U tests were used for nonnormally distributed data. Categorical variables were analyzed by using the chi-squared test, with Fisher's exact test invoked under conditions necessitating its use. This comprehensive statistical framework ensured the meticulous examination of the relationship between smoking habits and semen characteristics, thus facilitating a nuanced understanding of their interplay within the context of male infertility.

3 Results

Table 1 presents a detailed breakdown of the characteristics distinguishing men with primary infertility from men with secondary infertility among a study cohort of 1,938 participants. Men with primary or secondary infertility had an average age of 30.4 ± 4.6 years, with a notable age difference observed between the groups; specifically, individuals in the secondary infertility group were older (averaging 32.4 ± 5.3 years) compared to 29.7 ± 4.1 years in the primary infertility group. This age disparity was significant, with 10.6% of the patients in the secondary infertility group being over 40-years-old versus only 2.5% in the primary group. Lifestyle factors, including diet, alcohol consumption, duration of infertility, work hours, and sleep, showed no significant differences between the two groups in terms of their influence on infertility type. However, education level markedly differed; specifically, higher education was more prevalent in the primary infertility group. Interestingly, primary infertile men reported of a greater

TABLE 1 Descriptive and characteristic statistics.

Clinical characteristics	Total (n=1938)	Primary infertile men (n=1430)	Secondary infertile men (n=508)	P
Age (year), mean $\pm$ s.d.	30.4 $\pm$ 4.6	29.7 $\pm$ 4.1	32.4 $\pm$ 5.3	<0.001
<40, n (%)	95.3 (1848)	97.5 (1394)	89.4 (454)	<0.001
$\geq$ 40, n (%)	4.7 (90)	2.5 (36)	10.6 (54)	
BMI (kg/m ²), mean $\pm$ s.d.	24.9 $\pm$ 4.0	25.0 $\pm$ 4.0	24.9 $\pm$ 4.1	0.52
<24, n (%)	42.4 (822)	42.3 (605)	42.7 (217)	0.87
$\geq$ 24, n (%)	57.6 (1116)	57.7 (825)	57.3 (291)	
Nation, n (%)				0.13
Han	99.6 (1930)	99.7 (1426)	99.2 (504)	
Other	0.4 (8)	0.3 (4)	0.8 (4)	
Education, n (%)				<0.0001
Primary school	1.5 (29)	1.2 (17)	2.4 (12)	
Junior high school	20.9 (404)	18.7 (267)	27.0 (137)	
High school	18.7 (363)	18.3 (262)	19.9 (101)	
College/University	58.9 (1142)	61.8 (884)	50.8 (258)	
Duration of infertility, n (%)				0.08
$\leq$ 1 year	74.8 (1450)	75.7 (1082)	72.4 (368)	
>1 year, <3 year	13.4 (259)	13.5 (193)	13.0 (66)	
$\geq$ 3 year	11.8 (229)	10.8 (155)	14.6 (74)	
Alcohol status, n (%)				0.16
Drinkers	52.2 (1012)	51.3 (733)	54.9 (279)	
Non-drinkers	47.8 (926)	48.7 (697)	45.1 (229)	
Smoking status, n (%)				0.006
non-smokers	55.1 (1067)	57.1 (817)	49.2 (250)	
Moderate smoker	29.3 (568)	28.3 (405)	32.1 (163)	
Heavy smoker	15.6 (303)	14.6 (208)	18.7 (95)	
Frequency of night snack intake, n (%)				0.009
0/week	31.7 (614)	29.4 (420)	38.2 (194)	
1-3/week	64.7 (1254)	66.8 (956)	58.7 (298)	
>3/week	3.6 (70)	3.8 (54)	3.1 (16)	
Dietary habits, n (%)				0.27
Regular diet (three meals per day)	65.7 (1274)	65.0 (930)	67.7 (344)	
Irregular diet	34.3 (664)	35.0 (500)	32.3 (164)	
Work time, n (%)				0.21
<8 (h/d)	34.4 (667)	33.2 (475)	37.8 (192)	
8-10 (h/d)	47.7 (924)	49.3 (705)	43.1 (219)	
>10 (h/d)	17.9 (347)	17.5 (250)	19.1 (97)	
Sleep time, n (%)				0.12
$\leq$ 8 (h/d)	73.7 (1428)	74.6 (1067)	71.1 (361)	

(Continued)

TABLE 1 Continued

Clinical characteristics	Total (n=1938)	Primary infertile men (n=1430)	Secondary infertile men(n=508)	P
>8 (h/d)	26.3 (510)	25.4 (363)	28.9 (147)	
Semen parameters				P
Semen volume (ml),median (Q1,Q3)	3.1 (2.2,4.2)	3.1 (2.2,4.3)	3.0 (2.1,4.1)	0.17
Semen volume<1.5 (ml),n (%)	8.6 (166)	8.5 (122)	8.7 (44)	0.93
Sperm concentration (×10 ⁶ /ml),median (Q1,Q3)	67.7 (35.6,118.4)	65.3 (34.1,116.2)	72.4 (42.6,125.2)	0.0093
Sperm concentration<15×10 ⁶ /ml,n (%)	9.2 (179)	9.9 (141)	7.5 (38)	0.11
Progressive motility (%),median (Q1,Q3)	45.4 (33.1,57.7)	43.4 (30.0,55.1)	42.8 (28.7,54.1)	<0.0001
Progressive motility<32%,n (%)	28.1 (545)	29.9 (428)	23.0 (117)	0.003
Normal morphology (%),median (Q1,Q3)	6 (5,8)	7 (5,8)	6 (5,8)	0.33
Normal morphology<4%,n (%)	9.8 (179)	10.7 (143)	7.5 (36)	0.05
Asthenoteratozoospermia,n (%),n (%)	54.6 (1058)	55.5 (794)	52.0 (264)	0.17
Oligoasthenoteratozoospermia,n (%)	4.6 (90)	5.2 (75)	3.0 (15)	0.035
Leukocytic count (×10 ⁶ /ml),median (Q1,Q3)	0.1 (0.0,0.5) (n=998)	0.1 (0.0,0.5) (n=755)	0.1 (0.0,0.5) (n=243)	0.91
AsA (%),median (Q1,Q3)	2.0 (1.0,4.0) (n=821)	2.0 (1.0,4.0) (n=597)	2.0 (1.0,4.0) (n=224)	0.32

BMI, was calculated using P values that came from the chi-square test. If the data are regularly distributed, they are shown as the mean ± standard deviation, and for categorical variables, as n (%), and for continuous variables, as medians (Q1, Q3). 25th percentile is Q1. Q3 is the 75th percentile.

frequency of night snacking than their secondary counterparts. From a clinical perspective, primary infertile men also displayed superior sperm progressive motility and a lower incidence of smoking, yet they exhibited lower sperm concentrations, thus highlighting intricate relationships between lifestyle choices, clinical characteristics, and infertility type.

Table 2 shows the associations between various factors and primary versus secondary infertility. Significantly, the data demonstrated that heavy smokers were notably older than light smokers, with individuals who had quit smoking also displaying a tendency toward older age. A marked statistical correlation was identified among men experiencing infertility with respect to the frequency of nighttime snacking and consistency in dietary habits. Intriguingly, as smoking intensity increased, there was a noticeable increase in the incidence of irregular dietary patterns and late-night snacking, particularly among those with primary infertility, who reported of a greater prevalence of these behaviors than individuals with secondary infertility. Furthermore, an analysis of semen parameters demonstrated that a progressive reduction in semen volume was correlated with increased smoking frequency, which was a trend observed in both primary and secondary infertility cohorts. This pattern highlights the broader implications of lifestyle factors for reproductive health and indicates the potential for mitigating infertility risks through targeted lifestyle modifications. More specifically, there was a substantial relationship between smoking status and sperm concentration in primary infertile men (nonsmokers, moderate smokers, and heavy smokers), with sperm concentration decreasing progressively as smoking intensity

increased. Conversely, in the case of secondary infertility, a significant correlation was identified between sperm forward motility and cigarette smoking, thus demonstrating a progressive reduction in sperm motility with increasing cigarette consumption.

Table 3 presents the significant correlations observed between sperm concentration and various semen parameters. In the primary infertility group, a substantial correlation was identified between smoking and sperm concentration, even after adjusting for factors such as age, BMI, abstinence duration, alcohol consumption, infertility duration, intake of night-time snacks, dietary habits, and working hours. However, no significant associations were detected between smoking and other semen characteristics, including volume, spermatozoa count, and morphology. Within the group experiencing secondary infertility, smoking status was not significantly related to semen parameters such as semen volume or total sperm count. Nevertheless, a noteworthy correlation emerged between the extent of smoking and the rate of progressive sperm motility.

4 Discussion

In this comprehensive cross-sectional study involving 1,938 infertile men from China, we investigated the association between smoking habits and semen quality by stratifying participants into nonsmokers, moderate smokers (1-10 cigarettes per day), and heavy smokers (more than 10 cigarettes per day). Our findings highlight the significant impact of smoking on semen parameters and

TABLE 2 Descriptive statistics based on the smoking status.

	Primary infertile men(N=1430)				Secondary infertile men(N=508)			
	non-smokers (n=817)	Moderate smokers (n=405)	Heavy smokers (n=208)	P	non-smokers (n=250)	Moderate smokers (n=163)	Heavy smokers (n=95)	P
Age(year),mean ± s.d.	29.8 ± 3.9	29.2 ± 4.0	30.1 ± 4.8	0.04	32.4 ± 5.3	31.5 ± 5.3	33.7 ± 5.2	0.001
<40,n(%)	97.4(795)	98.3(398)	96.1(200)	0.28	89.6(224)	92.0(150)	84.2(80)	0.14
≥40,n(%)	1.5(21)	1.7(7)	3.8(8)		10.4(26)	8.0(13)	15.8(15)	
BMI(kg/m^2),mean ± s.d.	24.8 ± 3.7	25.3 ± 4.4	25.1 ± 4.4	0.24	24.6 ± 3.5	25.0 ± 4.4	25.7 ± 5.0	0.39
<24,n(%)	43.0(351)	49.4(165)	42.8(89)	0.74	42.8(107)	44.8(73)	36.8(35)	0.45
≥24,n(%)	57.0(465)	50.6(240)	57.2(119)		57.2(143)	55.2(90)	63.2(60)	
Duration of infertility, n(%)				0.11				0.18
≤1 year	76.2(622)	76.8(311)	71.6(149)		72.4(181)	72.4(118)	72.6(69)	
>1 year,<3 year	14.4(118)	11.9(48)	13.0(27)		13.2(33)	16.0(26)	7.4(7)	
≥3 year	9.4(77)	11.3(46)	15.4(32)		14.4(36)	11.6(19)	20.0(19)	
Frequency of night snack intake,n(%)				0.0001				<0.0001
0/week	33.5(274)	25.2(102)	21.2(44)		50.0(125)	28.2(46)	24.2(23)	
1-3/week	63.8(521)	70.4(285)	72.1(150)		47.6(119)	68.7(112)	70.5(67)	
>3/week	2.7(22)	4.4(18)	6.7(14)		2.4(6)	3.1(5)	5.3(5)	
Dietary habits,n(%)				<0.0001				<0.0001
Regular diet(three meals per day)	72.3(591)	59.5(241)	47.1(98)		80.4(201)	60.7(99)	46.3(44)	
Irregular diet	27.7(226)	40.5(164)	52.9(110)		19.6(49)	39.3(64)	53.7(51)	
Work time, n(%)				0.06				0.89
<8(h/d)	32.3(264)	34.3(139)	34.6(72)		38.0(95)	38.0(62)	36.8(35)	
8-10(h/d)	52.3(427)	46.1(187)	43.8(91)		44.0(110)	40.5(66)	45.3(43)	
>10(h/d)	15.4(126)	19.5(79)	21.6(45)		18.0(45)	21.5(35)	17.9(17)	
Sleep time, n(%)				0.36				0.06
≤8(h/d)	76.0(621)	72.3(293)	73.6(153)		75.6(189)	65.0(106)	69.5(66)	
>8(h/d)	24.0(196)	27.7(112)	26.4(55)		24.4(61)	35.0(57)	30.5(29)	
Semen parameters								
Semen volume(ml), median(Q1,Q3)	3.3(2.3,4.4)	3.0(2.2,4.1)	2.8(2.0,4.0)	0.01	3.2(2.2,4.5)	3.0(2.1,4.1)	2.8(1.8,3.7)	0.008
Semen volume<1.5(ml), n(%)	7.6(62)	8.6(35)	12.0(25)	0.67	6.8(17)	9.2(15)	12.6(12)	0.22
Sperm concentration (×10^6/ml), median(Q1,Q3)	68.6 (36.0,121.7)	60.7(31.4, 101.8)	59.2(28.8,116.2)	0.01	77.9 (45.6,129.4)	69.2(34.2, 120.8)	72.3(43.2,127.8)	0.18
Sperm concentration<15×10^6/ml, n(%)	7.7(63)	12.1(49)	13.9(29)	0.005	5.6(14)	8.6(14)	10.5(10)	0.24

(Continued)

TABLE 2 Continued

	Primary infertile men(N=1430)				Secondary infertile men(N=508)			
	non-smokers (n=817)	Moderate smokers (n=405)	Heavy smokers (n=208)	P	non-smokers (n=250)	Moderate smokers (n=163)	Heavy smokers (n=95)	P
Semen parameters								
Progressive motility(%), median (Q1,Q3)	41.5 (28.8,52.8)	43.4(28.5,55.0)	45.5(28.9,55.7)	0.25	48.1(36.3,58.5)	46.4(34.2,60.1)	44.7(29.3,56.3)	0.01
Normal morphology (%),median (Q1,Q3)	7(4,8)	6(5,8)	7(5,8)	0.7	6(5,8)	6(5,8)	6(4,8)	0.84
Normal morphology<4%,n(%)	23.7(193)	23.7(96)	19.7(41)	0.46	23.2(58)	16.6(27)	27.4(26)	0.10

P values are derived, unless otherwise noted, using analysis of variance.

demonstrates a marked correlation between tobacco use and diminished semen quality among both men with primary infertility and men with secondary infertility (13). Specifically, data analysis demonstrated that nonsmokers exhibited higher sperm concentrations than smokers, with a notable decrease in sperm concentration as smoking intensity increased. For instance, the median sperm concentration was 67.7 (35.6, 118.4) for the total cohort, with a discernible decrease among heavy smokers. Moreover, the study illuminated the differential effects of smoking on primary versus secondary infertility. In primary infertility cases, a significant association was found between smoking and semen concentration, wherein heavy smokers demonstrated the lowest sperm concentration and viability (3). Conversely, secondary infertility was notably linked to reduced sperm forward motility, thus showcasing a progressive decline with increased smoking levels (14). This dichotomy suggests that smoking has multifaceted detrimental effects on male reproductive health, depending on the infertility type. Our study also investigated the broader implications of lifestyle factors on infertility. Notably, heavy smokers were significantly older than light smokers, and the incidence of secondary infertility was greater among older participants, thus underscoring the compounded effect of age and long-term smoking on fertility outcomes. Furthermore, lifestyle patterns, including alcohol consumption, late-night snacking, and dietary habits, exhibited a significant correlation with smoking intensity, particularly accentuated in primary infertility cases (15). Interestingly, the primary infertility group, characterized by higher educational levels, showed more frequent late-night snacking habits, hinting at a potential link between socio-economic status, lifestyle patterns, and infertility. These patterns suggest that the cumulative impact of lifestyle factors, coupled with aging, might predispose individuals to secondary infertility, underscoring the need for a nuanced understanding of how lifestyle choices impact reproductive health over time.

This study significantly advances the discourse on the impact of smoking on male fertility, thus providing empirical evidence that underscores the detrimental effects of cigarette consumption on seminal quality. This scenario highlights the imperative for men facing infertility challenges to reconsider lifestyle choices

(particularly smoking habits) as part of their fertility treatment plan (16). Our research highlights the importance of a proactive stance in fertility clinics, wherein health care professionals should counsel patients on the benefits of quitting smoking to improve seminal quality and, consequently, fertility outcomes.

By delving into the nuanced relationship between lifestyle factors and male infertility, this investigation enriches the scholarly dialog and sets the stage for further research aimed at elucidating the complex dynamics that are occurring. The marked correlation between smoking and diminished semen parameters (specifically, sperm concentration in cases of primary infertility and sperm motility in cases of secondary infertility) emphasizes the critical need for targeted interventions and public health campaigns to counteract the harmful effects of smoking on reproductive health (17).

This study is pioneering in its thorough examination of smoking patterns among Chinese men suffering from primary and secondary infertility, whereby it categorized participants into smokers and nonsmokers and further distinguished smokers by intensity of consumption (18). Similar to the findings from Caster D et al.'s study, which reported significant reductions in sperm concentration among smokers compared to nonsmokers within a sample of 648 infertile males, our analysis extends these observations. We found that smoking adversely affects sperm viability and morphology to a lesser extent but significantly reduces sperm concentration, with a clear dose-response relationship observed between smoking intensity and sperm quality degradation (19). A particularly alarming result is the discovery that heavy smokers within the primary infertility cohort exhibited the lowest sperm concentration and viability, thus illustrating a direct correlation between smoking severity and a decline in sperm health.

Additionally, our findings demonstrated that individuals with secondary infertility were generally older and had a longer history of smoking, thus suggesting a cumulative effect of tobacco exposure over time. This prolonged engagement with smoking, coupled with age-related factors, contributes to an increased incidence of secondary infertility, thus indicating a compounded risk associated with long-term smoking habits (4). In summary, this investigation not only contributes valuable insights into the effects

TABLE 3 Probability ratios with 95% confidence intervals for aberrant semen scope at varying degrees of tobacco use.

	Primary infertile men			Secondary infertile men		
	non-smokers (n=817)	Moderate smokers(n=405)	Heavy smokers(n=208)	non-smokers (n=250)	Moderate smokers(n=163)	Heavy smokers(n=95)
Semen volume						
Crude	ref	1.15(0.74,1.76)	1.66(1.00,2.69)	ref	1.39(0.67, 2.87)	1.98(0.89, 4.30)
P		0.52	0.04		0.37	0.09
Adjusted	ref	1.13(0.71,1.76)	1.53(0.90,2.54)	ref	1.16(0.53, 2.52)	1.18(0.48, 2.77)
P		0.61	0.11		0.71	0.71
Sperm concentration						
Crude	ref	1.65(1.11,2.44)	1.94(1.20,3.07)	ref	1.58(0.73, 3.44)	1.98(0.83,4.60)
P		0.01	0.005		0.24	0.11
Adjusted	ref	1.60(1.05, 2.40)	1.88(1.14,3.05)	ref	1.38(0.61, 3.11)	1.65(0.65,4.06)
P		0.03	0.01		0.43	0.28
Total count						
Crude	ref	1.39(0.94, 2.04)	1.74(1.09,2.73)	ref	1.48(0.71,3.10)	1.92(0.836,4.26)
P		0.09	0.02		0.29	0.11
Adjusted	ref	1.39(0.92, 2.07)	1.76(1.08,2.81)	ref	1.27(0.58,2.75)	1.67(0.68,3.93)
P		0.11	0.02		0.55	0.25
Progressive motility						
Crude	ref	0.98(0.75, 1.27)	0.91(0.65,1.276)	ref	0.51(0.31,0.83)	0.54(0.29,0.96)
P		0.86	0.58		0.007	0.04
Adjusted	ref	1.10(0.845,1.44)	1.00(0.70,1.42)	ref	0.53(0.31,0.87)	0.53(0.28,0.99)
P		0.5	0.99		0.01	0.05
Total motility						
Crude	ref	0.88(0.69,1.13)	0.88(0.64,1.21)	ref	0.61(0.39,0.96)	0.79(0.46,1.3)
P		0.32	0.45		0.03	0.37
Adjusted	ref	0.95(0.73,1.23)	0.93(0.66,1.30)	ref	0.63(0.39,1.01)	0.79(0.44,1.37)
P		0.7	0.67		0.06	0.40
Nomal morphology						
Crude	ref	0.88(0.58,1.32)	1.26(0.77,2.01)	ref	0.41(0.15,0.98)	0.94(0.38,2.12)
P		0.55	0.34		0.06	0.89
Adjusted	ref	0.96(0.62,1.46)	1.46(0.87,2.38)	ref	0.37(0.13,0.93)	0.88(0.33,2.16)
P		0.86	0.14		0.05	0.79

Men having the following WHO-recognized semen quality values were used as the reference group for parameter analysis: semen volume ≥ 1.5 ml, sperm concentration $\geq 15 \times 10^6$ /ml, and progressive motility sperm $\geq 32 \times 10^6$ /ml. Crude: unmodified model Forage, BMI, period of abstinence, alcohol intake, length of infertility, how many nocturnal snacks consumed, dietary practices, and working hours were all changed in the model; ref stands for the reference.

of smoking on male fertility but also advocates for a holistic approach to fertility treatment, thus emphasizing lifestyle modifications as a crucial component of improving reproductive health outcomes.

In this study, we meticulously examined the interplay between several lifestyle and health factors, including age, body mass index

(BMI), alcohol consumption, duration of infertility, dietary patterns, and work and sleep duration, and their impact on semen quality (20). Through our analysis, we established pronounced correlations, notably with age, dietary habits, and smoking behaviors, which demonstrated their crucial influence on male fertility.

Our findings highlight the significant role of age in semen quality. With societal trends shifting toward delayed marriages, age has surfaced as being a paramount determinant affecting male reproductive health. The testes, which are pivotal for spermatogenesis (the formation of mature spermatozoa) and steroidogenesis (the production of testosterone, which is vital for male sexual functions) are impacted by the aging process. This study provides more information on how aging correlates with a progressive decrease in testosterone levels, which is indicative of a reduction in stromal cell activity responsible for testosterone synthesis (7). An age-related decrease in semen quality was evident, with individuals older than 40 years showing increased instances of abnormal semen volume, motility, viability, and sperm kinematics. This finding aligns with Agarwal et al.'s observations of diminished sperm concentration, motility, and volume as age advances, thus highlighting the undeniable impact of aging on fertility (15).

Furthermore, our data demonstrated a notable difference in age between men with primary versus secondary infertility, thus illustrating a demographic trend that supports the literature suggesting that secondary infertility is more prevalent among older men. Specifically, our analysis revealed that the mean ages of ex-smokers were 29.8 ± 3.9 years for men with primary infertility and 32.4 ± 5.3 years for men with secondary infertility. Similarly, the average age of heavy smokers in the primary infertility group was 30.1 ± 4.8 years, whereas that of their counterparts in the secondary infertility group was 33.7 ± 5.2 years. These findings highlight the potential cumulative effect of smoking over time, which is compounded by age-related factors, on fertility outcomes. It is important to clarify that our study did not conduct detailed age subgroup analyses to further explore these observed trends. The decision not to perform such analyses was based on the scope and objectives of our initial research design, which aimed to broadly assess the relationship between smoking habits and infertility without delving into the specific impacts within narrower age ranges. This approach, while insightful, suggests that further research is necessary to dissect the nuances of how age influences the relationship between smoking and fertility across different stages of male infertility. In contrast to the findings of Rehman R et al. (21), who reported no significant differences in age, BMI, or body fat between smokers and nonsmokers within the infertile cohort, our study demonstrated a distinct age trend among heavy smokers, thus indicating that those suffering from secondary infertility tended to be older. However, similar to their findings, we observed no significant correlation between BMI and male infertility in our cohort. This lack of significant BMI correlation, coupled with our insights into age differences, highlights the complex interplay of factors influencing male fertility and also highlights the need for a multifaceted approach in future research.

Our comprehensive analysis delved into the intricate relationship between various lifestyle and health parameters—such as age, Body Mass Index (BMI), alcohol consumption, duration of infertility, and specific dietary habits—and their impact on semen quality. While our investigation identified significant correlations with age, dietary practices, and smoking, offering new insights into the multifactorial influences on male fertility, it is important to note

that our study did not directly compare BMI between infertile patients and a control group of normal, fertile men. The decision not to compare BMI across these groups was driven by our focused research objectives, which aimed to understand the dynamics within an infertile cohort rather than contrast these with the general population. Although BMI showed no significant correlation with male infertility within our specific study population, this finding does not preclude the importance of BMI as a factor in male reproductive health more broadly. Previous studies have suggested varied impacts of BMI on semen quality, but our analysis contributes to the ongoing discourse by emphasizing that within our cohort of infertile men, BMI was not a distinguishing factor in fertility outcomes. By integrating these specific data points, our analysis not only deepens the understanding of lifestyle factors on male fertility but also underscores the importance of considering a range of factors—including age, diet, and smoking cessation—in improving reproductive outcomes (22). This study advocates for tailored interventions and comprehensive fertility evaluations that consider these critical factors, offering a pathway toward optimizing male reproductive health.

In contrast to previous studies that suggested that BMI has a negligible effect on semen quality, our research, which included an analysis of 206 men meeting our stringent criteria, did not establish a significant correlation between increased BMI and semen parameters. However, our study demonstrated pronounced associations between dietary patterns and semen quality. Cross-sectional research involving healthy Taiwanese men over 18 years of age highlighted the detrimental effects of high-sugar and high-carbohydrate diets on sperm motility and concentration, respectively ($P = 0.012$ and $P = 0.025$ for total sperm motility and progressive motility, respectively; $P = 0.001$ for sperm concentration). Furthermore, our findings suggest a negative impact of high sodium and saturated fat intake on sperm motility and overall sperm health (23), thus reinforcing the importance of nutritional choices in male fertility.

Particularly noteworthy is the positive correlation between sperm concentration and a healthy dietary pattern, as well as the frequency of sexual activity among men with suboptimal semen quality. This underscores the pivotal role of diet in reproductive health, which corresponds to Craig Niederberger's emphasis on the benefits of a nutritious diet for enhancing semen quality (24), especially in men with compromised semen parameters.

Despite the robustness of our findings, it is important to acknowledge the limitations inherent in our study's design. The regional specificity of our participant pool may restrict the generalizability of our conclusions. Additionally, the cross-sectional nature of our survey could introduce recall bias, and the reliance on self-reported data for smoking habits may not capture the full spectrum of tobacco exposure. The potential for residual confounding factors remains a challenge, as is common in observational studies, thus possibly affecting the observed associations.

However, the strengths of our study, including its prospective design, the clear differentiation between primary and secondary infertility, and the comprehensive data collection through detailed questionnaires—allow for a nuanced understanding of the interplay

between lifestyle factors and infertility (21). By considering both smoking and alcohol consumption and their cross-correlation, we were able to isolate their independent effects on fertility outcomes.

Overall, our research contributes significantly to the ongoing discourse on male reproductive health by demonstrating the adverse effects of smoking on semen quality among Chinese men with infertility. By demonstrating the clear link between smoking and diminished semen concentration in primary infertile men, as well as decreased sperm viability in secondary infertile cases, our study highlights critical areas for intervention and further research. These findings advocate for a holistic approach to fertility treatment, thus emphasizing the need for lifestyle modifications alongside medical interventions to optimize reproductive outcomes.

Data availability statement

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

Ethics statement

This study was approved by The First Affiliated Hospital of the USTC Ethical Committee (2021-RE-072). Written informed consent was obtained from all participants. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

SF: Data curation, Software, Writing – original draft, Conceptualization, Methodology, Project administration,

Resources. ZZ: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. HW: Data curation, Investigation, Methodology, Software, Writing – original draft. LL: Writing – review & editing. BX: Conceptualization, Project administration, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was supported by the National Key Research and Development Project (2019YFA0802600), the National Natural Science Foundation of China (82171599).

Acknowledgments

We are indebted to Xia Wu, Jingru Xu, and Yanyan Shang for their valuable contributions to the data collection.

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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OPEN ACCESS

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RECEIVED 11 March 2024

ACCEPTED 26 April 2024

PUBLISHED 16 May 2024

CITATION

Venditti M, Romano MZ, Boccella S,
Haddadi A, Biasi A, Maione S and Minucci S
(2024) Type 1 diabetes impairs the activity
of rat testicular somatic and germ cells
through NRF2/NLRP3 pathway-mediated
oxidative stress.
Front. Endocrinol. 15:1399256.
doi: 10.3389/fendo.2024.1399256

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Type 1 diabetes impairs the activity of rat testicular somatic and germ cells through NRF2/NLRP3 pathway-mediated oxidative stress

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Background: It is well known that metabolic disorders, including type 1 diabetes (T1D), are often associated with reduced male fertility, mainly increasing oxidative stress and impairing the hypothalamus–pituitary–testis (HPT) axis, with consequently altered spermatogenesis and reduced sperm parameters. Herein, using a rat model of T1D obtained by treatment with streptozotocin (STZ), we analyzed several parameters of testicular activity.

Methods: A total of 10 adult male Wistar rats were divided into two groups of five: control and T1D, obtained with a single intraperitoneal injection of STZ. After 3 months, the rats were anesthetized and sacrificed; one testis was stored at -80°C for biochemical analysis, and the other was fixed for histological and immunofluorescence analysis.

Results: The data confirmed that T1D induced oxidative stress and, consequently, alterations in both testicular somatic and germ cells. This aspect was highlighted by enhanced apoptosis, altered steroidogenesis and Leydig cell maturity, and impaired spermatogenesis. In addition, the blood–testis barrier integrity was compromised, as shown by the reduced levels of structural proteins (N-cadherin, ZO-1, occludin, connexin 43, and VANG2) and the phosphorylation status of regulative kinases (Src and FAK). Mechanistically, the dysregulation of the SIRT1/NRF2/MAPKs signaling pathways was proven, particularly the reduced nuclear translocation of NRF2, affecting its ability to induce the transcription of genes encoding for antioxidant enzymes. Finally, the stimulation of testicular inflammation and pyroptosis was also confirmed, as highlighted by the increased levels of some markers, such as NF-κB and NLRP3.

Conclusion: The combined data allowed us to confirm that T1D has detrimental effects on rat testicular activity. Moreover, a better comprehension of the molecular mechanisms underlying the association between metabolic disorders and male fertility could help to identify novel targets to prevent and treat fertility disorders related to T1D.

KEYWORDS

spermatogenesis, steroidogenesis, blood-testis barrier, INSL3, RXFP2, SIRT1, inflammasome, apoptosis

Introduction

Reproduction is an essential feature of all living organisms, as it allows them to produce fertile offspring, ensuring the survival of their species. A key event of this process is gametogenesis, which is comprehensive of the development and maturation of specialized cells, spermatozoa (SPZ), and oocytes. During gametogenesis, germ cells (GC) go through diverse stages like mitosis, meiosis, and differentiation, and the whole progression is finely and complexly regulated by a multitude of elements, including the expression of stage-specific genes and hormones produced by the hypothalamus–pituitary–gonads axis as well as local signaling modulators between the somatic and germinal compartments (1).

Due to the intrinsic complexity underlying reproductive homeostasis, and the countless factors involved, gametogenesis may be quite easily compromised, drastically lowering gamete quality and their ability to fertilize/be fertilized. Disorders in gametogenesis can lead to subfertility/infertility, which is a condition that affects a significant portion of the global population. Male infertility accounts for approximately 50% of the cases (2, 3), and it is mainly manifested with poor-quality sperm in terms of quantity (oligozoospermia), morphology (globozoospermia), and motility (asthenozoospermia) (4, 5).

Although, on one hand, approximately 30% of male infertility cases are still considered idiopathic, a wide range of factors can be counted among the known causes of reduced fertility (6), including genetic and hormonal abnormalities, exposure to environmental pollutants (including heavy metals, endocrine disrupting chemicals, and microplastics) (7, 8), lifestyle (smoking, alcohol consumption, and overweight/obesity) (9), and different disease, including cancer (10), varicocele (11), and metabolic disorders (12, 13).

Among the latter, diabetes mellitus (DM) is one of the most threatening due to its meaningful association with mortality and morbidity (14). DM, a chronic disease characterized by hyperglycemia consequent to insulin deficiency (type 1 diabetes, T1D) and/or insulin resistance (type 2 diabetes), is often associated with metabolic syndrome (15). Indeed DM is characterized by several manifestations, such as dyslipidemia, hypertension, oxidative stress, chronic inflammation, mitochondrial dysfunction, and endoplasmic reticulum stress.

DM, as well as other metabolic disorders, including obesity (16–19), mainly acts via two principal mechanisms: increased oxidative stress and impaired hypothalamus–pituitary–testis (HPT) axis, which has been associated with the increasing rates of male sub-infertility by provoking damage in testicular somatic (Leydig and Sertoli cells) and GC, with consequently altered spermatogenesis and reduced sperm parameters (20). In particular, the hypoglycemic state is mainly responsible for reactive oxygen species (ROS) hyperproduction and, consequently, oxidative stress via the generation of hydroxyl radicals by the autoxidation of glucose as well as the overproduction of advanced glycation end products created by nonenzymatic reactions between sugar and amino groups of proteins (21). It has been well recognized that oxidative stress is one of the main causes of infertility, independently of its source (22), since it induces testicular germ and somatic cells to produce excessive ROS, overwhelming the endogenous ROS scavenging systems and causing damage to many cellular macromolecules and organelles (21). In this regard, SPZ are particularly susceptible to oxidative damage because of the considerable amount of polyunsaturated fatty acids (PUFA) in their plasma membrane and the low concentration of antioxidant enzymes and DNA repair system in the cytoplasm and nucleus, respectively, leading to a significant decrease in sperm count and semen quality (21).

The major impact of oxidative stress on fertility has also been highlighted by the fact that many intervention strategies to face infertility are just addressed to reduce oxidative stress—for example, using antioxidant molecules (23–31). In this regard, our recent research focuses on the cellular and differentiative mechanisms that regulate spermatogenesis, which was perturbed using experimental models of oxidative stress (32, 33), to expand the knowledge of the molecular pathways involved in spermatogenesis.

In this paper, using a rat model of T1D obtained by treatment with streptozotocin (STZ), we confirmed previous works (reviewed in (20, 34)) and evaluated several additional parameters of testicular activity. We focused the attention on both the somatic and germinal compartments of the testis since Leydig and Sertoli cells' dysfunction was assessed via the analysis of steroidogenesis and Leydig cell (LC) maturity using, as a marker, the insulin-like factor 3 (INSL3)–relaxin family peptide receptor 2 (RXFP2) system as well

as the blood–testis barrier (BTB) integrity. Mechanistically, because many reports demonstrated the association of SIRT1 (35, 36), NRF2 (37, 38), MAPKs (39, 40), and inflammasome (41) pathways with cellular function altered by oxidative stress, we further verified whether these pathways may also be involved in the molecular mechanisms underlying the T1D rat testicular dysfunction.

Materials and methods

Animals, treatment, and sample collection

Two-month-old male Wistar rats (*Rattus norvegicus*, $n = 10$), weighing 220 ± 18.97 g, were acclimated in individual stainless steel cages under controlled conditions of light (12-h light and 12-h dark cycles), temperature ($24 \pm 2^\circ\text{C}$), and humidity ($55 \pm 20\%$) for 1 week before the beginning of the experiments. The animals had free access to food and water *ad libitum*. The rats were divided into two groups: the control (C; $n = 5$) receiving 5% citrate buffer solution (#211018; AppliChem GmbH, Darmstadt, Germany) and the treated group (T1D; $n = 5$) which received a single i.p. injection (65 mg/kg body weight) of STZ (#18883–66–4; Chem Cruz Biochemicals; Huissen, Netherlands). The STZ solution was freshly prepared in a cold 0.1-M citrate buffer (pH = 4.5). Body weight and serum glucose levels were assessed before STZ administration (day 0) and once per week until the end of the experimental observations. Measurements of glucose concentration were obtained from whole blood samples taken from the rat tail vein. The parameter, expressed in milligrams per deciliter, was determined by using a standard clinical blood glucometer set for testing glycemia (#Glucomen LX2; A. Menarini Diagnostics, Winnersh, United Kingdom).

After 3 months, all the rats were anesthetized by an i.p. injection of chloral hydrate (#15307; Sigma Aldrich; Milan, Italy) and then killed with a lethal dose of urethane (2 g/kg; #94300; Sigma Aldrich; Milan, Italy). Considering that the STZ treatment lasted for 3 months, we used 2-month-old rats, which are considered adults, to avoid their excessive aging.

From each animal, the testes were dissected and washed in preheated phosphate-buffered saline (PBS; P3813; Sigma Aldrich; Milan, Italy), and the left testis was immersed in Bouin's solution (#HT10132; Sigma Aldrich; Milan, Italy), while the right ones were quickly frozen in liquid nitrogen and stored at -80°C for histological and biochemical analysis, respectively.

The experimental procedures were approved by the Animal Ethics Committee of the University of Campania “L. Vanvitelli” of Naples and by the Italian Ministry for Health (protocol number 30/2021). Animal care complied with the Italian (D.L. 116/92) and European Commission (O.J. of E.C. L358/1 18/12/86) regulations on the protection of laboratory animals. All efforts were made to reduce both the animal number and suffering during the experiments.

Histology

The fixed testes were dehydrated in increasing ethanol concentrations before embedding in paraffin. Then, 5- μm -thick

paraffin sections were stained with hematoxylin (#MHS1; Sigma Aldrich; Milan, Italy) and eosin (HT110216; Sigma Aldrich; Milan, Italy) for histological evaluation. Slides were examined with a Leica microscope (Leica DM 2500, Leica Microsystems, Wetzlar, Germany). Photographs were taken using Leica DFC320 R2 Digital Camera. For histopathological analysis, 30 seminiferous tubules/animal, for a total of 150 tubules per group, were counted.

Evaluation of testicular superoxide dismutase and catalase activities and of thiobarbituric acid-reactive species levels

The enzymatic activities of superoxide dismutase (SOD) and catalase (CAT) were measured following the methods of Marklund and Marklund (42) and Claiborne (43), respectively. Enzymatic activity was expressed as units per milligram of protein (U/mg of protein).

Testis lysates (see “Protein extraction and western blot analysis”) were used to determine the thiobarbituric acid-reactive species (TBARS) levels, following a previous paper (44). The results were expressed as TBARS $\mu\text{M}/\mu\text{g}$ of extracted protein. Each measurement was performed in triplicate.

Testicular testosterone level measurement

Testosterone (T) levels were determined in the testis of both groups using a commercial kit (#582701; Cayman Chemical Company, Michigan, MI, USA) and using a previously published protocol for the sample's treatment (45). Testicular T levels were expressed as ng/g of tissue.

Protein extraction and western blot analysis

For total protein extraction, each testis was homogenized directly in RIPA lysis buffer (#TCL131; Hi Media Laboratories GmbH; Einhausen, Germany) containing 10 $\mu\text{L}/\text{mL}$ of protease inhibitors mix (#39102; SERVA Electrophoresis GmbH; Heidelberg, Germany) and then centrifuged at 14,000 g for 20 min. For nuclear/cytoplasmic cell fractionation, each testis was homogenized in a buffer containing 0.5% Nonidet P40 (#85124; Thermo Fisher Scientific, Waltham, MA, USA) and incubated on ice for 5 min. The samples were then centrifuged at 500 g for 5 min. The supernatant, representing the cytoplasmic fraction, was collected and stored for subsequent analysis. The pellet containing the nuclear fraction was washed three times with the lysis buffer and then resuspended in the same buffer before further centrifugation at 17,500 g. The resulting pellet represents the nuclear fraction (46). Lowry method was used to determine the protein concentration.

In total, 40 μg of total/cytoplasmic/nuclear protein extracts was separated into SDS-PAGE (9%–15% polyacrylamide; #A4983; AppliChem GmbH, Darmstadt, Germany) and treated as

described in Venditti et al. (47). For details concerning all the primary and secondary antibodies used, see [Supplementary Table S1](#). The amount of protein was quantified using ImageJ software (version 1.53 t; National Institutes of Health, Bethesda, USA). Each western blot (WB) was performed in triplicate.

Immunofluorescence analysis

For immunolocalization analysis, 5- μ m testis sections were dewaxed, rehydrated, and processed as described by Venditti et al. (48). [Supplementary Table S1](#) reports the details about all the antibodies used. The slides were mounted with Vectashield + DAPI (#H-1200-10; Vector Laboratories, Peterborough, UK) for nuclear staining and then observed under an optical microscope (Leica DM 5000 B + CTR 5000; Leica Microsystems, Wetzlar, Germany) with a UV lamp. The images were analyzed and saved with IM 1000 software (version 4.7.0; Leica Microsystems, Wetzlar, Germany). Photographs were taken using the Leica DFC320 R2 digital camera. Densitometric analysis of immunofluorescence (IF) signal intensity and counting of positive cells was performed with the Fiji plugin (version 3.9.0/1.53 t) of ImageJ Software counting 30 seminiferous tubules/animal for a total of 150 tubules per group. Each IF was performed in triplicate.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

The apoptotic cells were identified in paraffin sections through the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using DeadEndTM Fluorometric TUNEL System (#G3250; Promega Corp., Madison, WI, USA) following the manufacturer's protocol, with little modifications (49). Briefly, before the incubation with TdT enzyme and nucleotide mix, sections were blocked with 5% BSA (#A1391; AppliChem GmbH, Darmstadt, Germany) and normal goat serum (#S26-M; Sigma Aldrich; Milan, Italy) diluted 1:5 in PBS and then treated with Peanut agglutinin (PNA) lectin, to mark the acrosome. Finally, the cell nuclei were counterstained with Vectashield + DAPI. The sections were observed with the same microscope described in Section 2.7. To determine the % of TUNEL-positive cells, 30 seminiferous tubules/animal for a total of 150 tubules per group, were counted using the Fiji plugin (version 3.9.0/1.53 t) of ImageJ Software. TUNEL assay was performed in triplicate.

Statistical analysis

The values were compared by a Student's t-test for between-group comparisons using Prism 8.0, GraphPad Software (San Diego, CA, United States). Values for $p < 0.05$ were considered statistically significant. All data were expressed as the mean $\pm$ standard error mean (SEM).

Results

Effects of T1D on oxidative stress

First, to verify the effectiveness of the STZ treatment and the onset of a diabetic state, glycemia and body weight were recorded. [Supplementary Figure S1A](#) shows that the serum glucose levels significantly increased in T1D rats compared to the control ($p < 0.0001$; [Supplementary Figure S1A](#)) soon after 1 week of STZ treatment. Concomitantly, a decrease in body weight was observed in T1D rats compared to the controls ($p < 0.0001$; [Supplementary Figure S1A](#)), particularly after 5 weeks of the treatment.

As reported in [Figure 1A](#), T1D induced oxidative stress since a significant increase of testicular TBARS levels, an index of lipid peroxidation, compared to the control ($p < 0.001$; [Figure 1A](#)) was observed. This data was supported by the analysis of the activity of the antioxidant enzymes SOD ($p < 0.001$; [Figure 1B](#)) and CAT ($p < 0.01$; [Figure 1B](#)) compared to the control. In addition, SOD ($p < 0.05$; [Figures 1C, D](#)) and CAT ($p < 0.05$; [Figures 1C, E](#)) protein levels were downregulated in the testis of T1D rats compared to the controls.

The oxidative stress induced by T1D was further analyzed by measuring the testicular levels and localization of 4-hydroxynonenal (4-HNE), one of the most biologically relevant products of lipid peroxidation that can react with proteins to form 4-HNE-protein adducts (50).

The WB analysis showed that the 4-HNE levels were significantly higher in the testes of T1D rats than those of the control group ($p < 0.001$; [Figure 1F](#)). The IF analysis ([Figure 1G](#)) showed that control testis exhibited scattered 4-HNE-positive cells, specifically spermatocytes (SPC; arrowhead and inset) and Sertoli cells (SC; striped arrow). Contrarily, in the testis of T1D rats, the signal appeared, other than in the abovementioned cells, in spermatogonia (SPG; arrow), spermatids (SPT; dotted arrow), in the tail of luminal SPZ (triangle) as well as in the interstitial LC (asterisk). Fluorescence intensity analysis showed an evident increase of 4-HNE signal in the T1D group compared to the control ($p < 0.001$; [Figure 1H](#)).

Effect of T1D on apoptosis

[Figure 2](#) shows the effect of T1D on the apoptotic rate of germ and somatic cells. The WB analysis revealed an increase in p53 ($p < 0.01$; [Figures 2A, B](#)), Bax/Bcl-2 ratio ($p < 0.05$; [Figures 2A, C](#)), cytochrome c ($p < 0.01$; [Figures 2A, D](#)), and caspase-3 ($p < 0.01$; [Figures 2A, E](#)) protein levels in the T1D group compared to the control.

To support these data, a TUNEL assay was performed ([Figure 2F](#)). The findings revealed the presence of scattered apoptotic cells in the control group, mainly SPG (arrow; [Figure 2F](#)) and SPC (arrowhead; [Figure 2F](#)). T1D resulted in 112% increase in the number of TUNEL-positive cells ($p < 0.001$;

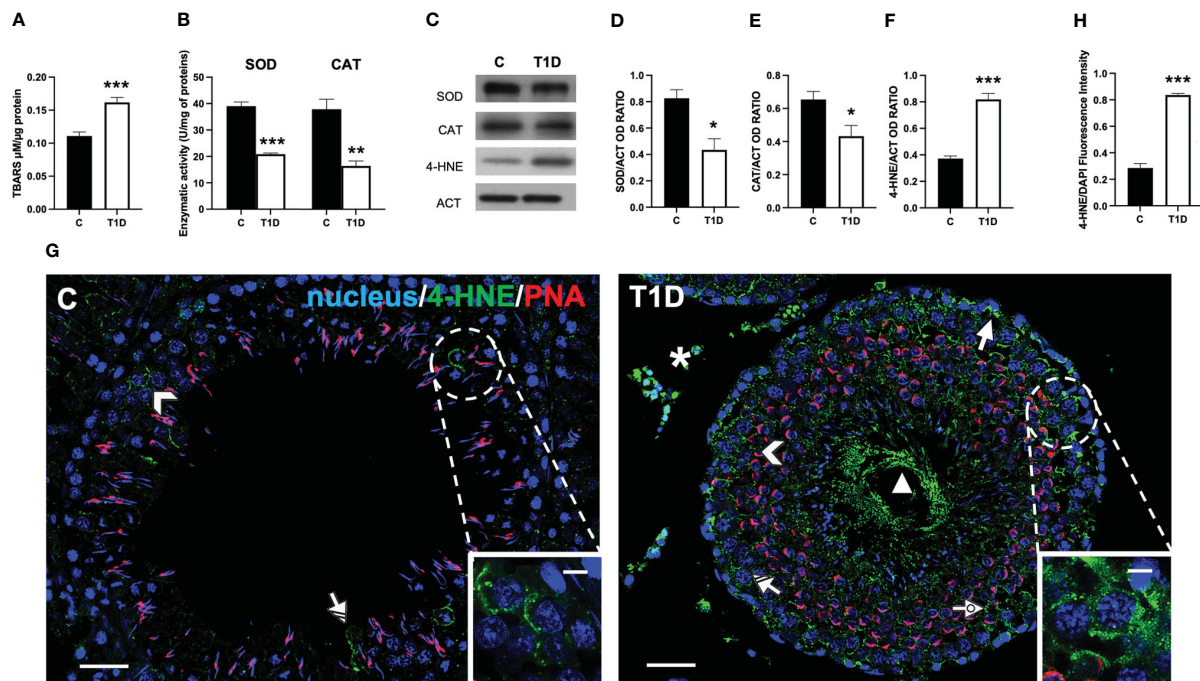


FIGURE 1

Analysis of oxidative stress parameters in control and T1D rat testis. **(A)** Testicular TBARS levels and **(B)** SOD and CAT enzymatic activities. **(C)** WB analysis of testicular SOD, CAT, and 4-HNE protein levels. **(D–F)** Histograms showing SOD, CAT, and 4-HNE relative protein levels, quantified using ImageJ and normalized to β -actin. **(G)** Testicular 4-HNE (green) immunolocalization. The slides were counterstained with PNA (red) and DAPI-fluorescent nuclear staining (blue). The images were captured at $\times 20$ (scale bars = 20 μ m) magnification and $\times 40$ (scale bars = 10 μ m) for the insets. Arrows, SPG; arrowheads, SPC; dotted arrows, SPT; triangle, SPZ tails; striped arrows, SC; asterisks, LC. **(H)** Histogram showing the quantification of 4-HNE fluorescence signal intensity. All values are expressed as means $\pm$ SEM from five animals in each group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

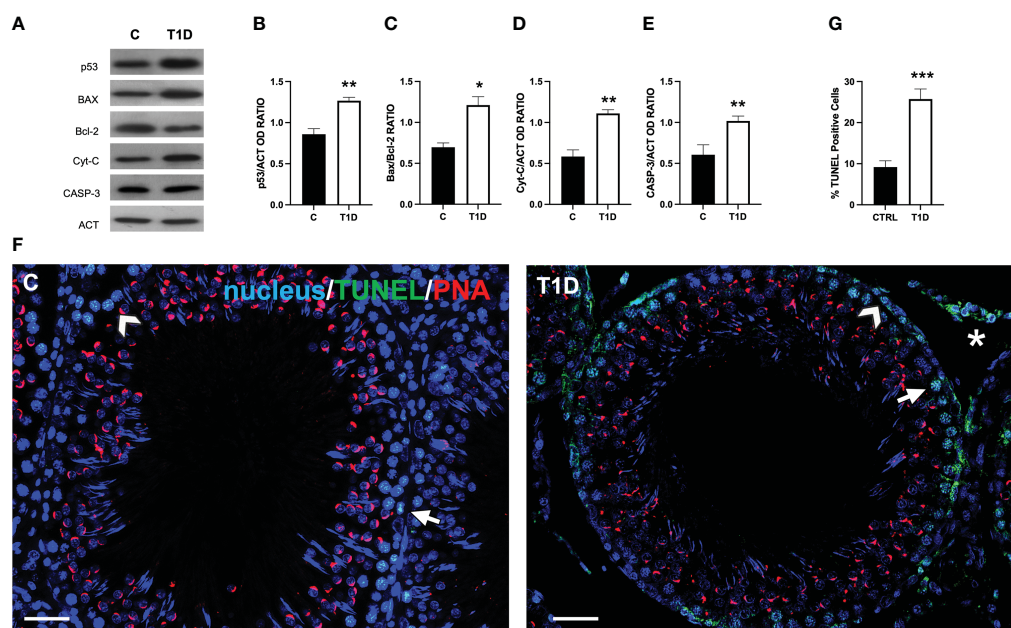


FIGURE 2

Analysis of apoptotic rate in control and T1D rat testis. **(A)** WB analysis of testicular p53, Bax, Bcl-2, cytochrome-C, and caspase-3. **(B–E)** Histograms showing the p53, Bax/Bcl-2 ratio, cytochrome-C, and caspase-3 relative protein levels quantified using ImageJ and normalized to β -actin. **(F)** Determination of apoptotic cells through the detection of TUNEL-positive cells (green). The slides were counterstained with PNA lectin (red) and with DAPI-fluorescent nuclear staining (blue). The images were captured at $\times 20$ (scale bars = 20 μ m) magnification. Arrows, SPG; arrowheads, SPC; asterisks, LC. **(G)** Histogram showing the percentage of TUNEL-positive cells. All the values are expressed as means $\pm$ SEM from five animals in each group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figures 2F, G), particularly of SPG, and LC in the interstitial compartment in comparison to the control.

Effects of T1D on the interstitial LC

Given the presence of apoptotic LC in the interstitial compartment of T1D rat testis, steroidogenesis was evaluated. Figure 3 shows that the testicular T levels in T1D rats were significantly reduced by about 54% compared to the controls ($p < 0.01$; Figure 3A). The effects of T1D on testicular steroidogenesis was also evaluated by analyzing the protein levels of steroidogenic acute regulatory protein (StAR), 3 β -hydroxysteroid dehydrogenase (3 β -HSD), cytochrome P450 17A1 (CYP17A1), and cytochrome P450 19A1 (CYP19A1) enzymes involved in T biosynthesis. The WB analysis confirmed that T1D altered the testicular hormonal milieu, as a decrease in StAR ($p < 0.05$; Figures 3B, C), 3 β -HSD ($p < 0.01$; Figures 3B, D), CYP17A1 ($p < 0.05$; Figures 3B, E), and CYP19A1 ($p < 0.01$; Figures 3B, F) protein levels, compared to the control, was observed. The impact of T1D on steroidogenesis was further confirmed by IF staining of StAR and 3 β -HSD, which is shown in Figure 3G. The two signals specifically localized into the interstitial LC (asterisk; Figure 3G and insets) in both groups; however, fluorescence intensity analysis showed a weaker signal for StAR and 3 β -HSD in T1D ($p < 0.01$; Figures 3H, I) compared to the control.

Finally, since the above-mentioned results clearly showed that T1D negatively impacts steroidogenesis occurring in LC, we

analyzed the protein level of INSL3, a small peptide hormone secreted by mature LC that reflects their differentiation and number in the testis of all mammals and of its receptor, RXFP2 (51). The WB analysis showed that both INSL3 ($p < 0.01$; Figures 3J, K) and RXFP2 ($p < 0.05$; Figures 3J, L) were downregulated in the T1D testis compared to the controls.

Effect of T1D on spermatogenesis

As shown in Supplementary Figure S2A, the testis from control rats exhibited a normal histological organization, as evidenced by GC in all the differentiative stages, including mature SPZ filling the lumen as well as LC and integral blood vessels in the interstitial compartment. The testes from T1D rats showed an altered structure, characterized by a deficiency of connections between GC (arrowhead) and, therefore, the presence of many empty spaces between them; in addition, the interstitial tissue was also affected since it appeared shrunken and the LC quite dispersed (asterisk). As shown in Supplementary Figures S2B, C, the analysis of two morphometric parameters further supported the histological results, as the tubules' diameter ($p < 0.001$; Supplementary Figure S2B), and the percentage of tubules' lumens full of SPZ ($p < 0.001$; Supplementary Figure S2C) were lower in the T1D group than in the controls.

To assess the effects of T1D on spermatogenesis, the protein levels of proliferating cell nuclear antigen (PCNA), phospho-histone H3 (p-H3), and synaptonemal complex protein 3 (SYCP3) were examined (Figures 4A–D). T1D provoked a significant decrease in

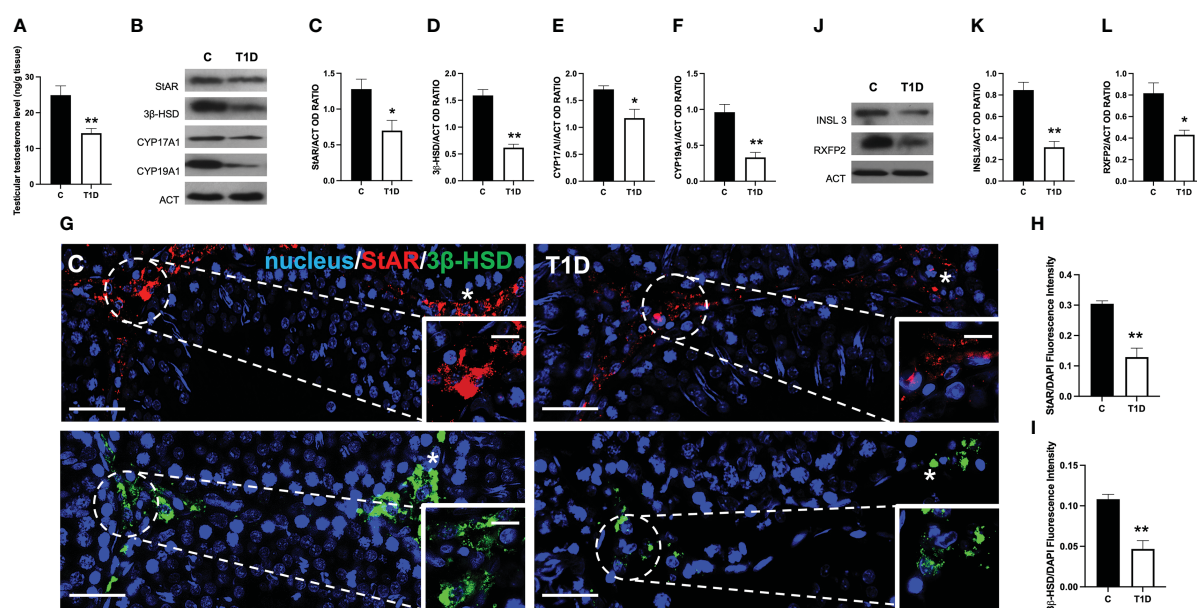


FIGURE 3

Analysis of LC activity in control and T1D rat testis. (A) Testicular Testosterone levels and (B) WB analysis of StAR, 3 β -HSD, CYP17A1, and CYP19A1 protein levels. (C–F) Histograms showing StAR, 3 β -HSD, CYP17A1, and CYP19A1 relative protein levels quantified using ImageJ and normalized to β -actin. (G) Testicular StAR (red) and 3 β -HSD (green) immunolocalization. The slides were counterstained with DAPI-fluorescent nuclear staining (blue). The images were captured at $\times 20$ (scale bars = 20 μ m) magnification and $\times 40$ (scale bars = 10 μ m) for the insets. Asterisks, LC. (H, I) Histogram showing the quantification of StAR and 3 β -HSD fluorescence signal intensity, respectively. (J) WB analysis of INSL3 and RXFP2 protein levels. (K, L) Histograms showing INSL3 and RXFP2 relative protein levels quantified using ImageJ and normalized to β -actin. All values are expressed as means $\pm$ SEM from five animals in each group. * $p < 0.05$; ** $p < 0.01$.

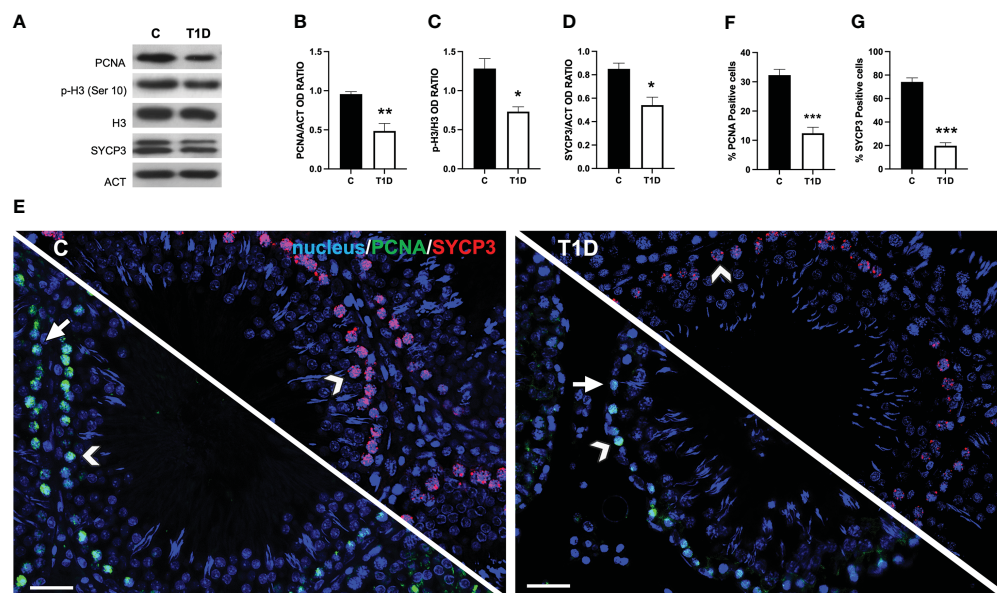


FIGURE 4

Analysis of spermatogenesis in control and T1D rat testis. (A) WB analysis of testicular PCNA, p-H3, H3, and SYCP3. (B–D) Histograms showing PCNA the p-H3/H3 ratio and SYCP3 relative protein levels quantified using ImageJ and normalized to β -actin. (E) Testicular PCNA (green) and SYCP3 (red) immunolocalization. The slides were counterstained with DAPI-fluorescent nuclear staining (blue). The images were captured at $\times 20$ magnification (scale bars = 20 μ m). Arrows, SPG; arrowheads, SPC. (F, G) Histograms showing the percentage of PCNA- and SYCP3-positive cells, respectively. All the values are expressed as means $\pm$ SEM from five animals in each group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

PCNA ($p < 0.01$; Figures 4A, B), p-H3 ($p < 0.05$; Figures 4A, C), and SYCP3 ($p < 0.05$; Figures 4A, D) protein levels compared to the controls.

Data coming from IF labeling of PCNA (green panel; Figure 4E) and SYCP3 (red panel; Figure 4E) showed a PCNA-specific

localization in the SPG (arrows) and SPC (arrowheads) in the testis of both groups; however, in T1D, a decrease of approximately 88% in PCNA-positive cells ($p < 0.001$; Figure 4F) was observed. Concerning SYCP3, it localized in the SPC nucleus (arrowheads; Figure 4E), and the percentage of SYCP3-positive cells decreased by

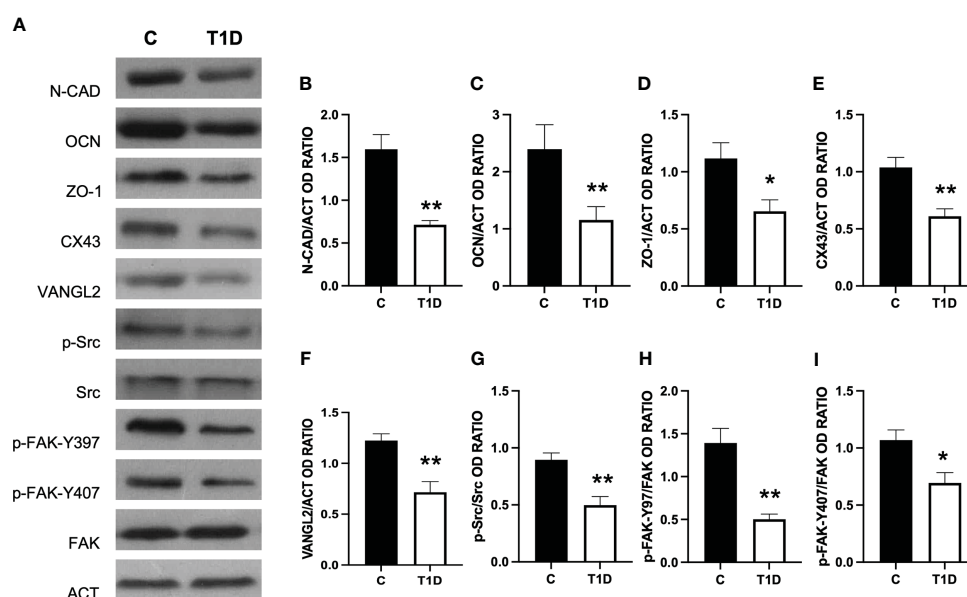


FIGURE 5

Analysis of blood–testis barrier markers in control and T1D rat testis. (A) WB analysis of testicular N-CAD, OCN, ZO-1, CX43, VANG2, p-Src, Src, p-FAK-Y397, p-FAK-Y407, and FAK in the testes of T1D animals. (B–I) Histograms showing N-CAD, OCN, ZO-1, CX43, and VANG2 relative protein levels and p-Src/Src, p-FAK-Y397/FAK, and p-FAK-Y407/FAK ratios quantified using ImageJ and normalized to β -actin. All the values are expressed as means $\pm$ SEM from five animals in each group. * $p < 0.05$; ** $p < 0.01$.

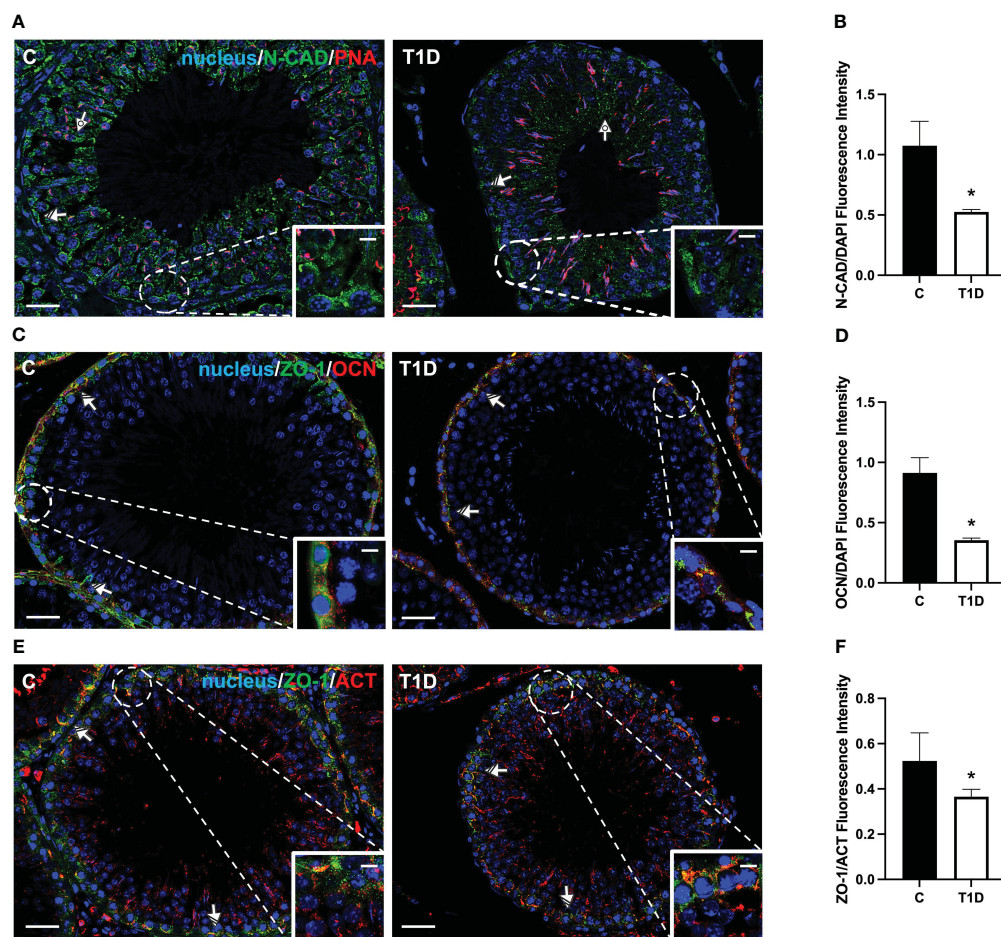


FIGURE 6

Immunofluorescent analysis of N-CAD, ZO-1, and OCN in control and T1D rat testis. **(A)** Testicular N-CAD (green) immunolocalization. The slides were counterstained with PNA lectin (red) and DAPI-fluorescent nuclear staining (blue). **(C)** Testicular ZO-1 (green) and OCN (red) immunolocalization. **(E)** Testicular ZO-1 (green) and β -actin (red) immunolocalization. All the slides were counterstained with DAPI-fluorescent nuclear staining (blue). All the images were captured at $\times 20$ (scale bars = $20\ \mu\text{m}$) magnification and $\times 40$ (scale bars = $10\ \mu\text{m}$) for the insets. Dotted arrows, SPT; striped arrows, SC. **(B, D, F)** Histograms showing the quantification of N-CAD, OCN, and ZO-1 fluorescence signal intensity, respectively. All the values are expressed as means $\pm$ SEM from five animals in each group. * $p < 0.05$.

115% in the T1D group compared to the control ($p < 0.001$; Figure 4G).

Effect of T1D on BTB integrity markers

T1D provoked considerable alterations in the BTB at both structural and regulatory proteins compared to control groups (Figures 5–7). Indeed T1D induced a significant reduction in the protein levels of N-cadherin (N-CAD; $p < 0.01$; Figures 5A, B), occludin (OCN; $p < 0.01$; Figures 5A, C), zonula occludens-1 (ZO-1; $p < 0.05$; Figures 5A, D), connexin 43 (CX43; $p < 0.01$; Figures 5A, E), and Van Gogh-Like 2 (VANG2; $p < 0.01$; Figures 5A, F) as well as in the phosphorylation status of p-Src ($p < 0.01$; Figures 5A, G), p-FAK-Y397 ($p < 0.01$; Figures 5A, H), and p-FAK-Y407 ($p < 0.05$; Figures 5A, I) compared to the control.

For a more comprehensive description of the effects exerted by T1D on N-CAD, OCN, ZO-1 (Figure 6), CX43, and VANG2 (Figure 7), an IF analysis was carried out. N-CAD, a protein

participating in the formation of cell adhesion complexes in BTB (52), localized at the SC interface (striped arrow; Figure 6A), as well as in their cytoplasmic extensions through the luminal compartment, connected to the heads of elongated SPT (dotted arrow; Figure 6A). It is worth noting that, in the testis of T1D rats, the N-CAD signal appeared less intense in both the basal and luminal compartments, where it was quite diffuse ($p < 0.05$; Figures 6A, B).

OCN (Figure 6C) and ZO-1 (Figure 6E) are a transmembrane membrane and a peripheral protein, respectively, connecting tight junction (TJ) components to the actin cytoskeleton (53). They specifically localized in the SC cytoplasm (striped arrow; Figures 6C, E; insets) in the two groups; however, the signal intensity decreased in the T1D rats ($p < 0.05$; Figures 6D, F) compared to the control.

CX43 is the principal testicular gap-junction protein allowing for intracellular communication (54). IF data showed that it localized in both GC, particularly in SPG (arrows; Figure 7A), and SPC (arrowheads; Figure 7A; insets), granting their synchronous differentiation, as well as in the basal cytoplasm of

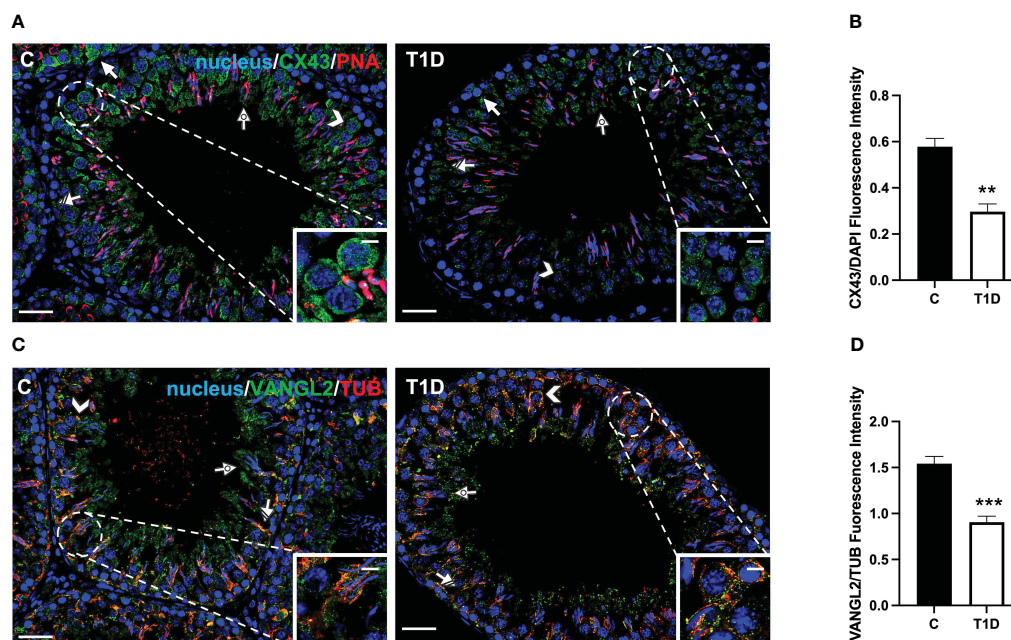


FIGURE 7

Immunofluorescent analysis of CX43 and VANGL2 in control and T1D rat testis. (A) Testicular CX43 (green) immunolocalization; the slides were counterstained with PNA lectin. (C) Testicular VANGL2 (green) and α -tubulin (red) immunolocalization. All the slides were counterstained with DAPI-fluorescent nuclear staining (blue). All the images were captured at x20 (scale bars = 20 μ m) magnification and x40 (scale bars = 10 μ m) for the insets. Arrows, SPG; arrowheads, SPC; dotted arrows, SPT; striped arrows, SC. (B, D) Histograms showing the quantification of CX43 and VANGL2 fluorescence signal intensity, respectively. All the values are expressed as means $\pm$ SEM from five animals in each group. ** $p < 0.01$; *** $p < 0.001$.

adjacent SC (striped arrow; Figure 7A) and in their protrusions surrounding the elongating SPT (dotted arrows; Figure 7A). T1D provoked an evident reduction in the fluorescent intensity in SC and GC compared to the control ($p < 0.01$; Figure 7B).

Lastly, VANGL2, a transmembrane protein belonging to the planar cell polarity family, controls the temporal and spatial cytoarchitecture at the basal and apical ectoplasmic specialization (ES) (55, 56). In the control testis, VANGL2 is expressed in SPC (arrowheads; Figure 7C) and, in co-localization with tubulin, in the SC cytoplasm (striped arrows; Figure 7C), and their distinct protrusions were extending toward the lumen and surrounding the SPT/SPZ heads (dotted arrows; Figure 7C, inset). In the T1D group, though VANGL2 localized in the cell types as mentioned above (Figure 7C), a weaker immunofluorescent signal was observed ($p < 0.001$; Figure 7D). Interestingly, this may also have effects on tubulin organization since microtubules partially lose their ordered, linear structure across the seminiferous epithelium.

Effect of T1D on testicular SIRT1/NRF2/MAPKs pathways

Considering that T1D induces testicular oxidative stress, as previously demonstrated and herein confirmed, we decided to further investigate some well-known molecular mechanisms involved in the cellular response to oxidative stress, such as the SIRT1/NRF2/MAPKs pathways (57–60). The results showed a reduction in SIRT1 protein level in T1D rat testis compared to the control ($p < 0.05$; Figures 8A, B); however, no significant

changes in FOXO1 levels were observed (Figures 8A, C). Regarding KEAP1, its protein expression significantly increased in the T1D group ($p < 0.01$; Figures 8A, D), while those of HO-1 ($p < 0.001$; Figures 8A, E) decreased compared with the control. Finally, the phosphorylation status of p38 ($p < 0.001$; Figures 8A, F) and JNK ($p < 0.001$; Figures 8A, G), was upregulated in the testis of the T1D group compared to the control.

To evaluate how T1D can modulate NRF2 nuclear translocation, we proceeded with a WB analysis performed on separated cytosolic and nuclear fractions. The results showed that T1D induced an increase of NRF2 in the cytoplasmic fraction ($p < 0.01$; Figures 8H, I), while its levels did not change in the nuclear fraction between the two groups.

To confirm these data, double-immunostaining of SIRT1 and NRF2 in the two groups was performed. In the control testis, SIRT1 specifically localized in SPG (arrow; Figure 8J), SPC (arrowhead; Figure 8J), and SPT (dotted arrow; Figure 8J and insets) nucleus. As for NRF2, it was present in the cytoplasm of the same cells (Figure 8J). In the testis of T1D rats, the intensity of the two signals showed an opposite behavior since it was higher for NRF2 ($p < 0.001$; Figures 8J, K) and weaker for SIRT1 ($p < 0.05$; Figures 8J, L), particularly in SPC (arrowhead; Figure 8J) and SPG (arrow; Figure 8J), respectively.

Effect of T1D on testicular inflammation and pyroptosis

To assess whether a T1D induced testicular inflammation and/or pyroptosis, several markers were evaluated. The results showed

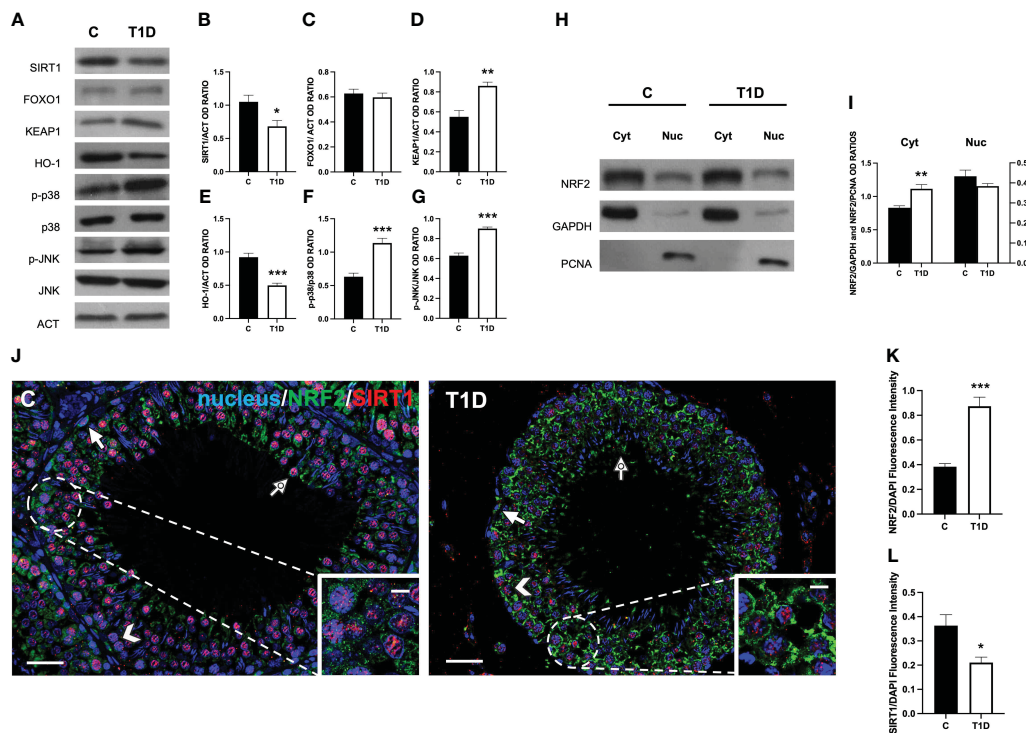


FIGURE 8

Analysis of SIRT1/NRF2/MAPKs pathways in control and T1D rat testis. (A) WB analysis of testicular SIRT1, FOXO1, KEAP1, HO-1, p-p38, p38, p-JNK, and JNK. (B–G) Histograms showing SIRT1, FOXO1, KEAP1, and HO-1 relative protein levels and p-p38/p38, and p-JNK/JNK ratios quantified using ImageJ and normalized to β -actin. (H) WB analysis of testicular NRF2 protein levels in cytoplasmic (Cyt) and nuclear (Nuc) fractions. (I) Histograms showing NRF2 relative protein levels quantified using ImageJ and normalized to GAPDH or PCNA. (J) Testicular NRF2 (green) and SIRT1 (red) immunolocalization. The slides were counterstained with DAPI-fluorescent nuclear staining (blue). The images were captured at $\times 20$ (scale bars = 20 μ m) magnification and $\times 40$ (scale bars = 10 μ m) for the insets. Arrows, SPG; arrowheads, SPC; dotted arrows, SPT. (K, L) Histograms showing the quantification of NRF2 and SIRT1 fluorescence signal intensity, respectively. All the values are expressed as means $\pm$ SEM from five animals in each group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

an increase in NF- κ B ($p < 0.001$; Figures 9A, B), IL-6 ($p < 0.01$; Figures 9A, C), NLRP3 ($p < 0.001$; Figures 9A, D), and caspase 1 ($p < 0.001$; Figures 9A, E).

As a confirmation of the above-mentioned data, an IF analysis of NF- κ B (Figure 9F) and NLRP3 (Figure 9H) was carried out. In the control testis, although the intensity of the signal was quite scarce, NF- κ B localized in the cytoplasm of SPG (arrow; Figure 9F), SPC (arrowhead; Figure 9F), SC (striped arrow; Figure 9F and insets), and interstitial LC (asterisk; Figure 9F). In the T1D testis, NF- κ B retained its localization in the cytoplasmic compartment of the cells mentioned above, showing a more intense fluorescent signal ($p < 0.001$; Figures 9F, G) compared to the control. It is worth noting that a nuclear signal in SPC (dotted arrow; Figure 9F) was also detected in T1D animals.

As for NLRP3, we obtained the most interesting, and quite unexpected, data. Indeed in the control testis, it localized in scattered cells, as SPC (arrowhead; Figure 9H) and at the center of the developing acrosome of round SPT, at the cap phase of acrosome biogenesis (dotted arrow and upper inset; Figure 9H), while it was absent in elongating SPT at the acrosome/maturation phase (lower inset; Figure 9H). In the T1D testis, NLRP3 was expressed in SPC (arrowhead; Figure 9H) and associated with the acrosome in round SPT (dotted arrow and upper inset; Figure 9H), showing a more extended and fluorescent intensity ($p < 0.001$;

Figure 9I) compared to the controls. In this case, NLRP3 was also absent in elongating SPT (lower inset; Figure 9H).

Discussion

Spermatogenesis is the well-ordered sequence of distinctive cellular phases, in which SPG gives rise to SPT via mitotic and meiotic divisions that, in turn, differentiate into SPZ through spermiogenesis. Such events are at the basis of the intrinsic complexity and delicacy of spermatogenesis, which also relies on the fact that many involved molecules (i.e., ions and hormones) play a dual role, depending on their relative concentration (61, 62), and among them, ROS are of primary importance. It should be remembered that endogenous ROS are mainly generated by mitochondrial cellular respiration, and during spermatogenesis, they act as signaling molecules, activating key pathways and playing physiological roles in HPT and SPZ function (63). Conversely, excessive ROS production, together with the compromised functionality of enzymatic and non-enzymatic antioxidant defenses, drives the cellular redox status toward a pro-oxidant environment, which damages the normal structure and activity of macromolecules and organelles (64).

It is well established that T1D, together with other metabolic disorders, is a major source of oxidative stress since hyperglycemia

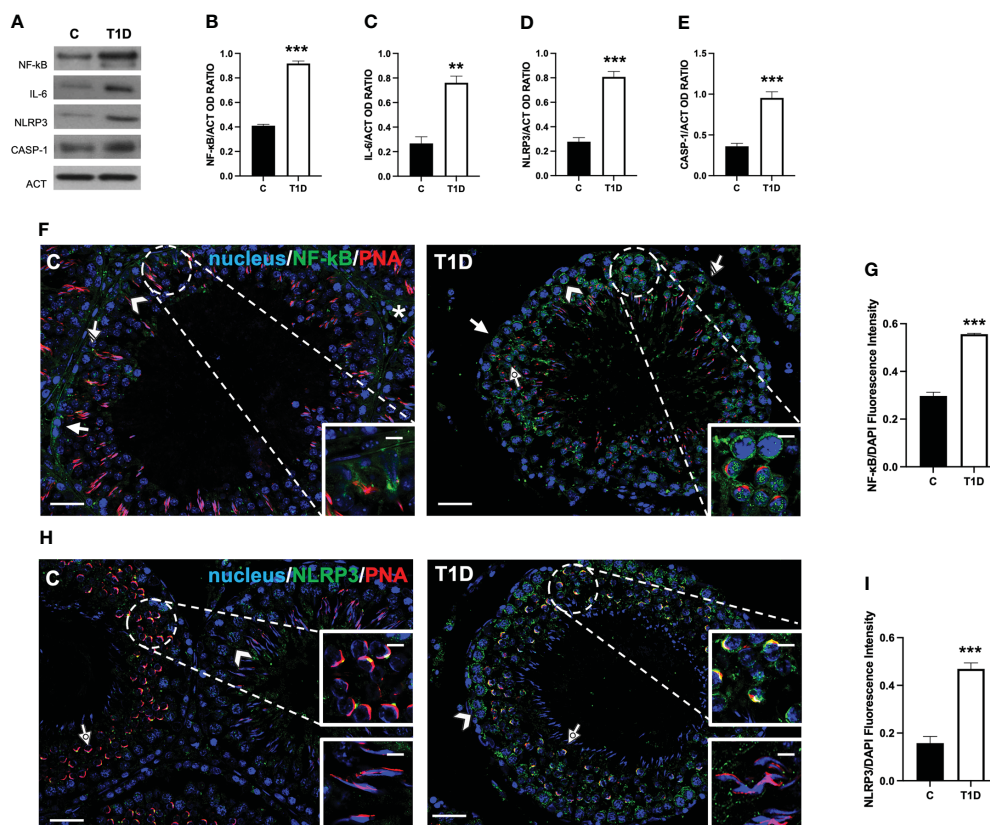


FIGURE 9

Analysis of pyroptosis in control and T1D rat testis. (A) WB analysis of testicular NF-κB, IL-6, NLRP3, and caspase-1. (B–E) Histograms showing NF-κB, IL-6, NLRP3, and caspase-1 relative protein levels quantified using ImageJ and normalized to β-actin. (F) Testicular NF-κB (green) immunolocalization. (H) Testicular NLRP3 (green) immunolocalization. All the slides were counterstained with PNA lectin (red) and with DAPI-fluorescent nuclear staining (blue). The images were captured at x20 (scale bars = 20 μm) magnification and x40 (scale bars = 10 μm) for the insets. Arrows, SPG; arrowheads, SPC; dotted arrows, SPT. (G, I) Histograms showing the quantification of NF-κB and NLRP3 fluorescence signal intensity, respectively. All the values are expressed as means ± SEM from five animals in each group. ***p* < 0.01; ****p* < 0.001.

activates “abnormal” pathways that promote ROS overproduction in many human tissues, testis included (65). In the last decades, the global incidence of T1D has increased, and although it has been usually considered to be a disease with onset during childhood, more adults than children are diagnosed each year (66).

In this paper, we first validated the successfulness of STZ treatment on testicular activity by verifying the typical features of T1D testis. Particularly, we confirmed the establishment of an oxidative stress status, as evidenced by the increased TBARS concentration, an indirect marker of lipid peroxidation, as well as the reduced activity and protein levels of the antioxidant enzymes SOD and CAT. Further evidence of this status was provided by the increase of 4-HNE, an end-product of lipid peroxidation that, reacting with proteins and DNA, can generate numerous forms of adducts (67), and due to this, it is a suitable parameter of oxidative stress. IF data also revealed that 4-HNE localized in all the cells composing the seminiferous epithelium as well as in interstitial LC. Quite expectedly, the most 4-HNE-reactive cells appeared to be the luminal SPZ since they are particularly susceptible to ROS due to inadequate antioxidant defenses and also to the presence of a high quantity of PUFA in their plasma membrane, which can be easily oxidized (68). Because of the increased oxidative stress, the results

confirmed an intensified apoptotic rate in both somatic and germ cells, suggested by the increased levels of pro-apoptotic proteins as well as of an enhanced number of TUNEL-positive cells (approximately 110%) in T1D testis.

Considering that many apoptotic LC in the interstitial compartment were observed, we examined the impact of T1D on their functionality and maturity by analyzing steroidogenesis and INSL3/RXFP2, respectively. The results indicated that testicular steroidogenesis was compromised, as evidenced by the reduced testicular T levels and impaired expression of several enzymes involved in its biosynthesis. It is worth remembering that INSL3 is a hormone specifically produced and secreted by LC, uncontrolled by the HPT axis, that provides information on their differentiation status and number (51); conversely, RXFP2 is INSL3’s receptor and is expressed mainly in testicular post-meiotic cells (69). In addition, several studies demonstrated that INSL3, by activating RXFP2, may regulate GC turnover and apoptosis, especially when spermatogenesis is under stress conditions (70), as that induced by T1D.

As discussed above, both T and INSL3 regulate indispensable steps for the normal progression of spermatogenesis; thus, their impaired levels (together with the induced apoptosis) are reflected

by the reduced expression of mitotic (p-H3; PCNA) and meiotic (SYCP3) markers as well as by the reduction of 88% and 115% PCNA- and SYCP3-positive cells, respectively. The combined data led us to hypothesize that T1D, through the induction of oxidative stress and disturbance of LC activity, reduces their ability to produce T and INSL3, which has a direct effect on spermatogenesis, as evidenced by the reduction in kinetic/dynamic activity of GC.

It is well known that during the progression leading SPG to differentiate into SPZ, which will be released into the tubules' lumen, cells are actively transported thanks to their intimate connection with SC, the somatic component of the seminiferous epithelium. Indeed SC creates a unique structure, the BTB, which divides the epithelium into two compartments: basal, where SPG and preleptotene SPC are located, and an apical one, in which all the other cell types reside. In addition, BTB is responsible for the establishment of an immune-privileged environment, allowing the testes to tolerate the antigenic factors expressed by the SPZ, protecting them from systemic immune attack (71). BTB is an extremely dynamic structure, placed between adjacent SC and SC-GC, and is based on structural, scaffolding, and signaling proteins that work coordinately through cycles of phosphorylation/dephosphorylation, endocytosis of membrane proteins, and their recycling to ensure the correct movement of GC (53).

Herein we confirmed that in the testis of T1D rats, the protein levels of N-CAD, ZO-1, OCN, and CX43 were reduced (72, 73). However, to our knowledge, this is the first report showing that T1D also affects the testicular levels of VANGL2, a protein located at the apical ES, as well as the activation of Src and FAK. VANGL2 regulates the spatial and temporal organization of actin microfilaments and microtubules, playing a significant role in controlling BTB integrity and SPT transit toward the lumen (56). IF data revealed an altered distribution of VANGL2 as well as that of tubulin in the SC cytoplasmic protrusions, strongly suggesting that T1D may also act on the cytoskeletal organization of testicular cells.

Regarding Src and FAK, their tandem phosphorylation allows interaction with other BTB components, such as OCN and ZO-1, regulating the GC transit and maintaining BTB integrity. Thus, as also previously demonstrated by other papers in T1D rodents (72, 73), we confirmed that BTB is compromised, emphasizing that its stability is fundamental for proper spermatogenesis. However, as a limitation of this study, these are indirect data, and an *in vivo* BTB integrity assay would offer direct evidence, solidifying the claim.

For a broader picture of the underlying molecular pathways of the T1D effects on testicular activity, we analyzed the contribution of some key factors notoriously involved in the oxidative stress response, namely, SIRT1, NRF2, and the MAPKs p38/JNK. The transcription factor NRF2 represents the central hub of this system since it is an adaptive cellular defense response to different stresses, including oxidative and metabolic ones (74). In normal conditions, NRF2 is "sequestered" in the cytoplasm by KEAP1, a ubiquitin E3 ligase, which promotes its degradation through ubiquitination; when an oxidative insult hits cells, the increased ROS production leads to NRF2 accumulation, its nuclear translocation, and the activation of ARE-dependent genes, including the antioxidant enzymes HO-1 and SOD (74). The regulation of NRF2 activity is multifaced since it has been demonstrated by previous reports that

other events may be involved, such as NRF2 deacetylation, promoted by SIRT1 (75), and/or its phosphorylation, induced by MAPKs (76). Herein we found that T1D downregulated testicular SIRT1 expression, while KEAP1 protein level and the phosphorylation status of p38 and JNK were upregulated. This, in turn, may lead to the deregulation of NRF2 signaling that, not translocating toward the nucleus, is not able to activate the cellular antioxidant defenses.

Worthy of note is that NRF2 seems to be a vital regulator of the molecular mechanisms that cause male infertility. In fact, several studies suggest that the function of NRF2 plays a crucial role in protecting spermatogenesis when an excessive amount of ROS is produced (77) as in the case of exposure to environmental pollutants (78, 79), aging (80), and metabolic disorder (17, 81). This protection, in turn, preserves specific sperm functions, particularly motility, which could otherwise be compromised under conditions of increased ROS exposure. This point is of interest since many intervention strategies, aimed to ameliorate/counteract prooxidant stimuli posed by different etiologies (such as disease or exposure to environmental pollutants), have as a target just the activation of the SIRT1/NRF2 pathways (77, 82).

All metabolic disorders, T1D included, are characterized by an intimate relationship between oxidative stress and inflammation: via the generation of a vicious cycle, oxidative stress stimulates inflammatory pathways, which, in turn, enhance ROS overproduction (83, 84). Due to this, we analyzed whether the induction of testicular inflammation was elicited by T1D. As well known, the principal actor involved in inflammation activation is transcription factor NF- κ B since most of the genes encoding for pro-inflammatory cytokines possess, in their promoter/enhancer regions, NF- κ B-binding sites (85). Here we found that T1D increased the testicular expression of both NF- κ B and the pro-inflammatory cytokine IL-6, thus confirming the activation of the inflammatory pathway.

NLRP3 is a gene under NF- κ B transcriptional control, the main component of the inflammasome (86). Inflammasome is a cytosolic, multiprotein complex which, once assembled, elicits the proteolytic cleavage and then activation of procaspase-1 into active caspase 1 that also induces the activation of other proinflammatory mediators, such as IL-1 β , or a specific proinflammatory cell death defined as pyroptosis (87). Our results showed that T1D also activated inflammasome, as well as testicular pyroptosis, probably in response to enhanced oxidative stress and inflammation, contributing to apoptosis of germ and somatic cells. These results are partially in line with other reports showing that oxidative status, induced by obesity (88), varicocele (89), and exposure to toxicants (90, 91), enhanced the expression of NLRP3 and of the downstream pathways, leading to testicular pyroptosis, inflammation, and, ultimately, to testicular dysfunction. However, in most of those studies, the main cellular source of NLRP3 production appeared to be the SC, and thus its correlation with perturbation with BTB integrity has been hypothesized; this point was supported by the fact that inhibiting NLRP3 pathway increased the expression of TJ components, consequently ameliorating the BTB integrity (92, 93).

Interestingly, although we could not detect any signal in SC cytoplasm, IF data revealed a peculiar NLRP3 localization in both control and T1D testis, indeed it was associated with the developing

acrosome system in the control, assuming a dot-shaped conformation in round SPT while, in T1D, its extension and signal intensity were increased, particularly in SPT, where a clear nuclear NF- κ B signal was also observed. Considering that acrosome biogenesis involves the generation of Golgi-derived vesicles (94) and that the Golgi apparatus seems fundamental for the recruitment and activation of NLRP3 (95, 96), we hypothesize that NLRP3 may also be somehow involved in the formation of this organelle. However, although fascinating, this hypothesis needs more in-depth investigation, and in-progress studies are focused just to clarify this aspect.

Conclusion

This study confirms that STZ-induced T1D negatively impacts rat testicular activity. We verified that T1D, by inducing oxidative stress, produces damage in both the somatic and germinal compartments, as evidenced by the alteration of several parameters, such as steroidogenesis, spermatogenesis, and BTB integrity. Mechanistically, these effects may be related to the dysregulation of the SIRT1/NRF2/MAPKs pathways and, particularly, by the increased cytoplasmic retention of NRF2, which is not able to activate adequate antioxidant defenses. In addition, induction of testicular inflammation and pyroptosis was also demonstrated, as highlighted by the increased levels of some markers, such as NF- κ B and NLRP3. Finally, the localization of NLRP3 in the acrosome region suggested its peculiar involvement in its biogenesis. The combined data encourages further studies to confirm not only this aspect but also on the development of strategies to be used in preventing/mitigating the effects of T1D and other metabolic disorders on human male fertility.

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 experimental procedures were approved by the Animal Ethics Committee of the University of Campania “L. Vanvitelli” of Naples and by the Italian Ministry for Health (protocol number 30/2021). The study was conducted in accordance with the local legislation and institutional requirements.

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Author contributions

MV: Conceptualization, Funding acquisition, Investigation, Visualization, Writing – original draft. MR: Investigation, Visualization, Writing – original draft. SB: Investigation, Methodology, Writing – original draft. AH: Investigation, Writing – original draft. AB: Investigation, Writing – original draft. SMA: Methodology, Writing – review & editing. SMi: Conceptualization, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Italian Ministry of University and Research (Grant No. PRIN to M.V., 2022, CUP B53D23011470006). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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.2024.1399256/full#supplementary-material>

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OPEN ACCESS

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RECEIVED 14 May 2024

ACCEPTED 30 July 2024

PUBLISHED 21 August 2024

CITATION

Zhao X, Nie J, Zhou W, Zeng X and Sun X
(2024) The metabolomics changes in
epididymal lumen fluid of CABS1
deficient male mice potentially
contribute to sperm deformity.
Front. Endocrinol. 15:1432612.
doi: 10.3389/fendo.2024.1432612

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The metabolomics changes in epididymal lumen fluid of CABS1 deficient male mice potentially contribute to sperm deformity

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Introduction: Epididymal lumen fluids provides a stable microenvironment for sperm maturation. Ca²⁺ binding protein CABS1 is known to maintain structural integrity of mouse sperm flagella during epididymal transit of sperm. Besides, CABS1 was reported to contain anti-inflammatory peptide sequences and be present in both human saliva and plasma. However, little is known about the role of CABS1 in regulation of the microenvironment of epididymal lumen fluids.

Methods: To further confirm the role of CABS1 in epididymis, we identified the expression of CABS1 in epididymal lumen fluids. Moreover, high performance liquid chromatography, coupled with tandem mass spectrometry technique was used to analyze the metabolic profiles and *in vivo* microperfusion of the cauda epididymis and inductively coupled plasma mass spectrometry (ICP-MS) assays was used to detect the concentration of metal ion of mouse cauda epididymal lumen fluids in CABS1 deficient and normal mice.

Results: The results showed that CABS1 is present in epididymal lumen fluids, and the concentration of calcium in epididymal lumen fluids is not changed in *Cabs1*^{-/-} male mice. Among 34 differential metabolites identified in cauda epididymis, 21 were significantly upregulated while 13 were significantly downregulated in KO cauda epididymis. Pathway analysis identified pyrimidine metabolism, inositol phosphate metabolism, arachidonic acid metabolism, purine metabolism and histidine metabolism as relevant pathways in cauda epididymis.

Discussion: The perturbations of mitochondrial dysfunction and inflammation may be the crucial reason for the poor performance of *Cabs1*^{-/-} sperm.

KEYWORDS

CABS1, epididymal lumen fluid, metabolomics, sperm maturation, LC-MS

1 Introduction

Testicular spermatozoa are morphologically complete when released, but largely immature and not yet capable of motility and sperm-egg recognition (1). During sperm transit in the epididymis, a series of changes are also taking place in different segment, such as the migration of cytoplasmic droplets (2), further changes in the morphology of sperm (3), changes in the lipid composition of sperm membranes (4). Sperm undergo a series of physicochemical and morphological changes from testis to epididymis, leading to the final attainment of full maturation.

The epididymis is divided into four main anatomical segments: the initial segment, caput, corpus, and cauda, which are specialized for its unique characteristics and functions and provides an optimal intraluminal microenvironment for sperm maturation. The luminal fluid consists of multiple components including proteins, RNA, inorganic ion, and small organic molecules, many proteins have been demonstrated to play significant role in sperm maturation (5). The precise regulation of secreted proteins which are in direct contact with the spermatozoa in the lumen of the epididymis is essential for the sperm maturation process. It has been found that over 700 proteins are added and more than 1000 proteins are removed from sperm during the transit from caput to the cauda in epididymis (6, 7). Abnormal epididymal function may account for a significant proportion of idiopathic male infertility, particularly in infertile patients with normal spermatogenic processes in the testes. The progress of metabolomics techniques has helped identify more metabolites, which yield a better understanding of the physiological processes at metabolite levels. In recent studies, over thousands of different metabolites were identified in different segment of epididymis lumen fluid using quantitative and qualitative comparisons.

The particular ionic composition in epididymal fluids is closely related to the regulation of luminal environment that helps sperm to maintain the quiescence state (8, 9). A decrease gradient of luminal Ca^{2+} was observed along the epididymal duct (10–12), the low calcium levels are believed to be essential to help keep the sperm in the dormant stage (13, 14). The aberrant epididymal luminal calcium homeostasis can lead to defective spermatozoa and impaired fertility (14–16). As a calcium-binding protein, whether CABS1 is involved in the regulation of Ca^{2+} homeostatic regulation or other components in the lumen is not clear.

The calcium-binding protein spermatid-associated 1 (CABS1) was found to be enriched in mammalian, mainly in elongated spermatid of testes and maturation sperm of epididymis in mice (17, 18). Moreover, our previous study showed that CABS1 had no effect on spermatogenesis in testis, while CABS1 was required for the maintenance of structural integrity of sperm flagella during epididymal transit (18). The increased percentage of sperm with a bent tail in the epididymis of *Cabs1* deficient mice suggested that the epididymal lumen fluid may account for the sperm tail deformation during the normal transit of spermatozoa in the epididymis. In an attempt to fully illuminate the role of CABS1 in regulation sperm maturation in epididymis, we employed *in vivo* microperfusion of the cauda epididymis and inductively coupled

plasma mass spectrometry (ICP-MS) assays and untargeted liquid chromatography-mass spectrometry (LC/MS) analysis on wild type and CABS1 deletion mice to reveal the changes in the epididymis microenvironment.

2 Materials and methods

2.1 Animals

The *Cabs1* KO C57/BL6 mice were generated as is shown in Zhang et al. (18), and WT mice were bred by ourselves. Healthy 4~5-month-old male C57/BL6 mice were sacrificed for collecting the epididymal luminal fluid. The experiment was conducted in accordance with the guidelines approved by the Institutional Animal Ethical Committee (IAEC) of Nantong University (S20230101-005, Nantong, China).

2.2 Epididymal fluid collection

WT (n=6) and KO (n=6) male mice were sacrificed, and the cauda epididymis was removed and immediately kept in 200 μL DPBS, several cracks were made in the tubules to facilitate the release of the epididymal fluid into the DPBS. After an incubation period at 37°C for 15 min, the DPBS containing the epididymal fluid and sperm was collected and centrifuged at 1500 rpm for 5 min and 12000 rpm for 10 min at 4°C. The resulting supernatants were collected and stored at -80°C for LC-MS analysis after fast freezing using liquid nitrogen.

2.3 Sample preparation

Samples were thawed on ice. All samples were concentrated in lyophilizer, and 100 μL of 80% methanol was added to the concentrated precipitate. The mixtures were vortexed for 1 min, sonicated for 30 min at 4°C, and placed at -20°C for 1 hour. Afterward, the mixtures were centrifuged at 12000 rpm for 15 min. A total of 200 μL of the supernatant was transferred to a 1.5 mL tube along with 5 μL 2-chloro-L-phenylalanine (1 mg/mL). Finally, the mixtures were transferred to a glass vial.

2.4 LC-MS analysis

LC-MS analysis was performed on a 15 μL aliquot of the pretreated epididymal cauda fluid samples using a LC-Q/TOF-MS platform (1290 Infinity LC, 6530 UHD and Accurate-Mass Q-TOF/MS; Agilent, Santa Clara, CA, USA) with the column (2.1 mm $\times$ 100 mm $\times$ 1.8 μm ; Agilent, Santa Clara, CA, US) held at 40°C. The mobile phase consisted of A (water with 0.05% formic acid) and B (acetonitrile). The flow rate was set to 0.4 mL/min, and the column temperature was maintained at 40°C. The gradient elution program

was as follows: 5% B for 0 to 1 min, 95% B for 1 to 13.5 min, and then stable at 5% B for 13.6 to 16 min.

To identify differential metabolites in the epididymal cauda fluid of WT and KO mice, both positive and negative modes of electrospray ionization source (ESI) were employed. The ionization mode was set with a capillary voltage of 3.0 kV (+) and 3.2 kV (-), a heater temperature of 300°C, sheath gas flow of 45 arb, auxiliary gas flow of 15 arb, sweep gas flow of 1 arb, a capillary temperature of 350°C, and an S-Lens RF level of 30% (+) or 60% (-).

2.5 Bioinformatics and statistical analysis

The data were analyzed using SIMCA-P 14.1 software package (Sartorius Stedim Data Analytics AB, Umea, Sweden). Principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were performed on the data. The first principal component of the variable importance in the projection (VIP) was utilized to identify differential metabolites, where the VIP value exceeded 1 and the p-value of the Student's t-test was <0.05. Additionally, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg/>) was used to identify the enriched metabolic pathways of differential metabolites.

2.6 *In vivo* microperfusion of the cauda epididymis and inductively coupled plasma mass spectrometry assays

To collect the epididymis lumen fluid and prevent the contamination of calcium ions in the blood, we employed *in vivo* microperfusion, following established methods (16). Briefly, adult male mice were anaesthetized via intraperitoneal injection of sodium pentobarbital. The distal epididymis was exposed through an abdominal incision under a dissecting microscope, and the vas deferens was cannulated with a microtubule. Another microtubule was inserted into the lumen of the cauda tubule, and paraffin oil was infused at a rate of approximately 100 microliters per minute using an infusion pump (Genie Touch, Kent Scientific). The collected sperm-fluid samples were initially weighed and then diluted 10 times (w/v) with 10 mM Tris-HCL (pH 7.4), followed by centrifugation at 3000 x g. The resulting supernatant was transferred to a new eppendorf tube. An equal volume of nitric acid (69%, Damas-beta) to the supernatant and sperm pellet, respectively, was added for digestion. Samples were boiled for 15 min at 100°C and then stored for ICP-MS at 4°C. A quadrupole-based NexION 2000 S ICP mass spectrometer equipped with a TYPE C ST3 nebuliser and a quartz cyclone spray chamber (PerkinElmer) was utilized throughout the experiment. Prior to analysis, the ICP-MS underwent adjustment using a multi-element standard solution of 10 mg/L. Samples were diluted with deionized water and measured in dynamic reaction cell

(DRC) mode using NH₃ as the reaction gas to eliminate interferences. 1 µg/L of Ge was used as an internal standard.

2.7 ATP content in epididymal sperm

Sperm ATP content was measured using an enhanced ATP assay kit, S0027 (Beyotime Biotechnology, Shanghai, China). To accomplish this, 200 µL of cell lysis reagent was added to the sperm collected from one mouse cauda. The sperm suspension was subsequently vortexed and then incubated at room temperature for 5 min. The resulting cell lysate was centrifuged at 12000 g for 5 min, and the supernatant was utilized for ATP quantification following the manufacturer's instructions. The concentration of ATP was calculated based on an ATP standard curve and expressed as nmol/10 µg protein.

2.8 Western blot

After induction of anesthesia, the epididymis was removed and different segment of epididymides were immediately kept in 100 µL DPBS, several cracks were made in the tubules to facilitate the release of the epididymal fluid. After an incubation period at 37°C for 15 min, the DPBS containing the epididymal fluid and sperm was collected and centrifuged at 1500 rpm for 5 min and 12000 rpm for 10 min at 4°C. The resulting supernatants were collected. The protein present in epididymal luminal fluid was precipitated using acetone and then centrifuged at 12000 rpm for 30 min at 4°C, the resulting supernatants were discarded, and the precipitate was dissolved in radio immunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, Shanghai, China), which contained 2% SDS. This step was performed along with the epididymal fluid to isolate sperm proteins. The proteins were separated on SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. To block the membranes, 5% non-fat milk was used for 2 hours at room temperature. Primary and secondary antibodies were used for probing the membranes. The primary antibodies included rabbit anti-CABSI (produced by ourselves) at a dilution of 1:500, α -Tubulin (#11224-1-AP, Proteintech, Wuhan, China) and GAPDH (#10494-1-AP, Proteintech, Wuhan, China) at a dilution of 1:5000. And the secondary antibodies were diluted into 1:10000. The protein bands were visualized using Amersham Imager 600 (GE Healthcare).

2.9 Statistical analysis

Results are expressed as the mean $\pm$ standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism (version 7.0), and sets of data were compared using a one-tailed Student's t-test. * P <0.05 was regarded as statistically significant, and ** P <0.01 was considered extremely statistically significant.

3 Results

3.1 Genetic deletion of *Cabs1* doesn't impact elemental contents in luminal fluid

To verify the function of CABS1 in the epididymis lumen, we assessed the presence of CABS1 and the elemental composition in the luminal fluid. Western blot analysis confirmed the abundant presence of CABS1 in the epididymal luminal fluid (Figure 1). The elemental contents (calcium Ca, magnesium Mg, zinc Zn, and iron Fe) in the luminal fluid from the luminal perfused cauda-vas deference tubules of KO or WT mice were detected using ICP-MS. Among the commonly detected elements, no significant differences were observed in the elemental content of the luminal fluid, including calcium in the cauda (unpaired t-test, ns, $P > 0.05$) (Figure 2).

3.2 PCA and OPLS-DA analysis of metabolites identified in caudal epididymal luminal fluid

To investigate the role of CABS1 in regulating the micro-environmental homeostasis of the epididymis, we compared the metabolites between WT and KO mice. The similarity and differences among the WT and KO mice were analyzed using multivariate analysis, primarily including the PCA and OPLS-DA, which allowed us to assess the stability of the overall analytical process and distribution between samples. The PCA score plots clearly distinguished the samples from different groups, with samples within the same group forming closer clusters, and the two groups being well separated (Figures 3A, D). To minimize intra-group errors and random errors, an OPLS-DA supervised model was utilized to discern inter-group differences and identify differential compounds. The OPLS-DA score plots demonstrated strong clustering of the two groups, with all samples falling within the 95% confidence interval (Hotelling's T-squared ellipse) (Figures 3B, E). The R^2 and Q^2 values from the permutation test indicated that the OPLS-DA model exhibited good repeatability and predictive capabilities (Figures 3C, F).

3.3 Screening and identification of differential metabolites

Multivariate statistical analysis was conducted to identify differential metabolites associated with sperm deformity. This analysis employed the Student t-test to compare metabolite profiles in the epididymal luminal fluid between WT and KO mice. The results of this analysis can provide valuable insights into the metabolic pathways involved in sperm deformity. Based on the screening criteria, including VIP values > 1 from OPLS-DA and P-values < 0.05 from the t-test, we identified 34 differential metabolites between WT and KO mice. Among these, 21 metabolites were up-regulated and 13 were down-regulated in the epididymal luminal fluid of the cauda in *Cabs1* KO mice. The differential metabolites included fatty acids, amino acids, nucleosides, cholic acids, phospholipids, and other metabolites (Table 1).

3.4 Pathway enrichment and metabolic pathway analysis of the potential metabolic mechanism

Following the identification of differential metabolites between WT and KO mice, we conducted an analysis of the enriched metabolic pathways using the KEGG database. The heatmap used to visualize the affected metabolites is displayed in Figure 4A. The pathway analysis revealed that these differential metabolites were enriched in 15 metabolic pathways (Table 2). Figures 4B, C shows that the differential metabolites were primarily involved in histidine metabolism, nicotinate and nicotinamide metabolism, starch and sucrose metabolism, pyrimidine metabolism, and tryptophan metabolism. The matching status, P-value, $-\ln(P)$, and impact of each pathway are listed in Table 2.

3.5 Deletion of CABS1 causes abnormal level of prostaglandins and acylcarnitines

Among the differential metabolites, the levels of free L-carnitine and acylcarnitines, such as hexanoylcarnitine, acetyl-L-carnitine

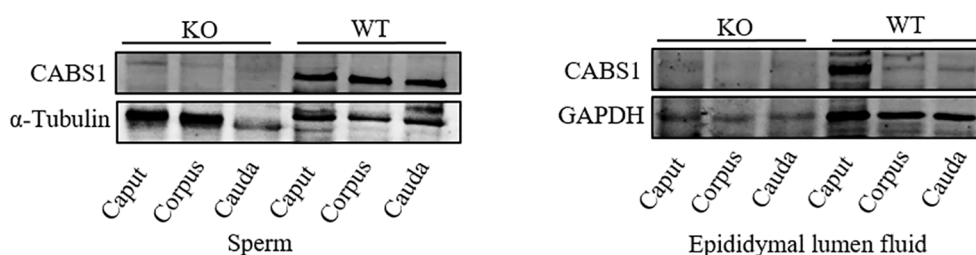


FIGURE 1

The expression levels of CABS1 in the epididymis. Immunoblot of CABS1 proteins in sperm and lumen fluid of the epididymis from WT and KO mice. The bands of expected size of CABS1 (~68 kDa), α -Tubulin (~55 kDa) and GAPDH (~36 kDa). α -Tubulin and GAPDH serves as the loading control.

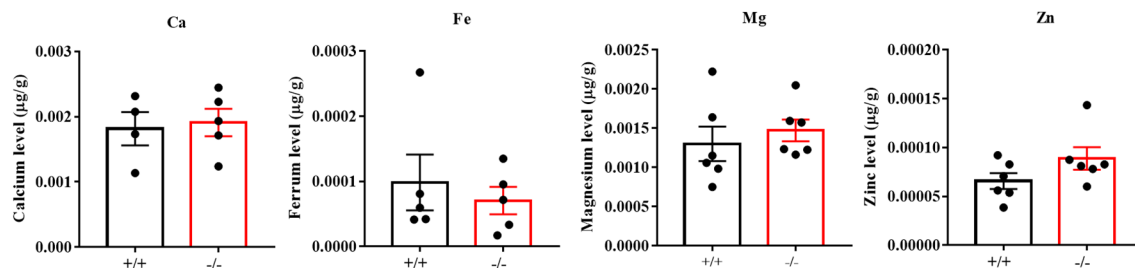


FIGURE 2

The contents of the elemental (calcium Ca, magnesium Mg, zinc Zn, and iron Fe) in the luminal fluid separated from the luminally perfused cauda-vas deference tubules of KO or WT mice ($n=4$ to 6 biological replicates). Amongst the detected elements, the content of Ca, Mg, Zn, and Fe showed no significant differences in the cauda luminal ($P>0.05$).

and propionylcarnitine were all significantly increased in KO mice (Figure 5A). L-carnitine and acylcarnitines, known to be involved in β -oxidation, provide ATP for sperm motility. To further substantiate these findings, we measured ATP levels in sperm. Consistent with the observed metabolic alterations, our results revealed a significant decrease in ATP levels in sperm from KO mice (Figure 5B). Moreover, Prostaglandins and their metabolite, which are known as important inflammatory factors, such as PGD2, PGE2, PGJ2, 15-Deoxy- $\Delta^{12,14}$ -prostaglandin D2, ent-Prostaglandin F2 α and 13,14-Dihydro-15-keto-PGE2, were significantly increased in KO mice (Figure 5C).

4 Discussion

In our study, we identified the presence of the sperm-specific protein CABS1 in the epididymal luminal fluid. Additionally, our

findings indicate that the deletion of CABS1 does not affect the elemental contents. However, it does have a significant impact on the levels of prostaglandins and acylcarnitines in the epididymal luminal fluid. Given acylcarnitines' crucial role in lipid metabolism, we also quantified the ATP in sperm, our results showed that decreased ATP levels in CABS1 knockout mice may offer an explanation for their decreased motility, adding another layer of understanding to the complex factors that regulate sperm function and male fertility.

During sperm maturation in the epididymis, the dynamics of the luminal fluid components play a vital role. Interestingly, in mice with the absence of CABS1, the sperm morphology in the testes remains normal. However, abnormalities in sperm flagellar structure occur during their transit through the epididymis, and notably, this defect is independent of changes in epididymal osmotic pressure (18). Given the presence of CABS1 in the epididymal luminal fluid, further investigations were conducted to

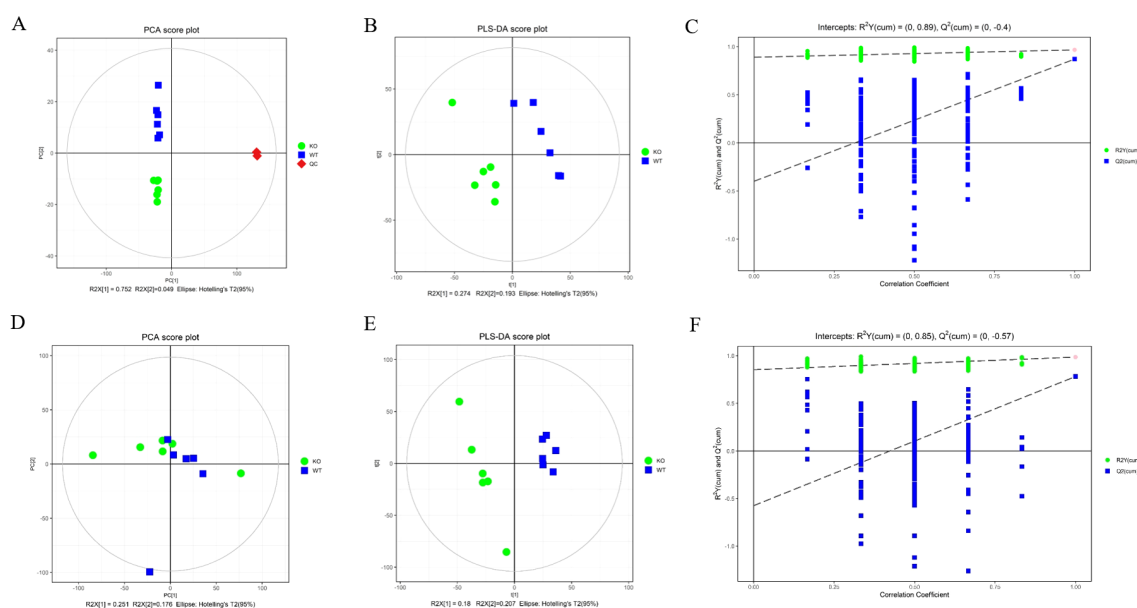
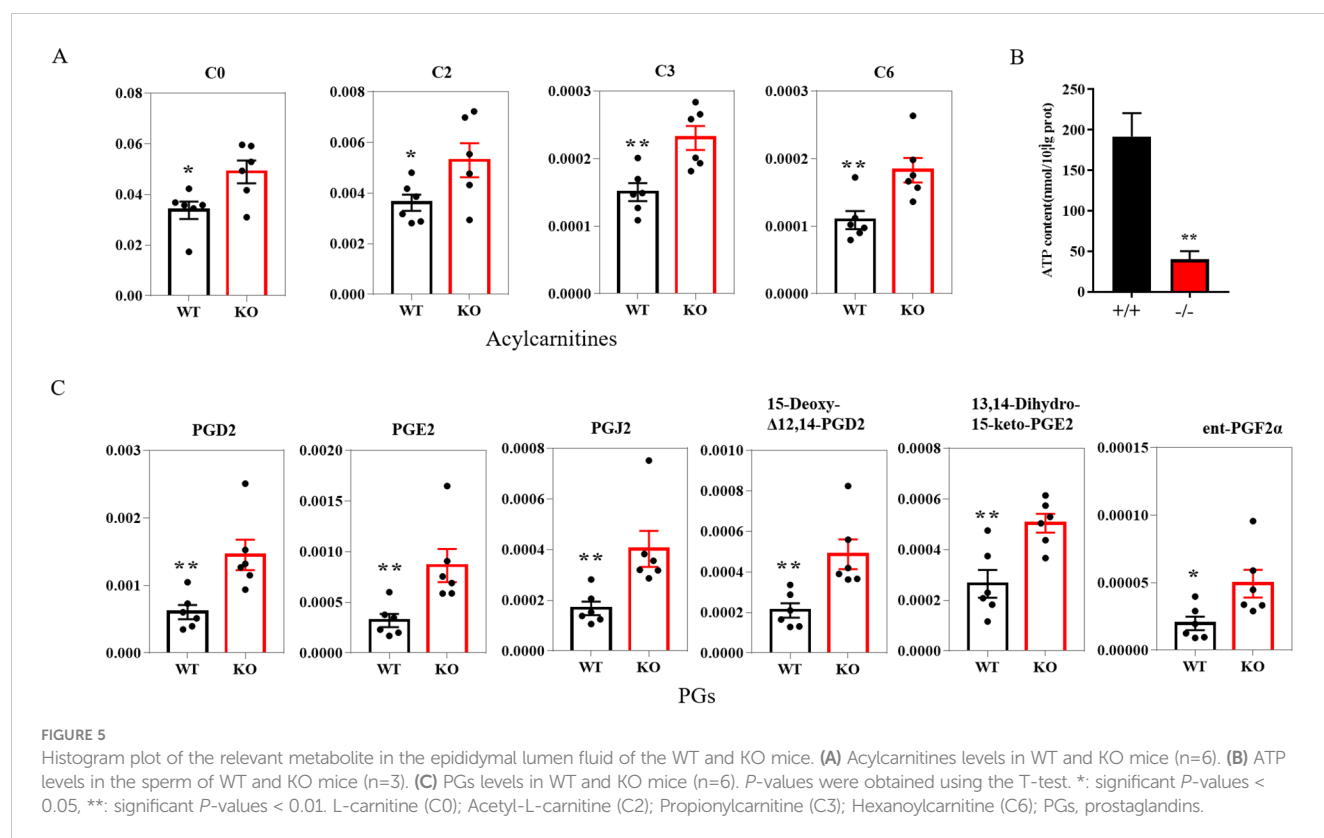


FIGURE 3

Principal component analysis (PCA) score plots (A, D), orthogonal projections to latent structures-discriminate analysis (OPLS-DA) score plots (B, E), and permutation tests of OPLS-DA mode (C, F) of epididymal fluid metabolomics analysis. (A–C) ESI+ model. (D–F) ESI- model.

TABLE 1 Differentiating metabolites between WT and KO mice in epididymal lumen fluids.

Mode	No.	Metabolites	Fold change	VIP	P-VALUE
PGs					
ESI-	1	Prostaglandin J2	2.39	1.989799	0.011599
ESI-	2	Prostaglandin D2	2.39	1.988324	0.006764
ESI-	3	15-Deoxy-Δ12,14-prostaglandin D2	2.31	1.979999	0.006856
ESI-	4	Prostaglandin E2	2.69	1.953277	0.011912
ESI-	5	13,14-Dihydro-15-keto-PGE2	1.91	1.762489	0.004588
ESI-	6	ent-Prostaglandin F2α	2.47	1.747608	0.028156
Acylcarnitines					
ESI+	7	Hexanoylcarnitine	1.68	1.5969	0.008472
ESI+	8	Acetyl-L-carnitine	1.46	1.404584	0.047536
ESI+	9	L-Carnitine	1.45	1.388692	0.023368
ESI+	10	Propionylcarnitine	1.53	1.640512	0.004706
Nucleosides					
ESI+	11	Hypoxanthine	1.37	1.555766	0.021995
ESI-	12	Thymidine	1.57	1.832369	0.018468
ESI-	13	Uridine	1.29	1.622794	0.021227
ESI-	14	Guanosine-5'-monophosphate	2.56	1.767177	0.041752
ESI+/-	15	Inosine	2.34	1.877633	0.016096
Amino acids and dipeptide					
ESI+	16	L-Methionine sulfoxide	0.46	1.97596	0.002997
ESI+	17	Gly-Tyr	1.1	1.723937	0.005169
ESI-	18	gamma-Glutamylleucine	0.66	1.486205	0.03755
ESI-	19	Leucylproline	0.68	2.032455	0.028172
ESI-	20	D-Glutamine	0.67	1.973361	0.025406
organic acid					
ESI-	21	13-HPODE	0.45	2.313781	0.000442
ESI-	22	Tetradecanedioic acid	0.55	2.045986	0.027065
ESI-	23	3-Methylglutaric acid	0.58	2.032535	0.00371
ESI-	24	Hexadecanedioic acid	0.68	1.668895	0.04492
ESI-	25	Azelaic acid	0.51	1.659492	0.046438
ESI-	26	CITRAMALATE	1.32	1.565828	0.025785
ESI-	27	Orotic acid	1.48	1.718916	0.036149
ESI+/-	28	Suberic acid	0.5	2.069452	0.019755
others					
ESI+	29	Nicotinamide	1.51	1.478073	0.017481
ESI+	30	Histamine	0.6	1.762852	0.002594
ESI+	31	N-Methylhistamine	0.51	2.107697	0.000019
ESI-	32	D-Glucose 6-phosphate	1.45	1.813342	0.029537
ESI-	33	Dihydroxyacetone phosphate	1.6	1.779492	0.007019
ESI-	34	L-Kynurenine	0.74	1.760346	0.019652



indicate an inflammatory state in the microenvironment of epididymal luminal fluids, which significantly contributes to reduced sperm motility. Therefore, we conducted a metabolomic analysis to identify changes in the epididymal luminal fluid. As anticipated, significant increases were observed in metabolites such as carnitine and prostaglandins (Figure 5). Based on these findings, it is hypothesized that the alterations in the metabolites of the epididymal luminal fluid may be another crucial factor contributing to changes in the expression of sperm flagellar proteins.

Phospholipid metabolites have been identified as inflammatory factors. Arachidonic acid (AA), a major product of phospholipid hydrolysis, plays a significant role in various pathophysiological processes. Furthermore, AA can be converted into various prostaglandin species, with PGD₂, along with prostaglandins PGE₂, PGI₂, and 15-d PGJ₂, reported to regulate the inflammation process (21–23). The synthesis of PGD₂ in the genitalia is controlled by the enzyme lipocalin-type prostaglandin D₂ synthase (L-PGDS) (24), which is highly expressed in the testis and epididymis of mice (25, 26). Reduced expression of L-PGDS has been found in the seminal plasma of oligozoospermic men compared to normozoospermic men (27), indicating its key role in sperm development and maturation. Additionally, PGJ₂ has been reported as a metabolite of PGD₂ (28), and the metabolites of PGD₂ can influence diverse cellular functions.

The absence of tryptophan catabolism along the kynurenine pathway has been shown to produce an inflammatory state in the epididymis (29). Moreover, a significant increase in sperm number and the proportion of sperm with abnormal morphology have been observed in mice deficient in indoleamine 2,3-dioxygenase (IDO),

the rate-limiting enzyme of tryptophan catabolism through the kynurenine pathway (29). Kynurenine, a tryptophan-derived metabolite, has reduced concentration in the cauda epididymis fluid of *Cabs1* KO mice compared to WT mice. It is reported that CABS1 is also present in salivary glands and is associated with stress and anti-inflammation (19, 20). The altered metabolomic fingerprinting of inflammatory factors demonstrates an inflammatory state in the epididymis of *Cabs1*-deleted mice.

Lipids are the vital components of sperm, containing cholesterol, phospholipids, and glycolipids. The maturation of sperm involves changes in the lipid composition of the sperm membrane. As spermatozoa move from the caput to the cauda region, there is an increase in membrane fluidity due to a remodeled ratio of cholesterol to phospholipid (30, 31). The increased membrane fluidity in cauda spermatozoa is crucial for sperm movement, acrosome reaction, and egg fusion (31). Additionally, the ratio of polyunsaturated: saturated fatty acids (PUFAs) can contribute to changes in membrane fluidity (30). PUFAs can also alter the composition and organization of mitochondrial membranes, leading to increased production of reactive oxygen species (ROS) and subsequent peroxidation of membrane phospholipids and mitochondrial dysfunction (32). In our study, metabolomic fingerprinting analysis revealed significant decreases in suberic acid, azelaic acid, tetradecanedioic acid, hexadecanedioic acid, and 13-HPODE in KO mice, all of which are lipid or oleochemical products. Disorders of oxidative phosphorylation have been associated with oxidized linoleic acid (33). 13-HPODE, as one of the most common dietary peroxidized lipids, induces inflammation *in vivo* (34) and may be involved in mitochondrial

dysfunction-related disorders (35). Abnormalities in lipid metabolism can lead to spermatogenic dysfunction and consequently male infertility.

Furthermore, our study identified high levels of L-carnitine in CABS1-deficient mice. Free L-carnitine is known to be taken from blood plasma and concentrated in the epididymal lumen. The uptake of L-carnitine in the sperm plasma membrane plays a protective role in mitochondrial function, participating in the intermediary metabolism of fatty acids and β -oxidation of long-chain fatty acids (36, 37). Acylcarnitines, fatty acid metabolites, are used as markers for errors in fatty acid oxidation and energy metabolism. Short-chain acylcarnitines (C2-C5) are the most abundant group in the body (38), and altered concentrations have been linked to various diseases and pathologies (39–41). As short-chain acylcarnitines, acetyl-L-carnitine and propionylcarnitine play vital roles in energy production by providing acetyl groups for β -oxidation (42). Carnitine is considered to positively affect sperm motility and maturation by supplying abundance energy and acting as an antioxidant to decrease ROS in mitochondria (43). However, excessive acylcarnitine may reflect inadequate fatty acid oxidation and mitochondrial dysfunction, potentially leading to metabolic disease (44). The increased level of L-carnitine, acetyl-L-carnitine and propionylcarnitine may indicate perturbations in the β -oxidation of fatty acids (45). The quantification of ATP in sperm further supports the notion that disordered energy metabolism may partially account for the decreased sperm motility.

5 Conclusion

In our research, we investigated the differences in metabolite profiles of epididymal luminal fluid in male mice with and without CABS1. The analysis of metabolome profiles, combined with ATP quantification, suggested that mitochondrial dysfunction and inflammation may be key factors contributing to the impaired sperm function in *Cabs1* knock-out mice. Further research is required to elucidate the specific role of these metabolites in sperm maturation. This study established a connection between infertility and the metabolite profiles of epididymal luminal fluid, providing insight into the molecular mechanisms of male infertility.

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 animal studies were approved by Institutional Animal Ethical Committee (IAEC) of Nantong University (S20230101-005, Nantong, China). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

XLZ: Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Validation, Writing – original draft, Writing – review & editing. JN: Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. WZ: Data Curation, Investigation, Validation, Writing – original draft, Writing – review & editing. XHZ: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. XS: Conceptualization, Data curation, Funding acquisition, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was supported by grants from Jiangsu Provincial Research Hospital (YJXYY202204-YSB44 to XS), Key Laboratory of Reproductive Health Diseases Research and Translation of Ministry of Education from Hainan Medical University (SZLAB202307 to XS), the Society and livelihood Project of Nantong City (MS22022119 to XS), Health Commission of Nantong City (MS2023019 to XS), the Natural Science Foundation of Jiangsu Province (BK20210840 to XLZ), the Startup R&D funding from Nantong University (135420631136 to XLZ), the National Natural Science Foundation of China (32271167 to XHZ), Jiangsu Innovation and Entrepreneurship Talent Plan (JSSCRC20211543 to XHZ), the Social Development Project of Jiangsu Province (NO. BE2022765 to XHZ), and the Society and livelihood Project of Nantong City (No. MS22022087 to XHZ).

Acknowledgments

We would like to extend our sincere thanks to Baoli Zhang and Winnie Shum from ShanghaiTech University for their assistance with the quantification of calcium in epididymal luminal.

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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OPEN ACCESS

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RECEIVED 21 August 2024

ACCEPTED 28 October 2024

PUBLISHED 19 November 2024

CITATION

Wan Y, Chen X, Chen Z, Liu W, Li S and Hua J (2024) Impact of *Ureaplasma urealyticum* infection on semen parameters and *in vitro* fertilization outcomes in infertile men. *Front. Endocrinol.* 15:1484007. doi: 10.3389/fendo.2024.1484007

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Impact of *Ureaplasma urealyticum* infection on semen parameters and *in vitro* fertilization outcomes in infertile men

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Introduction: *Ureaplasma urealyticum* (UU) is a common pathogen associated with genital tract infections in infertile males. However, its impact on semen quality, embryo development, and *in vitro* fertilization (IVF) outcomes remains underexplored. This study aims to evaluate the effect of male UU infection on semen parameters, embryo development, pregnancy outcomes, and neonatal health in infertile couples.

Methods: A retrospective analysis was conducted on 1,215 infertile couples at the First Hospital of USTC. Participants were divided into two groups based on the male partner's UU infection status: UU-positive (n=249) and UU-negative (n=966). Semen parameters (sperm concentration, motility, morphology, anti-sperm antibodies, DNA stainability) were assessed. Embryo development was evaluated through fertilization rates and blastocyst formation rates. Pregnancy outcomes (clinical pregnancy, live birth rates, miscarriage rate) and neonatal health (gestational age, birth weight, Apgar scores, preterm delivery) were also compared.

Results: Semen parameters, including sperm concentration, motility, and morphology, were similar between the UU-positive and UU-negative groups. However, the UU-positive group had significantly higher levels of anti-sperm antibodies (ASA) ($p=0.020$) and higher DNA stainability (HDS) ($p=0.014$). Despite these differences, embryo quality, as measured by fertilization rates and blastocyst formation rates, was not significantly different between the two groups. Pregnancy outcomes, including clinical pregnancy and live birth rates, were also comparable. While the UU-positive group had a slightly higher miscarriage rate, this difference was not statistically significant. Neonatal outcomes, including gestational age, birth weight, Apgar scores, and preterm delivery rate, did not differ significantly between the two groups.

Discussion: The study suggests that while male UU infection may adversely affect certain semen parameters, its impact on IVF outcomes—such as embryo quality,

pregnancy rates, and neonatal health—appears to be minimal. These findings support the continued use of IVF as a viable and safe option for infertile couples with male UU infection, as it does not significantly influence reproductive or neonatal outcomes.

KEYWORDS

Ureaplasma urealyticum, male infertility, semen parameters, oxidative stress, neonatal outcomes, *in vitro* fertilization

Introduction

Male infertility contributes to nearly 50% of all infertility cases (1), with a wide range of factors influencing male reproductive potential, including infections of the genital tract. *Ureaplasma urealyticum* (UU) is a common pathogen associated with genital tract infections and has been implicated in male infertility (2–4). Although the adverse effects of reproductive tract infections have been well studied in female infertility patients (5), the influence of male UU infections on semen quality and *in vitro* fertilization (IVF) outcomes remain controversial. Some studies suggest that UU infection can negatively affect sperm concentration, motility, and morphology, thereby reducing fertility potential (6, 7). However, other studies have reported that seminal UU infection may not have a substantial negative impact on semen quality, pregnancy rates, or outcomes of assisted reproduction (8).

Some studies have suggested that male UU infection may impair not only semen parameters but also embryo quality and pregnancy outcomes, potentially leading to higher rates of miscarriage and lower live birth rates (9, 10). The pathophysiological mechanisms behind these effects include the induction of inflammatory responses and oxidative stress within the male reproductive tract, resulting in increased sperm DNA fragmentation and decreased nuclear maturity (8). However, other studies have reported conflicting results, showing no significant association between UU infection and adverse reproductive outcomes, suggesting the need for further investigation to clarify the role of UU in male infertility and IVF success (11, 12).

Given the conflicting evidence, this research seeks to thoroughly assess the effect of male UU infection on semen and embryo quality, as well as pregnancy outcomes in infertile couples undergoing IVF treatment. This study aims to provide a clearer understanding of how male UU infection influences IVF reproductive outcomes. The findings could have important implications for the management of male infertility and the optimization of assisted reproductive technology (ART) protocols to improve the chances of successful conception and healthy live births.

Patients and methods

Patients

A total of 1,215 infertile couples who underwent IVF cycles at the First Hospital of USTC were enrolled in this study between January 2020 and June 2023. The inclusion criteria for this study were as follows: female factor infertility (such as tubal infertility, endometriosis, ovulatory disorders, and cervical factor infertility), certain male factors (such as mild to moderate oligoasthenoteratozoospermia), and unexplained infertility (with normal ovarian function assessment, tubal patency evaluation, and semen analysis). The 1,215 infertile couples were divided into two groups based on the male partner's UU infection status: UU-positive (n=249) and UU-negative (n=966). Female participants were screened and excluded based on the presence of UU, *Mycoplasma hominis*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, fungal infections, and common gynecological inflammatory markers. Male participants were screened for and excluded based on the presence of UU, *Mycoplasma hominis*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and fungal infections. In the UU-positive group, most male patients were asymptomatic, while a minority experienced mild discomfort associated with urination or ejaculation.

Semen parameters

Semen samples were collected from patients following a period of 2–7 days of abstinence by masturbation into sterile containers. Standard semen parameters, including sperm volume, concentration, and percentage of spermatozoa with forward motility (PR) and total motility (PR+NP), were assessed in a specialized seminal laboratory using computer-assisted sperm analysis (CASA) (SAS Medical, Beijing, China). The assessment of sperm concentration and motility was carried out by the Saes SAS-II sperm quality analyzer (SAS Medical, Beijing, China). Sperm morphology, leukocyte count, and anti-sperm antibodies were

determined through Diff-Quick staining, benzidine peroxidase staining, and mixed antiglobulin reaction, respectively (Anke Biotechnology, Hefei, China). The morphological parameters of sperm and peroxidase-positive cells were analyzed under a microscope (LEICA DM2500, Wetzlar, Germany). All analyses were conducted and evaluated following the World Health Organization (WHO) manual (5th edition). Fluorescence signals from spermatozoa stained with acridine orange were analyzed using the BD Accuri C6 flow cytometer (BD, San Jose, USA). The resulting data were processed using the sperm DNA fragmentation index (DFI) viewer software (Cellpro, Ningbo, China), yielding values for high DNA stainability (HDS) and sperm DFI. The HDS value reflects the percentage of immature nuclear spermatozoa, while the DFI represents the extent of damage to sperm nuclear chromatin.

Semen STD detection

All semen samples were subjected to UU culture and drug sensitivity testing following the guidelines provided by the manufacturer (Zhong Ai Sheng Hebei Biotechnology Co. Ltd., Xingtai, China). The semen specimens were inoculated into a liquid medium and subsequently distributed into wells containing indicators, such as urea and arginine, to detect the presence of potential pathogens. The culture plates were incubated at a controlled temperature of $36 \pm 1^\circ\text{C}$. UU presence was determined by observing a color change in the medium, which occurs when UU metabolizes urea, leading to an increase in pH and a shift in the medium's color from orange-yellow to red after 24 hours of incubation. For positive samples, further identification and quantification were performed, with 10^4 colony-forming units (CFU) per milliliter or higher being considered a positive result. To identify co-infections with other microorganisms, standard laboratory procedures were employed. *Mycoplasma hominis*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and various fungi were screened using specific diagnostic methods. *Chlamydia trachomatis* was detected using latex immunochromatography assays (Abogen Biosciences Co. Ltd., Hangzhou, China). For *Neisseria gonorrhoeae*, standard culturing techniques were applied (Autobio Biosciences Co. Ltd., Zhengzhou, China). Fungal infections were identified through fungal slide culture methods (Tianda Diagnostic Reagents Co., LTD, Hefei, China).

IVF procedure and pregnancy follow-up

Female partners underwent controlled ovarian stimulation (COS) treatment. Each female was administered 8000 IU of recombinant hCG (Guangdong Lizhu Group Lippo Biochemicals Co., Ltd.) or 250 µg of Azer (Gonapeptyl, Ferring Pharmaceuticals) subcutaneously on the same day when more than two dominant follicles measured ≥ 18 mm in diameter. Eggs were retrieved 34–36 hours later. Fertilization assessment was conducted 16–20 hours post-retrieval, followed by a 24-hour observation period for

cleavage and embryo scoring. One to two high-quality embryos were selected for intrauterine transfer on day 3 after retrieval, while blastocyst transfer involved the selection of one blastocyst for transfer on day 5. Elective transfers were performed using frozen embryos. Blood and urinary hCG levels were measured 12–14 days post-embryo transfer. In the event of a biochemical pregnancy diagnosis, clinical confirmation was obtained at 30 days via ultrasound examination, which revealed the presence of a gestational sac, germ bud, and fetal heartbeat *in utero*. Subsequent monitoring included the early, mid, and late stages of pregnancy as well as delivery outcomes and newborn health status.

Statistical analysis

Statistical analyses were performed using R version 4.3.0 (R Core Team, Vienna, Austria). Continuous variables following a normal distribution were represented as mean $\pm$ standard deviation (SD), while those not normally distributed were reported as median (interquartile range, IQR). Categorical variables were represented as frequency (percentage). Student's t-test was applied for normally distributed continuous variables, whereas the Mann-Whitney U test was employed for non-normally distributed parameters. Pearson's chi-square or Fisher's exact test was used for qualitative data analysis. A p-value < 0.05 was considered statistically significant.

Results

Baseline characteristics

The analysis of baseline demographic and clinical characteristics in this study's population, comprising 1,215 male patients from infertile couples, divided into UU-positive (n=249) and UU-negative (n=966) groups. There were no significant differences between the two groups in terms of age, body mass index (BMI), duration of infertility, and infertility types (all $p>0.05$). Additionally, there were no significant differences between the two groups in terms of basal follicle-stimulating hormone (FSH) levels in the female partners ($p=0.235$), endometrial thickness on hCG days ($p=0.423$), and the number of days taken for ovulation induction ($p=0.321$). In summary, the two groups did not differ statistically regarding general clinical data parameters (Table 1).

Semen parameters

The comparison of semen parameters between the UU-positive and UU-negative groups of male infertile patients showed no significant differences in abstinence time ($p=0.744$) or semen volume ($p=0.566$). Sperm concentration was similar between the two groups ($p=0.706$). The median progressive motility was marginally greater in the UU-positive group (41.77%) compared to the UU-negative group (39.22%), though this difference did not reach statistical significance ($p=0.079$). Similarly, total motility was

TABLE 1 The general clinical data characteristics of the two groups.

Variables	Total (n=1215)	UU-negative (n=966)	UU-positive (n=249)	<i>p</i>
Male age (years; mean ± SD)	32.25 ± 5.04	32.22 ± 5.09	32.38 ± 4.88	0.651
Female age (years; mean ± SD)	33.60 ± 5.88	33.60 ± 5.82	33.57 ± 6.12	0.950
BMI of male (kg/m ² ; mean ± SD)	24.80 ± 3.39	24.74 ± 3.22	25.05 ± 3.98	0.254
BMI of female (kg/m ² ; mean ± SD)	22.75 ± 3.27	22.83 ± 3.29	22.45 ± 3.18	0.102
Duration of infertility (years; mean ± SD)	2.91 ± 2.34	2.89 ± 2.28	3.00 ± 2.59	0.529
Types of infertility				0.665
Primary infertility (n (%))	532 (43.79)	426 (44.10)	106 (42.57)	
Secondary infertility (n (%))	683 (56.21)	540 (55.90)	143 (57.43)	
Female basic FSH (mIU/ml; mean ± SD)	7.56 ± 2.63	7.51 ± 2.56	7.75 ± 2.87	0.235
Female endometrial thickness (mm; mean ± SD)	10.54 ± 2.64	10.57 ± 2.70	10.42 ± 2.42	0.423
Ovulation induction (days; mean ± SD)	9.63 ± 4.16	9.57 ± 4.13	9.87 ± 4.31	0.321

UU, *Ureaplasma urealyticum*; SD, standard deviation; BMI, body mass index; FSH, follicle stimulating hormone.

slightly higher in the UU-positive group (48.57%) than in the UU-negative group (46.16%), but there was no statistically significant difference ($p=0.088$). Leukocyte count and sperm morphology were consistent across both groups ($p=0.323$ and $p=0.293$, respectively). However, anti-sperm antibody (ASA) levels showed a statistically significant difference, with the UU-positive group having a higher median ASA percentage ($p=0.020$). Additionally, HDS, which reflects sperm nuclear immaturity, was notably elevated in the UU-positive group (median 6.54%) compared to the UU-negative group (median 5.75%) ($p=0.014$). Among the UU-positive group, DFI was slightly higher, but this difference was not statistically significant ($p=0.276$). In summary, although most semen parameters were similar, the UU-positive group exhibited higher ASA levels and HDS values (Table 2).

IVF embryo and pregnancy parameters

The comparison of embryo quality and pregnancy outcomes between UU-positive and UU-negative groups showed no significant differences in the normal fertilization rate (67.72% vs. 66.17%; $p=0.133$). The rate of high-quality embryos was comparable between the groups, with 54.49% in the UU-positive group and 55.13% in the UU-negative group ($p=0.629$). The blastocyst formation rate was also similar, at 48.87% for the UU-positive group and 49.05% for the UU-negative group ($p=0.909$). The embryo implantation rates were 38.69% for the UU-positive group and 39.73% for the UU-negative group ($p=0.688$). In the UU-positive group, the clinical pregnancy rate was 61.45%, compared to 63.56% in the UU-negative group ($p=0.537$).

TABLE 2 Semen parameters of the two group of male infertile patients.

Variables	Total (n=1215)	UU-negative (n=966)	UU-positive (n=249)	<i>p</i>
Abstinence time (days; median (IQR))	4.00 (3.00, 7.00)	4.00 (3.00, 7.00)	4.00 (3.00, 7.00)	0.744
Semen volume (ml; median (IQR))	3.00 (2.10, 4.00)	3.00 (2.10, 4.00)	3.00 (2.00, 4.00)	0.566
Sperm concentration (×10 ⁶ /ml; median (IQR))	77.04 (46.38, 127.68)	78.61 (47.18, 127.32)	75.40 (43.89, 131.94)	0.706
Progressive motility (%; median (IQR))	39.63 (29.05, 51.52)	39.22 (27.96, 51.59)	41.77 (31.35, 51.38)	0.079
Total motility (%; median (IQR))	46.60 (34.36, 59.45)	46.16 (33.89, 59.25)	48.57 (36.69, 59.90)	0.088
Leukocyte count (×10 ⁶ /ml; median (IQR))	0.16 (0.08, 0.35)	0.16 (0.08, 0.35)	0.16 (0.09, 0.35)	0.323
AsA (%; median (IQR))	2.00 (1.00, 4.00)	2.00 (1.00, 4.00)	2.00 (1.00, 5.00)	0.020
Normal morphology (%; median (IQR))	6.00 (4.00, 7.00)	6.00 (4.00, 7.00)	6.00 (4.00, 8.00)	0.293
DFI (%; median (IQR); n=139)	12.92 (9.34, 18.33)	12.79 (9.35, 18.18)	14.28 (9.00, 18.69)	0.276
HDS (%; median (IQR); n=139)	6.01 (4.66, 7.86)	5.75 (4.62, 7.68)	6.54 (5.14, 8.74)	0.014

IQR, interquartile range (25% and 75% percentiles); AsA, antisperm antibody; DFI, DNA fragmentation index; HDS, high DNA stainability.

TABLE 3 Embryo quality and pregnancy outcomes parameters.

Variables	Total (n=1215)	UU-negative (n=966)	UU-positive (n=249)	<i>p</i>
the number of oocytes (n (%))	11.19 ± 7.26	11.38 ± 7.33	10.44 ± 6.95	0.068
Normal fertilization rate, n (%)	9034 (66.47)	7274 (66.17)	1760 (67.72)	0.133
High-quality embryo rate, n (%)	4969 (55.01)	4010 (55.13)	959 (54.49)	0.629
Blastocyst formation rate, n (%)	2966 (49.02)	2383 (49.05)	583 (48.87)	0.909
Embryo implantation rate, n (%)	900 (39.53)	729 (39.73)	171 (38.69)	0.688
Clinical pregnancy rate, n (%)	767 (63.13)	614 (63.56)	153 (61.45)	0.537
Miscarriage rate, n (%)	117 (15.25)	86 (14.01)	31 (20.26)	0.054
Live birth rate, n (%)	650 (53.49)	528 (54.66)	122 (48.99)	0.110

Although the miscarriage rate was higher in the UU-positive group (20.26%) than in the UU-negative group (14.01%), it was not statistically significant ($p=0.054$). The live birth rate was 48.99% in the UU-positive group and 54.66% in the UU-negative group ($p=0.110$) (Table 3).

IVF neonatal outcomes

The average gestational age was 38.07 weeks in the UU-positive group and 38.31 weeks in the UU-negative group ($p=0.291$). The

preterm delivery rate was slightly higher in the UU-positive group at 20.49% compared to 18.94% in the UU-negative group ($p=0.695$). The distribution of delivery types indicated that normal deliveries were more common in the UU-positive group (40.98%) than in the UU-negative group (33.33%), but this difference was not statistically significant ($p=0.110$). Both groups had nearly identical rates of singleton pregnancies, with 84.43% in the UU-positive group and 84.09% in the UU-negative group ($p=0.927$). The distribution of neonatal sex was consistent across both groups, with no significant difference ($p=0.978$). Apgar scores were also similar, with a mean of 9.83 in the UU-positive group and 9.81 in the UU-negative group

TABLE 4 Neonatal outcomes in this study.

Variables	Total (n=1215)	UU-negative (n=966)	UU-positive (n=249)	<i>p</i>
Gestational age (weeks; mean ± SD)	38.26 ± 2.20	38.31 ± 2.16	38.07 ± 2.38	0.291
Preterm delivery rate (n (%))	125 (19.23)	100 (18.94)	25 (20.49)	0.695
Type of delivery (n (%))				0.110
Normal delivery	226 (34.77)	176 (33.33)	50 (40.98)	
Cesarean delivery	424 (65.23)	352 (66.67)	72 (59.02)	
Singleton pregnancies rate, n (%)	547 (84.15)	444 (84.09)	103 (84.43)	0.927
Twin pregnancies rate, n (%)	103 (15.85)	84 (15.91)	19 (15.57)	
Neonatal sex rate (n (%))				0.978
Female neonate	341 (45.29)	277 (45.26)	64 (45.39)	
Male neonate	412 (54.71)	335 (54.74)	77 (54.61)	
Apgar score (mean ± SD)	9.81 ± 0.51	9.81 ± 0.52	9.83 ± 0.48	0.685
Neonatal weight (kg; mean ± SD)	3.07 ± 0.66	3.08 ± 0.66	3.04 ± 0.67	0.559
Neonatal height (cm; mean ± SD)	49.07 ± 2.64	49.07 ± 2.63	49.04 ± 2.71	0.878
Very low birth weight (< 1500 g) n (%)	12 (1.59)	9 (1.47)	3 (2.13)	0.478
Low birth weight (1500-2499 g) n (%)	126 (16.73)	99 (16.18)	27 (19.15)	0.394
Normal birth weight (2500-3999 g) n (%)	588 (78.09)	479 (78.27)	109 (77.31)	0.803
Macrosomia (≥ 4000 g) n (%)	27 (3.59)	25 (4.08)	2 (1.41)	0.125

IQR, interquartile range (25% and 75% percentiles); SD, standard deviation.

($p=0.685$). The newborns' height and weight were similar across the groups, showing no notable discrepancies ($p=0.879$ and $p=0.559$, respectively). The incidence of very low birth weight and low birth weight was marginally elevated in the UU-positive group (2.13% vs. 1.47%, $p=0.478$ and 19.15% vs. 16.18%, $p=0.394$), but the differences were not statistically significant. Compared to the UU-negative group (4.08%), macrosomia occurrence was lower in the UU-positive group (1.41%), however, this difference did not reach statistical significance ($p=0.125$) (Table 4).

Discussion

Approximately 15% of male infertility patients present with concurrent genital tract infections (13). Colonization by *Mycoplasma* in the urogenital tract is common, with a significant number of asymptomatic carriers, especially for UU in the male reproductive system (14, 15). However, there is ongoing debate regarding the effect of male reproductive system infection, particularly semen UU infections, on semen quality and pregnancy outcomes (16). Several studies have suggested that genital tract infections can lead to oligoasthenozoospermia as well as abnormal sperm DFI and HDS (6). However, other studies have found no apparent correlation between genital tract infection, male semen parameters, and adverse pregnancy outcomes (17).

This study investigates the impact of UU infection on various semen parameters, embryo quality, pregnancy outcomes, and neonatal outcomes within a cohort of 1,215 male patients, divided into UU-positive and UU-negative groups. The results indicate no significant differences in baseline characteristics between these groups, while male semen analysis revealed higher ASA levels and high DNA stainability (HDS) in the UU-positive group. In contrast, other semen parameters showed no significant differences. Similarly, the study found no substantial differences in IVF embryo quality, pregnancy outcomes, and neonatal outcomes between the UU-positive and UU-negative groups. These findings suggest that while UU infection may affect specific semen parameters, it does not significantly impact overall IVF reproductive outcomes.

The observed differences in semen parameters, particularly in ASA levels and HDS, are consistent with previous studies highlighting the potential pathogenic role of UU in male infertility (18) reported that UU and *Ureaplasma parvum* (UPA) infections could impair semen motility and induce inflammation, contributing to male infertility. The increase in ASA levels in the UU-positive group supports the hypothesis that UU infection can trigger an immune response, leading to the production of antibodies against sperm, which could impair sperm function and reduce fertility potential (18). Moreover, the higher HDS values observed in the UU-positive group align with findings by Aghazarian et al. (19), who demonstrated that UU infection is associated with increased DNA damage in sperm and reduced sperm quality. This suggests that UU may contribute to male infertility through mechanisms involving both immunological responses and direct damage to sperm DNA.

Despite the differences in ASA levels and HDS, the overall semen parameters, including sperm concentration, motility, and morphology, did not show significant differences between the UU-positive and UU-negative groups. Fu et al. (3) observed that although UU infection is associated with decreased motility and concentration of sperm, these effects might not be significant in all cases. The lack of significant differences in most semen parameters could be due to the variability in individual responses to UU infection, as well as differences in the severity and duration of infection. Furthermore, studies have shown that other factors, such as co-infections with other urogenital pathogens and individual genetic susceptibility, might influence the extent of the impact of UU on semen quality (20, 21). Although the UU-positive and UU-negative groups showed significant differences in HDS, the proportion of HDS in the UU-positive group remained under 15% threshold that is commonly thought to impact embryo quality (22). Additionally, studies conducted on mice and humans have revealed that oocytes might repair DNA damage (23).

The study also assessed the impact of UU infection on IVF outcomes, including embryo quality, pregnancy rates, and neonatal outcomes. The findings indicate no significant differences in these parameters between the UU-positive and UU-negative groups, suggesting that UU infection may not substantially affect the success of IVF treatments. This aligns with the findings of previous studies that have shown mixed or inconclusive evidence regarding the impact of UU on reproductive outcomes. For instance, Bai et al. (8), found no significant differences in IVF outcomes between men with and without UU infection, while other studies have reported slight reductions in pregnancy rates and increased miscarriage rates in UU-positive couples. The present study's findings contribute to the growing body of evidence suggesting that while UU may impact semen quality, its effects on IVF outcomes may be less pronounced, particularly when other factors, such as the quality of oocytes and embryos, are optimal.

The neonatal outcomes examined in this study, including gestational age, preterm delivery rates, birth weight, and Apgar scores, also showed no significant differences between the UU-positive and UU-negative groups. This finding is consistent with the results of several studies that have reported no substantial impact of UU infection on neonatal health (24, 25). However, it is essential to note that while UU infection may not directly affect neonatal outcomes, the potential for long-term health effects on offspring due to subclinical infections or immune responses in the mother and fetus cannot be entirely ruled out (26). It is noteworthy that these microorganisms have the potential to cause a range of urogenital infections, including urethritis, prostatitis, epididymitis, and orchitis. Consequently, male patients testing positive for UU should be considered for eradication therapy with suitable antibiotic regimens. Further research is needed to explore the potential long-term consequences of UU infection during pregnancy and its implications for neonatal and child health.

Despite the comprehensive nature of this study, several limitations should be considered. First, the cross-sectional design limits the ability to establish causal relationships between UU infection and the observed outcomes. Longitudinal studies with

larger sample sizes and follow-up data would provide more robust evidence regarding the impact of UU on male fertility and reproductive outcomes. Additionally, the culture method was unable to provide an absolute quantitative analysis of UU, limiting the ability to precisely measure its load in clinical samples. The amount of UU may be associated with disease outcomes. Therefore, more accurate quantification could improve our understanding of its clinical significance. Although common pathogens known to impact male fertility were excluded, some uncommon bacterial species were not ruled out. Additionally, the study did not account for potential confounding factors, such as the presence of other urogenital pathogens, the duration and severity of UU infection, and the patients' previous treatment history, which could influence the results. Finally, the reliance on standard semen parameters may not capture the full extent of the impact of UU on sperm function, and more advanced diagnostic techniques, such as proteomics and genomics, could provide deeper insights into the molecular mechanisms underlying UU-related infertility.

In summary, our study's results suggest that male UU infection may have some adverse effects on semen parameters, but IVF technology remains a viable and safe option for infertile couples with male UU infection.

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 humans were approved by the Ethical Committee of the First Affiliated Hospital of USTC (Approval ID: 2022-RE-261). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

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Author contributions

YW: Data curation, Writing – original draft, Conceptualization, Methodology, Project administration, Resources. XC: Formal analysis, Investigation, Methodology, Software, Writing – original draft. ZC: Investigation, Software, Writing – original draft. WL: Data curation, Writing – original draft. SL: Investigation, Writing – original draft. JH: Project administration, Writing – review & editing, Supervision, Funding acquisition.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was funded by the National Natural Science Foundation of China (grant numbers 82301800 and 82101685).

Acknowledgments

We thank all the IVF patients and the reproductive center involved in the research.

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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OPEN ACCESS

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RECEIVED 28 March 2024

ACCEPTED 14 October 2024

PUBLISHED 16 December 2024

CITATION

Xie Q, Jiang X, Zhao M, Xie Y, Fan Y, Suo L
and Kuang Y (2024) Effect of freezing and
thawing on ejaculated sperm and subsequent
pregnancy and neonatal outcomes in IVF.
Front. Endocrinol. 15:1408662.
doi: 10.3389/fendo.2024.1408662

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Effect of freezing and thawing on ejaculated sperm and subsequent pregnancy and neonatal outcomes in IVF

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Background: Techniques for sperm cryopreservation have exhibited their potential in male fertility preservation. The use of frozen–thawed sperm in *in vitro* fertilization (IVF) cycles is widespread today. However, many studies reported that cryopreservation might have adverse effects on sperm DNA integrity, motility, and fertilization, probably due to cold shock, intra- and extracellular ice crystals, and excess reactive oxygen species (ROS). Studies suggested that freezing and thawing impaired sperm viability and might adversely affect subsequent fertilization and pregnancy outcomes. The potential damage to fertilization and subsequent embryonic development and offspring health raises the concern on sperm cryopreservation. However, the above mentioned studies are limited to intracytoplasmic sperm injection (ICSI) cycles, while IVF is a more natural and patient-friendly method. IVF requires a higher quality of sperm than ICSI. However, the effect of freezing and thawing on sperm used for IVF remains unknown. Therefore, we aim to investigate the effect of freezing and thawing on ejaculated sperm and subsequent pregnancy and neonatal outcomes in IVF.

Methods: This retrospective cohort study at a tertiary-care academic medical center included 447 women who used paternal frozen–thawed ejaculated sperm and 31,039 women who used paternal freshly ejaculated sperm for IVF and underwent frozen–thawed blastocyst transfer from January 2011 to September 2021. To balance the baseline characteristics of the two groups, patients using frozen sperm were matched with control groups using a propensity score matching algorithm with a ratio of 1:3.

Results: Although sperm motility decreased from 82.04% to 75.70% ($P < 0.001$) after the freezing–thawing process, the fertilization rate (68.27% for frozen sperm and 67.54% for fresh sperm), number of viable embryos (1.98 and 2.16), clinical pregnancy rate (44.7% and 51.8%), and live birth rate (40.3% and 42.4%) were comparable between the two groups (all $P > 0.05$). For neonatal outcomes, no between-group differences were observed in offspring gender, gestational age,

birthweight, and the rate of preterm birth (21.7% and 12.9%), low birthweight neonates (19.2% and 16.0%), and birth defects (0.0% and 0.8%) (all $P > 0.05$).

Conclusions: Frozen–thawed sperm had lower sperm motility but resulted in comparable embryonic, pregnancy, and neonatal outcomes versus fresh sperm in IVF cycles.

KEYWORDS

frozen sperm, *in vitro* fertilization, assisted reproduction, pregnancy outcomes, neonatal outcomes

1 Introduction

Since the first successful pregnancy from frozen–thawed human sperm (1), cryopreservation of sperm has helped thousands of patients with azoospermia and severe oligozoospermia (2, 3). Thanks to the rapid development of cryopreservation techniques, the applied range of frozen sperm has expanded to a larger population nowadays and made it possible for male fertility conservation, sperm donation, and regular assisted reproductive technology (ART) treatment (4, 5).

Application of frozen–thawed sperm in regular *in vitro* fertilization (IVF) treatments is considered essential and patient-friendly. Freezing sperm in advance can offer both patients and doctors flexible schedules, avoid oocyte waste in case of unpredictable early ovulation, and help relieve tensions that may lead to failed sperm retrieval.

However, some studies have pointed out that the freezing–thawing process would impair sperm motility, DNA integrity, and fertilization capacity, probably due to cold shock, intracellular and extracellular ice crystals, and excessive ROS (6–14). Meanwhile, some clinical studies focused on intracytoplasmic sperm injection (ICSI) cycles suggested that the freezing–thawing process did impair sperm motility but had no detrimental effect on subsequent fertilization and pregnancy outcomes (15–18). Compared with ICSI, IVF is a more natural and patient-friendly way but has a higher requirement for sperm density and motility. Some studies showed a little lower live birth rate in IVF cycles than in ICSI cycles (11–13) (19). However, to our best knowledge, the effect of the freezing–thawing process on clinical outcomes in IVF cycles remained unclear.

Taken together, the aim of this study is to assess the effect of frozen–thawed sperm in IVF cycles on embryonic, pregnancy, and neonatal outcomes.

2 Materials and methods

2.1 Ethical approval

This study was approved by the Ethics Committee (Institutional Review Board) of the hospital (SH9H-2020-T400-1).

2.2 Study design and patients

We performed this retrospective cohort study at the Department of Assisted Reproduction of Shanghai Ninth People's Hospital affiliated with Shanghai Jiao Tong University School of Medicine. We studied all women who underwent IVF with paternal ejaculated sperm and subsequent frozen–thawed blastocyst transfer from January 2011 to September 2021. After excluding patients with core data missing or repeated cycles during the study period, women using frozen–thawed sperm were matched with those who used fresh sperm through propensity score matching.

2.3 Sperm cryopreservation and laboratory protocols

The standard operating procedure of freezing and thawing sperm in our center remained unchanged during the study period. To freeze sperm, we added an equal volume of cryoprotectant to ejaculated sperm and placed it at 4°C for 20 min at first and then in liquid nitrogen vapor for 10 min. The sperm was immersed in liquid nitrogen until use. To thaw sperm, the samples were placed at 37°C for 3 min. For semen preparation, fresh sperm was prepared after liquefaction, and frozen sperm was prepared after thawing. First, semen was centrifuged using isolate UP and isolate DOWN. Then, we added 1-mL human tubal fluid (HTF; Irvine Scientific, USA) with 5%(v/v) serum substitute supplement (SSS; Irvine Scientific, USA) to the pellet and let the sperm swim up at 37° for 60 min. The sperm quality was assessed under a microscope.

IVF was performed 4–6 h after oocyte retrieval. Sperm was added into the drop of HTF with 10% (v/v) SSS. Fertilization was assessed 16–20 h later, and two pronucleus would be considered as normal fertilization. The zygotes were cultured in a humidified atmosphere containing 5% O₂ and 6% CO₂ at 37°C.

The endometrial preparation for FET has been previously reported in detail (20). Briefly, women with regular ovulatory cycles, irregular menses, or a history of thin endometrium underwent modified natural cycles, mildly stimulated cycles, or artificial cycles, respectively.

As previously reported (21), embryo vitrification was performed with a Cryotop carrier system (Kitazato Biopharma Co.), and dimethyl-sulfoxide-ethylene glycol-sucrose was used as cryoprotectants. A descending concentration gradient of sucrose (1 to 0.5 to 0 mol/L) was used for embryo thawing.

2.4 Outcome measures and definitions

The main outcomes included fertilization rate (%), number of viable embryos, clinical pregnancy rate (%), and live birth rate (%). Other outcomes included concentration and motility after freezing-thawing process, viable rate per oocyte retrieved (%), number of good embryos, good embryos rate per oocyte retrieved (%), biochemical pregnancy rate (%), implantation rate (%), and miscarriage rate (%).

Normal fertilization was defined as the presence of two pronucleus after IVF. Clinical pregnancy was defined as the presence of a gestational sac as detected by ultrasound examination at 7 weeks post-FET. The implantation rate was defined as the proportion of the number of gestational sacs among the number of embryos transferred. Live birth was defined as the delivery of any viable infant at more than 28 weeks of gestation.

2.5 Statistical analysis

We performed propensity score matching to account for differences in baseline characteristics. Women using frozen sperm were matched with controls who used fresh sperm by using the nearest matching pattern with a ratio of 3. The propensity scores were calculated by logistic regression with 10 covariates including female age (continuous), male age (continuous), female BMI (continuous), type of infertility (primary or secondary), infertility duration (continuous), gravidity (0 or ≥ 1), parity (0 or ≥ 1), female infertility diagnosis (tubal, mixed/other factors, unexplained), sperm concentration before preparation (continuous), sperm motility before preparation (continuous), treatment protocol (PPOS, mild stimulation, GnRH-ant protocol, long protocol, short protocol and natural cycles), treatment year (2011–2013, 2014–2015, 2016–2018 and 2019–2020), and number of oocytes retrieved (continuous).

Continuous variables were presented as mean with standard deviation, and categorical variables were presented as frequencies with percentages. First, we test normality by the Shapiro–Wilk test and the homogeneity of variances by Levene's test. According to the results of the above mentioned tests, continuous variables were compared by using *t*-test or *U*-test as appropriate. For categorical variables, between-group differences were analyzed by using the chi-square test or Fisher exact test as appropriate.

Statistical analysis was performed using R statistical programming language (version 4.0.3; R Foundation for Statistical Computing, Vienna, Austria), and statistical significance was set at $P < 0.05$.

3 Results

The flowchart of this study is presented in Figure 1. Briefly, a total of 54,785 cycles using IVF were screened from our database. After excluding 22,415 repeated cycles and 884 cycles whose core data were missing, 31,486 cycles remained. A total of 447 cycles with frozen sperm were matched with 1,341 cycles with fresh sperm at a ratio of 3. Supplementary Figure S1 shows the distribution of propensity scores before and after matching.

3.1 Baseline characteristics

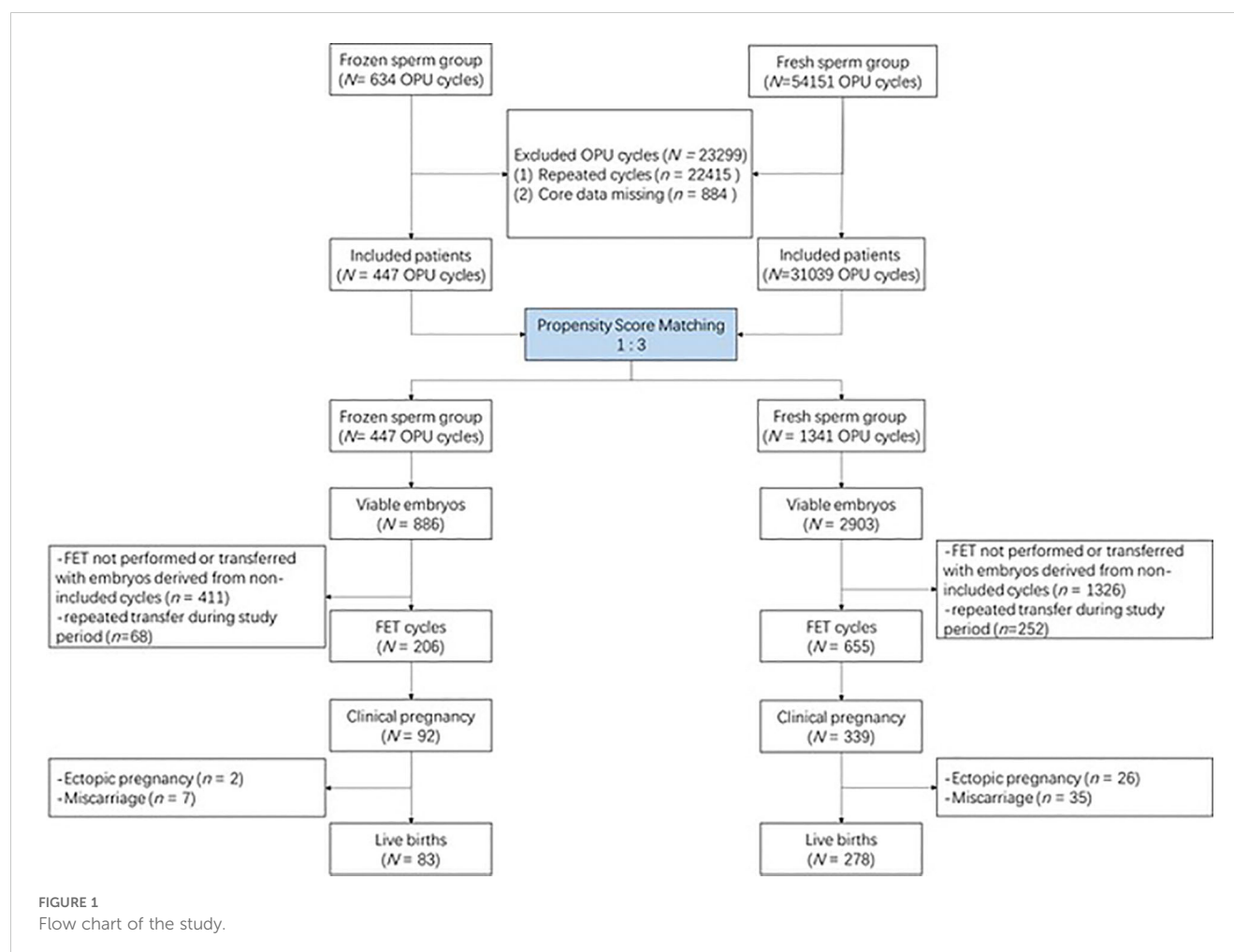
As demonstrated in Table 1, no significant between-group differences were found in the post-matching analysis with regard to all baseline characteristics, including female age, male age, female body mass index, infertility type, infertility duration, female infertility diagnosis, gravidity, parity, treatment protocol, OPU year, as well as sperm concentration and motility before preparation (all $P > 0.05$).

3.2 Laboratory outcomes

In our center, sperm were frozen before the preparation step. After thawing, the sperm would be measured first, prepared, and measured again. As presented in Table 2, sperm motility decreased from 82.04% to 75.70% ($P < 0.001$) after the freezing–thawing process, while the sperm concentration decreased slightly from 30.92 to 29.68 $\times 10^6$ /mL ($P = 0.001$). After *in vitro* fertilization, the fertilization rates between the two groups were comparable (68.27% for frozen sperm and 67.54% for fresh sperm, $P = 0.704$). The number of viable embryos also was not impaired by the freezing–thawing process of sperm (1.98 for frozen sperm and 2.16 for fresh sperm, $P = 0.168$). Similarly, there were no significant between-group differences when the number and rates of good embryos and blastocysts were analyzed (all $P > 0.05$).

3.3 Pregnancy outcomes

Table 3 shows the pregnancy outcomes of frozen–thawed embryos derived from included OPU cycles. No between-group differences were observed in the number of transferred embryos, embryo stage at transfer, and endometrial preparation protocol. Among 206 cycles using frozen sperm, 102 cycles (49.5%) led to clinical pregnancy, while 369 cycles (56.3%) of 655 cycles using fresh sperm resulted in clinical pregnancy ($P = 0.102$). The biochemical pregnancy rate and implantation rate were also comparable between the frozen sperm and fresh sperm groups (all $P > 0.05$). Similarly, the live birth rate per FET cycle was 40.3% in the frozen sperm group and 42.4% in fresh sperm cycles ($P = 0.642$).



3.4 Neonatal outcomes

As demonstrated in Table 4, 62 singletons and 42 twins were born from frozen sperm, and 219 singletons and 118 twins were born from fresh sperm. In both singletons and twins, no between-group differences were observed in offspring gender, gestational age, and birthweight (all $P > 0.05$). For adverse neonatal outcomes, the comparisons did not reveal any significant differences in preterm birth (<37 weeks), low birthweight (<2500 g), and major congenital malformation (all $P > 0.05$).

4 Discussion

In this retrospective study, we found that although sperm motility was impaired after the freezing–thawing process, frozen–thawed sperm led to that are comparable with those of pregnancy and neonatal outcomes fresh sperm.

4.1 Strength and weakness

The sample size of 447 cycles using frozen–thawed sperm for IVF is the largest, to our best knowledge, in this topic. Besides that,

propensity score matching, a highly specialized follow-up system, and careful selection of the study population made the statistical model more reliable and less biased.

However, this study is limited by the retrospective design, which cannot exclude unknown confounders. Prospective studies with a larger sample size are needed to confirm our results. Another limitation is that the results could be restricted to frozen–thawed embryo transfer cycles.

4.2 Comparison with previous studies

Techniques for sperm cryopreservation have been widely used in patients with azoospermia and severe oligozoospermia. Many studies have analyzed the effect of frozen sperm for ICSI in these patients, and three meta-analyses have summarized them well. The first one analyzed 17 researches with 1,476 ICSI cycles performed before 2004 in patients, with obstructive or non-obstructive azoospermia (17) and showed that, compared with the fresh sperm group, the clinical pregnancy rate decreased in the frozen–thawed epididymal sperm group (RR: 1.20; 95% CI: 1.0–1.42) and the implantation rate decreased within the frozen testicular sperm group (RR: 1.75; 95% CI: 1.10–2.80), while the fertilization rates were comparable among the three groups (17). The second meta-analysis including 574 ICSI cycles in patients with nonobstructive azoospermia was published in 2014

TABLE 1 Baseline characteristics by group.

	Frozen sperm (N=447)	Fresh sperm (N=1,341)	P-value
Female age	36.85 ± 5.48	36.81 ± 5.50	0.908
Male age	39.35 ± 6.90	39.35 ± 6.71	0.778
Female body mass index	21.86 ± 3.22	21.78 ± 3.04	0.722
Primary infertility	172 (38.5)	524 (39.1)	0.867
Duration of infertility	4.21 ± 4.00	4.15 ± 3.97	0.811
Female infertility diagnosis			0.928
Tubal	126 (28.2)	369 (27.5)	
Mix/other factors	291 (65.1)	886 (66.1)	
Unexplained	30 (6.7)	86 (6.4)	
Gravidity ≥1 (%)	273 (61.1)	809 (60.3)	0.823
Parity ≥1 (%)	80 (17.9)	242 (18.0)	1.000
Treatment protocol (%)			0.966
PPOS	232 (51.9)	722 (53.8)	
Mild stimulation	93 (20.8)	276 (20.6)	
GnRH-ant protocol	31 (6.9)	81 (6.0)	
Long protocol	38 (8.5)	103 (7.7)	
Short protocol	26 (5.8)	78 (5.8)	
Natural cycle	27 (6.0)	81 (6.0)	
OPU year (%)			0.790
2011–2013	150 (33.6)	425 (31.7)	
2014–2015	149 (33.3)	443 (33.0)	
2016–2018	110 (24.6)	342 (25.5)	
2019–2021	38 (8.5)	131 (9.8)	

Data are presented as mean ± standard deviation or number (percentage).
PPOS, progestin-primed ovarian stimulation; OPU, oocyte pick-up.

and reported no difference in any clinical outcomes between frozen and fresh sperm (15). The latest one including 17 studies with 1,261 cycles was performed in 2018 and also reported a similar result—that is, cryopreservation did not affect the fertilization rate or the live birth rate (16).

The above mentioned evidence has indicated that using frozen–thawed sperm in ICSI might not affect the outcomes in patients with azoospermia. However it still cannot represent the effect on IVF in patients with normal sperm. First, sperm from patients with obstructive or non-obstructive azoospermia was usually retrieved from the epididymis or testis instead of by masturbation. Thus, we cannot exclude the possibility that the difference in terms of sperm origin would cause different tolerance levels to cryopreservation. Besides that, IVF requires higher sperm quality, which might be seriously impaired during the freezing–thawing process.

Another major application of frozen–thawed sperm is fertility preservation in cancer patients, which helps patients freeze their sperm before antineoplastic therapy and receive ART treatment later. Due to the specific study population of cancer patients, the sample sizes were usually very small. One study with 30 ICSI cycles reported that half cycles led to clinical pregnancies finally, which is comparable to the control group only with tubal factor infertility (22). Another three studies demonstrated differences between IVF and ICSI. The first one including 29 patients demonstrated slightly higher fertilization rate and pregnancy rate in ICSI cycles than in IVF cycles and ultimately five live births from the 26 IVF cycles (19.2%) and four from the 19 ICSI cycles (21.1%) (19). The second study including 75 cycles reported a similar fertilization rate (49% vs 51%) but with a lower live birth rate (34% vs 40%) in IVF than in ICSI (12). The last one with a comparatively larger sample size observed that the fertilization rate and delivery rate of frozen sperm dropped by half in 54 IVF cycles than in 169 ICSI cycles, and they also reported a comparable pregnancy rate of 118 cancer patients (56.8%) using sperm frozen before therapy to other male-factor patients in ICSI cycles (11).

Contrary to previous studies, frozen–thawed sperm led to fertilization rates and live birth rates that are similar with those of

TABLE 2 Cycle characteristics and outcomes by group.

	Frozen sperm (N=447)	Fresh sperm (N=1,341)	P-value
Sperm characteristics			
Concentration before preparation × 10 ⁶ /mL	66.17 ± 22.93	66.87 ± 22.30	0.128
Motility before preparation (%)	31.61 ± 12.83	31.88 ± 10.72	0.166
Concentration after preparation × 10 ⁶ /mL	29.68 ± 14.09	30.92 ± 12.77	0.001
Motility after preparation (%)	75.70 ± 19.42	82.04 ± 17.01	<0.001
Embryonic characteristics			
No. of oocytes retrieved			0.997
1–3	205 (45.9)	618 (46.1)	
3–10	182 (40.7)	541 (40.3)	
11–15	36 (8.1)	107 (8.0)	

(Continued)

TABLE 2 Continued

	Frozen sperm (N=447)	Fresh sperm (N=1,341)	P-value
Embryonic characteristics			
≥16	24 (5.4)	75 (5.6)	
No. of normal fertilized oocytes	2.93 ± 3.41	3.21 ± 3.68	0.184
Normal fertilization rate (%)	68.27 (35.89)	67.54 (36.14)	0.704
No. of viable embryos	1.98 ± 2.38	2.16 ± 2.47	0.168
Viable embryo rate per oocyte retrieved (%)	44.26 (37.33)	44.76 (37.07)	0.721
No. of good embryos	1.70 ± 2.13	1.95 ± 2.56	0.104
Good embryo rate per oocyte retrieved (%)	38.77 (37.69)	39.65 (36.80)	0.481
No. of viable blastocysts	0.32 ± 0.85	0.33 ± 0.91	0.789
Blastocyst rate per embryo cultured for blastulation (%)	15.98 (26.87)	17.37 (30.47)	0.988

Data are presented as mean ± standard deviation or number (percentage). Sperm were prepared after freezing–thawing. IVF, *in vitro* fertilization. P < 0.05 was considered as statistically significant and shown in bold.

the fresh sperm group in this current study. Sperm included in the abovementioned studies were frozen many years ago for fertility preservation. The long-term cryopreservation for fertility preservation and the underdeveloped freezing–thawing procedure

many years ago might be attributed for the impaired fertilization rate and subsequent live birth rate. Besides that, freezing–thawing techniques have developed rapidly over the years. New methods including antioxidants, vitrification, freezing in seminal plasma, and so on attempted to minimize the damage to sperm potential (23–26).

TABLE 3 Pregnancy outcomes of frozen–thawed embryos originating from the two regimens.

	Frozen sperm	Fresh sperm	P-value
No. of cycles	206	655	
Cycle characteristics			
No. of embryos transferred per cycle			0.876
Single	60 (29.1)	185 (28.2)	
Double	146 (70.9)	470 (71.8)	
Embryo stage at transfer			1.000
Cleavage stage	180 (87.4)	574 (87.6)	
Blastocyst stage	26 (12.6)	81 (12.4)	
Endometrial preparation			0.815
Mild stimulation	66 (32.0)	207 (31.6)	
Hormone replacement therapy	87 (42.2)	265 (40.5)	
Natural cycle	53 (25.7)	183 (27.9)	
Pregnancy outcomes per cycle			
Biochemical pregnancy rate	102/206 (49.5)	369/655 (56.3)	0.102
Clinical pregnancy rate	92/206 (44.7)	339/655 (51.8)	0.090
Implantation rate	121/352 (34.4)	422/1125 (37.5)	0.317
Miscarriage rate	7/92 (7.6)	35/339 (10.3)	0.561
Live birth rate	83/206 (40.3)	278/655 (42.4)	0.642

Data are presented as number (percentage). FET, frozen–thawed embryo transfer.

4.3 Possible mechanism

In line with previous studies, sperm motility was impaired after freezing–thaw process. The difference in sperm motility between the fresh and the frozen–thawed sperm is 6.34%. It should be noted that we evaluated frozen–thawed sperm motility after the isolation and swim-up procedure, and therefore this parameter could more accurately reflect the fertilization situation.

Many studies have described the possible mechanisms of freezing damage to sperm. Intracellular and extracellular ice crystals forming during the freezing–thawing process would damage organelle structure and function (27, 28). A Rhodamine 123-based study found that mitochondrial activity decreased by nearly half after the freezing–thawing process (29). Besides that, oxidative stress during cryopreservation is another vital factor that damages sperm quality (6, 7, 30). Decreased motility after thawing showed a close association with increased oxidation–reduction potential, which is related to damaged axonemal structure and plasma membrane (30). Moreover, many studies have reported that cryopreservation of sperm led to increased DNA fragmentation (7, 10, 31), which is related to an increased risk of miscarriage and lower live birth rate (26, 32–36).

Although freezing damage is still unavoidable nowadays, much literature reported that normal sperm is more resistant to freezing damage than poor-quality sperm. Donelly and colleagues compared the tolerance level of ejaculated sperm to cryopreservation in patients with and without male factor infertility (37). The DNA integrity of frozen–thawed sperm was impaired seriously (decreased by 24–40%) in infertile patients but stayed intact in fertile patients (19). Zhang *et al.* analyzed the relationship between the sperm parameters before cryopreservation and the recovery rate of

TABLE 4 Neonatal outcomes of live-born infants.

	Singletons			Twins		
	Frozen sperm	Fresh sperm	<i>P</i> -value	Frozen sperm	Fresh sperm	<i>P</i> -value
No. of children	62	219		42	118	
Male offspring	33 (53.2)	113 (51.6)	0.934	20/42 (47.6)	60/118 (50.8)	0.857
Gestational age (weeks)	38.13 ± 1.78	38.61 ± 1.41	0.126	36.00 ± 2.61	36.14 ± 1.78	0.954
Birthweight (g)	3,219.03 ± 511.23	3,312.83 ± 544.33	0.278	2,458.40 ± 582.22	2,520.08 ± 456.29	0.903
Adverse neonatal outcomes, <i>n</i> (%)						
Preterm birth (<37 weeks)	9 (14.5)	13 (5.9)	0.051	9/21 (42.9)	23/59 (39.0)	0.959
Low birthweight (<2,500 g)	3 (4.8)	13 (5.9)	1.000	17/42 (40.5)	41/118 (34.7)	0.634
Major congenital malformations	0 (0.0)	2 (0.9)	1.000	0/118 (0.0)	1/118 (0.8)	1.000

Data are presented as mean ± standard deviation or number (percentage).

progressive motility and found that the sperm quality, including concentration, progressive motility, and morphology, before freezing exhibited a positive correlation with the recovery rate of progressive motility after thawing (38). Studies in rhesus monkeys (39) and goats (40) also observed similar phenomena.

Lastly, the ultimate aim of sperm cryopreservation is to deliver a healthy baby. What does it matter if sperm motility is reduced as long as it does not affect embryo development and live birth? Eastick and colleagues used time-lapse microscopy to observe the development of embryos derived from fresh or frozen sperm and found a difference in the morphokinetic parameters and embryo development between the two groups (4). In this study, we prepared the sperm by isolation and swim-up to select and enrich healthy sperm. The motility of frozen–thawed sperm was only 6.34% lower than that of fresh sperm, which is also sufficient for successful IVF, and the final live birth rate was comparable. In the fresh sperm group, 655 transfers led to 278 babies (42.4%), while 206 transfers led to 83 babies (40.3%) in the frozen–thawed sperm group. Also, no between-group difference was observed in neonatal outcomes.

It should be noted that the live birth rate (41.9%) in this current study is much lower than the previous report (50.74%). The possible reason could be the advanced female age in this study (36.8 years old) than in the previous study (31.8 years old). The preference for frozen–thawed sperm in older couples probably resulted from clinical inconvenience and affordable payments. Therefore, instead of directly comparing patients using frozen or fresh sperm, we performed propensity score matching to minimize the differences in baseline characteristics and make the results more solid. Although the treatment and laboratory procedure remained consistent during the whole study period, we cannot deny the technique improvements in 10 years. To correct the effect of time, we included treatment year as a covariate into the matching model, and the primary results remained stable.

5 Conclusion

Frozen–thawed sperm resulted in embryonic and pregnancy outcomes comparable with those of fresh sperm in IVF and subsequent frozen–thawed embryo transfer cycles.

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 humans were approved by the Ethics Committee (Institutional Review Board) of Shanghai Ninth People's Hospital (SH9H-2020-T400-1). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

QX: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Software, Validation, Writing – original draft, Writing – review & editing. XJ: Data curation, Formal analysis, Investigation, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. MZ: Formal analysis, Investigation, Methodology, Project administration, Writing – original draft. YX: Resources, Software, Writing – original draft. YF: Supervision, Validation, Writing – original draft. LS: Conceptualization, Funding acquisition, Resources, Writing – review & editing. YK: Conceptualization, Project administration, Resources, Validation, Visualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Science and Technology Commission of Shanghai Municipality (22Y21900800, 21JC1403900).

Acknowledgments

The authors would like to express sincere gratitude to Professor Ben W. Mol, Department of Obstetrics and Gynecology, Monash University, for his generous help in study design and revision of manuscript.

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.2024.1408662/full#supplementary-material>

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OPEN ACCESS

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RECEIVED 08 September 2024

ACCEPTED 12 December 2024

PUBLISHED 07 January 2025

CITATION

Yao G, Dou X, Chen X, Qi H, Chen J, Wu P, Li J, Liang S, Han Z, Bai S and Hu X (2025) Association between sperm DNA fragmentation index and recurrent pregnancy loss: results from 1485 participants undergoing fertility evaluation. *Front. Endocrinol.* 15:1493186. doi: 10.3389/fendo.2024.1493186

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Association between sperm DNA fragmentation index and recurrent pregnancy loss: results from 1485 participants undergoing fertility evaluation

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Objective: Several male factors have been reported to play a role in recurrent pregnancy loss (RPL). The aim of this study is to explore the relationship between semen parameters, sperm DNA fragmentation index (DFI) and RPL.

Method: A total of 1485 participants were recruited from a university hospital between April 2020 and August 2022. Six hundred and thirtyfour men from couples with RPL were assigned to the case group, while 851 men from couple without RPL who underwent fertile evaluation were assigned to the control group. Semen parameters including sperm DNA fragmentation, were assessed.

Results: No statistically significant differences in semen parameters, sperm kinematics and DFI were observed between the case group and the control group. A higher proportion of men in the case group had a DFI > 30% compared to those in the control group; however, this difference was not statistically significant. Restricted cubic spline analysis revealed no significant non-linear relationships between continuous DFI and risk of RPL.

Conclusion: Our study indicates that there is no significant relationship between DFI and RPL risk. Further prospective studies are needed to explore the impact of DFI on fertility outcomes in couples experiencing RPL.

KEYWORDS

DNA fragmentation index, recurrent pregnancy loss, semen quality, DFI, RPL

Introduction

Approximately 15% of reproductive-age couples suffer from infertility globally, with male factors accounting for half of these cases (1, 2). Conventional semen analysis is a standard method for evaluating semen quality, including parameters such as volume, sperm concentration, motility and morphology. However, this method is limited to evaluating basic parameters and additional sperm functions, such as DNA integrity, acrosome reaction and capacitation, require further assessment. Sperm DNA integrity is a critical parameter for male reproduction. Due to the complex organization of haploid genome, the DNA within the sperm nucleus is often altered by protamine-mediated compaction (3). Previous studies have demonstrated that sperm DNA fragmentation can adversely affect fertilization and embryo development (4–6). DNA Fragmentation Index (DFI) is a parameter used to assess the integrity of sperm DNA. Specifically, DFI measures the percentage of spermatozoa exhibiting DNA fragmentation, which can be an indicator of sperm quality and fertility potential. High DFI values are associated with increased sperm DNA damage, which may result from factors like oxidative stress, apoptosis, or autophagy (7).

Recurrent pregnancy loss (RPL) is defined as two or more clinically recognized pregnancy losses before 20–24 weeks of gestation, including both embryo and fetal loss (8). According to previous epidemiological studies, the prevalence of RPL is 1–4% among all patients who achieve pregnancy (9). Several factors have been suggested to contribute to pathogenesis of RPL, including maternal age, genetic defects, uterine pathology, endocrine disorders, and infectious agents. However, the exact etiology of RPL in approximately 50–75% of patients remains unclear (10). Although most known causes of RPL are related to female factors, recent studies have also indicated that semen quality may influence the risk of RPL. A systematic review and meta-analysis including 16 studies and 2969 couples suggested that increased DFI was associated with an increased risk of RPL (11). Additionally, several studies reported male partners of couples with RPL were more likely to have high DFI compared with those with low DFI (12, 13). Importantly, new guidelines from the European Society of Human Reproduction and Embryology (ESHRE), the American Urological Association (AUA), and the American Society for Reproductive Medicine (ASRM) recommend sperm DFI as a parameter for evaluating RPL (14–16).

Assays for analyzing sperm DNA integrity have been developed in recent years, including the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, the comet assay, the sperm chromatin dispersion (SCD) assay, and the sperm chromatin structure assay (SCSA). In a recent review, the authors provided a comprehensive summary of the techniques, advantages, and disadvantages of the TUNEL, SCSA, SCD, and Comet assays (17). The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay is noted for its sensitivity, reliability, and minimal interrater variability. However, its disadvantages include high cost, the need for specialized equipment, and extensive training requirements. The sperm chromatin dispersion (SCD) assay is advantageous due to its

simplicity and minimal equipment requirements, though it is hindered by a high degree of interobserver variability. The comet assay offers sensitivity and reproducibility, but suffers from interobserver variability, the necessity for an experienced operator, and the use of variable protocols. Finally, the sperm chromatin structure assay (SCSA) is characterized by a standardized protocol, reproducibility, and the ability to examine a large number of cells, though it also faces limitations in terms of high cost, the need for specialized equipment, and specific training requirements. Among these tests, SCSA is utilized for assessing the DFI in several large cohorts, although there is an ongoing effort to establish a consensus approach that is both highly reliable and cost-effective. Data from SCSA indicate that DFI has a strong positive correlation with pregnancy outcomes and miscarriage (18). In this study, which includes a relatively large sample size, we measured sperm DFI using SCSA to investigate the association between DFI and RPL risk.

Methods

Participants

Between April 2020 and August 2022, a total of 1485 male partners of infertile couples at the Reproductive Center of The First Affiliated Hospital of University of Science and Technology of China (USTC) and the female partners aged between 22 and 40 years, were included in this retrospective study. Couples who conceived their previous natural pregnancy within 1 year and experienced RPL were assigned to the case group, while couples without RPL and at least one previous pregnancy or live birth were assigned to the control group. All control group underwent fertility treatments for female factor infertility such as tubal factors. Semen parameters, including sperm DNA fragmentation were assessed, and clinical characteristics, such as demographic factors, lifestyle factors, and medical history, were collected for all participants. Couples diagnosed with causes of RPL, such as abnormal karyotypes and mullerian ducts, diabetes, hyperprolactinemia, thyroid disorders and positive for the antiphospholipid antibodies, antinuclear antibodies, lupus antibodies and anti β_2 glycoprotein antibodies, were excluded from the study. The exclusion criteria in male partners of infertile couples included azoospermia, testicular cancer, cryptorchidism and genetic defects related to the male reproductive tract. This study was approved by the Ethical Committee of The First Affiliated Hospital of USTC (No. 2023-RE-196).

Routing semen analysis

All semen sample collected at couples with previous pregnancy or live birth less than one year. Semen parameters (e.g., semen volume, sperm concentration, motility, and morphology) were assessed according to the WHO criteria (37). Ejaculates were collected by masturbation, followed by liquefaction at 37°C for at least 30 minutes. Semen parameters, including sperm concentration, progressive motility, and total motility, were analyzed using a computer-assisted sperm analysis (CASA)

system (SAS-II, SAS Medical, Beijing, China). Sperm morphology was assessed following Diff-Quick staining (Anke Biotechnology, Hefei, China) using a light microscope (CX33, Olympus Corporation, Tokyo, Japan). Leukocytes were evaluated following peroxidase staining (Anke Biotechnology, Hefei, China). Antisperm antibodies (AsAs) were assessed using the mixed antiglobulin reaction (MAR) test (Anke Biotechnology, Hefei, China).

Detection of sperm DFI

The sperm DFI was assessed using the sperm chromatin structure assay (SCSA, Celula, Chengdu, China). Semen samples were diluted with TNE buffer (Tris-HCl, NaCl, and ethylenediaminetetraacetic acid (EDTA)), followed by treatment with an acidic detergent solution (TritonX-100, NaCl, and HCl, pH 1.2). After 30 seconds, staining buffer (acridine orange, citric acid, Na₂HPO₄, EDTA, NaCl, pH 6.0) was added. The samples were analyzed using a flow cytometer (Celula, Chengdu, China) and a minimum of 5000 cells were examined per sample. Chromatin damage was assessed using acridine orange staining, where green fluorescence indicated double-stranded DNA and red fluorescence indicated single-stranded and denatured DNA (19).

Statistical analysis

A systematic review and meta-analysis reported that there is a mean difference of DFI of 11% between RPL and fertile control group (20). Additionally, a recent study from China showed DFI higher than 30% in unexplained RPL group was 42% (13). By setting power at 90%, alpha error at 5%, the estimated sample size was 796 with 398 in each group. Qualitative variables were described as frequencies (percentages), and quantitative variables were described as the mean $\pm$ standard deviation (SD) if normally distributed and medians (interquartile range, IQR) if not. Pearson's chi-square test and Student's *t* test were used for parametric comparisons, and the Mann-Whitney *U* test was utilized for nonparametric comparisons. Logistic regression and multivariate regression model were used to examine the association between DFI and RPL. Covariates initially included age, BMI, education, smoking, alcohol consumption, chronic diseases, urogenital infections and varicocele. Restricted cubic spline (RCS) was performed to assess for the dose-response relationships between RPL and DFI. A *P* value < 0.05 was considered to indicate statistical significance. All statistical analyses were performed using Prism 9.0.

Results

Clinical characteristics of 634 men from infertile couples affected by RPL and 851 men from couples who underwent fertile evaluation are reported in Table 1. Age and BMI did not show any statistically significant differences between the case and control groups. Lifestyle factors, including smoking and alcohol

consumption, were similar between the two groups. No significant differences were observed in educational level, sex frequency, history of chronic diseases and urogenital infections. The duration of infertility (*P* < 0.001) and the prevalence of varicocele (*P* = 0.004) were significantly higher in the case group compared to the control group.

Semen parameters were further compared between two groups. No statistically significant differences in semen volume, sperm concentration, progressive motility, total motility, TMSC, morphology, AsAs, DFI, HDS and leukocytes were found between men in the case group and those in the control group (Table 2). In addition, the proportion of men with abnormal semen parameters did not differ significantly between the two groups. A higher proportion of men with DFI > 30% was observed in the case group compared to the control group (6.3% vs. 5.2%), but this difference did not reach statistical significance. Similarly, the study groups were comparable in terms of sperm kinematics, including VCL, VSL, VAP, BCF, ALH, MAD, LIN, WOB, and STR (Table 3).

The association between abnormal semen parameters and the risk of RPL was further investigated using logistic regression analysis (Table 4). No significant associations were observed in either the crude or adjusted models. We further performed multivariate regression models for evaluating the association between sperm DFI and RPL. However, no correlation between DFI and RPL were found (adjusted OR 1.01, 95% CI (1.00-1.03), *P* = 0.09, Table 5). RCS was performed to assess the dose-response relationships between RPL and continuous DFI (Figure 1). The DFI was included as a natural cubic spline function, adjusting for age, BMI, education, smoking, drinking, sex frequency, chronic disease, duration of infertility, urogenital infections, varicocele, semen volume, sperm concentration, total motility, progressive motility, TMSC and normal morphology. No significant non-linear relationships were observed between continuous DFI and the risk of RPL.

Discussion

Recent studies have suggested a positive correlation between sperm DFI and abnormal pregnancy history. In this study, a total of 1485 men from couples with RPL and those without RPL was recruited, and semen quality, including sperm DFI was evaluated. However, our results indicated that no significant relationship was observed between sperm DFI and the risk of RPL.

Several clinical characteristics, including lifestyle and medical history, have been associated with RPL. A recent study by Esquerre-Lamare et al. recruited 60 participants (33 men from couples with RPL and 27 men from couples with a history of live birth less than the one year) and assessed environmental and family factors as well as semen parameters (21). The results showed that the RPL group were more likely to have a higher male BMI and a family history of infertility. They also found no significant differences in andrological histories and clinical examinations (e.g., cryptorchidism, varicoceles, vas deferens, epididymal position) between cases and controls. However, our study showed that the mean BMI of men did not differ between the study groups, which is consistent with several

TABLE 1 Clinical characteristics in men from the case group and the control group.

Characteristics	Total (n=1485)	Control (n=851)	Case (n=634)	P value
Age, mean ± s.d.	32.2 ± 5.6	32.4 ± 5.8	32.0 ± 5.4	0.29
BMI ^a , mean ± s.d.	24.7 ± 3.4	24.7 ± 3.3	24.7 ± 3.5	0.78
Smoking status, n (%)				0.13
Nonsmoker ^b	865 (58.2)	510 (59.9)	355 (56.0)	
Smoker	620 (41.8)	341 (40.1)	279 (44.0)	
Drinking status, n (%)				0.60
Nondrinker ^c	677 (45.6)	393 (46.2)	284 (44.8)	
Drinker	808 (54.4)	458 (53.8)	350 (55.2)	
Education, n (%)				0.25
Middle school or lower ^d	299 (20.1)	162 (19.1)	137 (21.6)	
High school	253 (17.1)	139 (16.3)	114 (18.0)	
College/University	933 (62.8)	550 (64.6)	383 (60.4)	
Sex frequency (weekly), n (%)				0.55
<1	174 (11.7)	93 (10.9)	81 (12.8)	
1-2	973 (65.5)	563 (66.2)	410 (64.7)	
>2	338 (22.8)	195 (22.9)	143 (22.5)	
Chronic diseases, n (%) ^e				0.27
No	1309 (88.1)	757 (89.0)	552 (87.1)	
Yes	176 (11.9)	94 (11.0)	82 (12.9)	
Duration of infertility (year), n (%)				<0.001
<1	742 (50.0)	471 (55.4)	271 (42.7)	
1-2	375 (25.2)	207 (24.3)	168 (26.5)	
>2	368 (24.8)	173 (20.3)	195 (30.8)	
Urogenital infections, n (%) ^f				0.74
No	1277 (86.0)	734 (86.3)	543 (85.7)	
Yes	208 (14.0)	117 (13.7)	91 (14.3)	
Varicocele, n (%)				0.004
No	1372 (92.4)	801 (94.1)	571 (90.1)	
Yes	113 (7.6)	50 (5.9)	63 (9.9)	

BMI, body mass index; s.d, standard deviation.
^aCalculated as weight in kilograms divided by height in meters squared.
^bincluded never smoking and no smoking during the past 3 months.
^cincluded never drinking and no drinking during the past 3 months.
^dincluded primary school and junior high school.
^eincluded diabetes, hypertension, and hyperlipidemia.
^fincluded epididymitis, prostatitis, balanoposthitis, and seminal vesiculitis.

previously published results (13, 22). In addition, our study found that men from couples with RPL were more likely to have varicocele compared to men from couples without RPL. This finding is consistent with Busnelli et al., who reported a higher prevalence of varicocele in men with RPL compared to the men without RPL (14.9% vs. 8.0%), although this difference did not reach statistical significance (23).

Traditional semen analysis provides a quantitative assessment of sperm concentration, motility and morphology (24). Several studies have shown a trend of low sperm motility and high

TABLE 2 Semen parameters in men from the case group and the control group.

Semen parameters	Total (n=1485)	Control (n=851)	Case (n=634)	P value
Volume (ml), median (IQR)	3.2 (2.3-4.2)	3.2 (2.2-4.3)	3.1 (2.3-4.1)	0.87
< 1.5 ml, n (%)	107 (7.2)	69 (8.1)	38 (6.0)	0.12
Concentration (million/ml), median (IQR)	68.2 (38.0-120.0)	67.7 (37.3-123.2)	69.5 (39.6-118.3)	0.73
< 15 million/ml, n (%)	96 (6.5)	53 (6.2)	43 (6.8)	0.67
Progressive motility (%), median (IQR)	40.4 (28.6-52.3)	39.4 (27.3-52.0)	42.1 (29.8-52.7)	0.09
< 32%, n (%)	471 (31.7)	285 (33.4)	186 (29.3)	0.09
Total motility (%), median (IQR)	45.6 (32.6-58.6)	44.5 (31.5-58.5)	47.7 (33.6-58.8)	0.12
< 40%, n (%)	577 (38.9)	346 (40.7)	231 (36.4)	0.10
TMSC (million), median (IQR)	94.9 (39.1-204.0)	92.0 (36.1-202.4)	97.0 (44.5-205.2)	0.33
< 9 million, n (%)	91 (6.1)	53 (6.2)	38 (6.0)	0.85
Normal morphology (%), median (IQR; n)	7.0 (5.0-8.0; 1437)	7.0 (5.0-8.0; 826)	7.0 (5.0-8.0; 611)	0.38
< 4%, n (%)	106 (7.4)	61 (7.4)	45 (7.4)	0.99
Leukocytes($\times 10^6$ /ml), median (IQR; n)	0.08 (0.03-0.29; 873)	0.04 (0.01-0.08; 511)	0.06 (0.03-0.28; 362)	0.17
AsAs (%), median (IQR; n)	1.0 (0.0-3.0; 575)	1.0 (0.0-3.0; 334)	2.0 (0.0-4.0; 241)	0.20
DFI (%), median (IQR)	11.9 (7.6-17.9)	11.9 (7.6-17.8)	11.9 (7.7-18.3)	0.41
>30%, n (%)	84 (5.7)	44 (5.2)	40 (6.3)	0.35
HDS (%), median (IQR)	6.6 (4.9-8.8)	6.6 (4.8-8.8)	6.6 (5.0-8.9)	0.34

IQR, interquartile range; TMSC, total motile sperm count; AsAs, antisperm antibodies; DFI, DNA fragmentation index; HDS, high DNA stainability.

abnormal sperm morphology in men from couples with RPL (12, 25, 26). However, similar to our findings, many studies that examined the correlation between routine semen parameters and RPL found no significant correlation (27, 28). Since normal sperm genetic material is essential for successful fertilization, as well as embryo and fetal development, sperm DNA integrity is considered a key parameter for evaluating clinical pregnancy outcomes. Several

meta-analyses have reported that sperm DFI is associated with RPL (29, 30). However, these meta-analyses may be underpowered due to the limited number of studies and sample sizes included (20). Results from our study are consistent with Zhang et al. that reported no significant difference in DFI between RPL group and control group (27). Additionally, the researchers followed up with the participants for one year and observed a trend toward higher DFI

TABLE 3 Sperm kinematics in men from the case group and the control group.

Sperm kinematics	Total (n=1485)	Control (n=851)	Case (n=634)	P value
VCL (μ m/sec), median (IQR)	32.5 (23.3-43.9)	32.0 (22.7-43.5)	33.3 (24.0-44.7)	0.18
VSL (μ m/sec), median (IQR)	13.7 (9.1-18.5)	13.2 (8.8-18.5)	14.2 (9.6-18.6)	0.19
VAP (μ m/sec), median (IQR)	17.5 (12.0-23.5)	16.9 (11.7-23.4)	18.1 (12.6-23.7)	0.18
BCF (Hz), median (IQR)	8.3 (5.7-11.6)	8.0 (5.4-11.6)	8.7 (5.9-11.6)	0.13
ALH (μ m/sec), median (IQR)	2.3 (2.0-2.4)	2.2 (2.0-2.4)	2.3 (2.0-2.5)	0.25
MAD (degrees), median (IQR)	13.9 (11.1-16.6)	13.7 (10.9-16.6)	13.9 (11.3-16.7)	0.30
LIN, median (IQR)	38.7 (32.8-43.9)	38.4 (32.5-43.8)	39.1 (33.2-44.2)	0.26
WOB, median (IQR)	50.8 (45.3-55.1)	50.6 (45.1-55.0)	51.2 (45.5-55.4)	0.25
STR, median (IQR)	74.8 (64.4-79.4)	74.4 (63.9-79.2)	75.3 (65.1-79.6)	0.14

VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; BCF, frequency of beat cross; ALH, mean amplitude of lateral head displacement; MAD, mean angular displacement; LIN, linearity coefficient; WOB, wobble coefficient; STR, straightness coefficient.

TABLE 4 Odds ratios (95% confidence intervals) for abnormal semen parameters in men from couples with RPL.

Semen parameters	Case	
	OR (95% CI)	P
Crude		
Volume < 1.5 ml	0.72 (0.48-1.08)	0.12
Concentration < 15 million/ml	1.10 (0.72-1.66)	0.67
Progressive motility <32%	0.82 (0.66-1.03)	0.09
Total motility <40%	0.84 (0.68-1.03)	0.10
TMSC < 9 million	0.96 (0.62-1.47)	0.85
Normal morphology < 4%	1.00 (0.67-1.48)	0.99
DFI > 30%	1.24 (0.79-1.92)	0.35
Adjusted		
Volume < 1.5 ml	0.75 (0.49-1.13)	0.17
Concentration < 15 million/ml	1.00 (0.96-1.00)	0.99
Progressive motility <32%	0.80 (0.64-1.01)	0.06
Total motility <40%	0.80 (0.64-1.00)	0.05
TMSC < 9 million	0.89 (0.57-1.38)	0.60
Normal morphology < 4%	0.91 (0.60-1.38)	0.67
DFI > 30%	1.24 (0.78-1.95)	0.36

RPL, recurrent pregnancy loss; TMSC, total motile sperm count; DFI, DNA fragmentation index; OR, odds ratio; CI, confidence interval.
Adjusted model: adjusted for age, BMI, education, smoking, alcohol consumption, chronic diseases, urogenital infections, and varicocele.

in men from couples with subsequent abnormal pregnancy outcomes compared to those from couples with ongoing pregnancies. This suggests that DFI may be a potential semen parameter for predicting future reproductive outcomes.

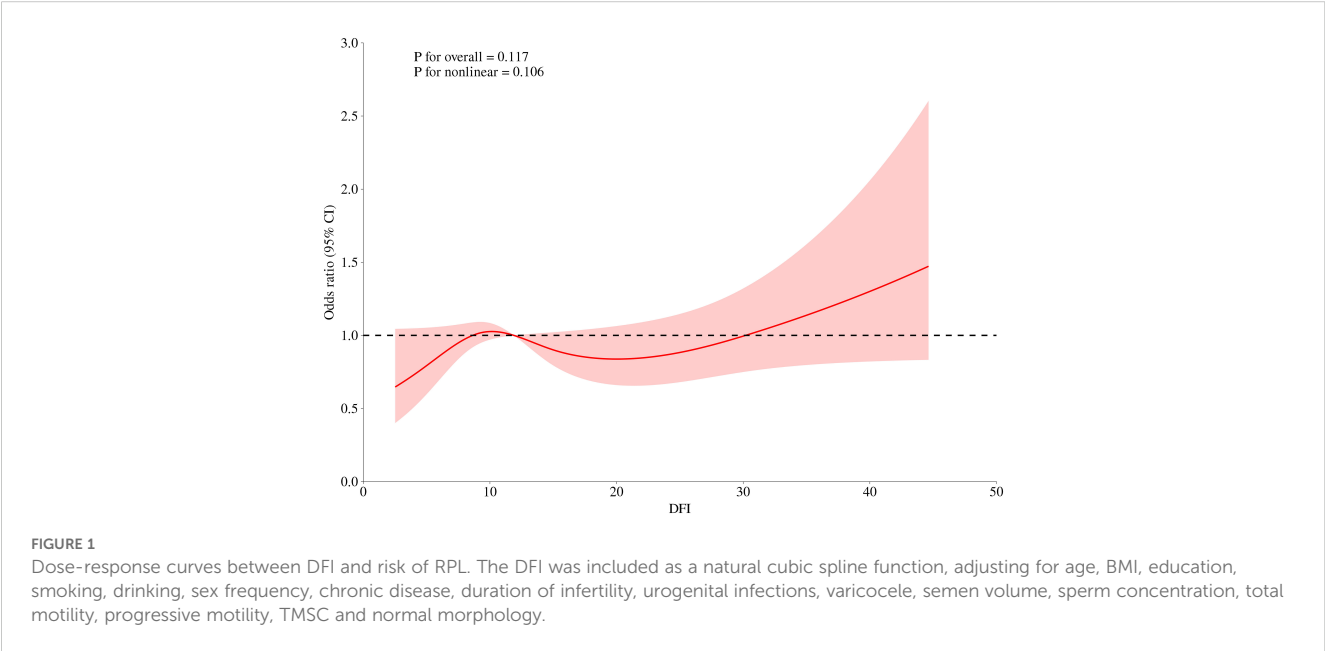
TABLE 5 Multivariate regression models for the association between sperm DFI and RPL.

DFI	RPL	
	OR (95% CI)	P
Crude	1.01(1.00-1.02)	0.23
Adjusted	1.01 (1.00-1.03)	0.09

DFI, DNA fragmentation index; RPL, recurrent pregnancy loss; OR, odds ratio; CI, confidence interval. Adjusted model: adjusted for age, BMI, education, smoking, alcohol consumption, chronic diseases, urogenital infections, varicocele, semen volume, sperm concentration, total motility, progressive motility, TMSC and normal morphology.

A cut-off DFI > 30% was reported as a severe DNA fragmentation in several studies (13, 31, 32). Consistent with other studies, our study also showed a trend of a higher proportion of sperm DFI ≥ 30% in men from the RPL group compared to those from the control group (22, 33). Interestingly, the RCS model indicated that when DFI exceeded 30%, the risk of RPL appeared to increase. Damaged DNA in the sperm may cause embryos to fail in their early stages, contributing to pregnancy loss (34). In addition, sperm DNA contributes to the development of placenta. Fragmented DNA can impair trophoblast development, leading to inadequate placental function, which is a known cause of pregnancy loss (35). Furthermore, DNA fragmentation may disrupt epigenetic marks in sperm, leading to errors in gene expression regulation during embryogenesis. This can result in developmental abnormalities and increase the risk of miscarriage (36). These findings suggest that a threshold DFI, rather than continuous DFI, may be more useful for assessing the correlation between DFI and RPL.

This study has several strengths. First, the relatively large sample size provides substantial statistical power. Additionally, our study included potential confounding factors (e.g. lifestyles and medical history) that have been previously reported to be associated with RPL. However, this study has several limitations. First, the study is limited by its



retrospective design and potential selection bias. Second, all participants were recruited from couples undergoing fertility evaluation at a single clinic. Third, although several potential confounders were adjusted for, residual confounding and unknown factors could not be ruled out. It is noted that the relationship between sperm DNA fragmentation and recurrent pregnancy loss cannot be fully understood without considering female factors. Maternal age, uterine environment, oocyte quality, and immune response all play significant roles in moderating the impact of DFI on pregnancy outcomes. Fourth, the SCSA assay cannot measure DNA strand breaks directly. Finally, different cut-off values for DFI may produce biased results.

In conclusion, our study found no significant relationship between sperm DFI and the risk of RPL. Further prospective studies are needed to investigate the impact of DFI on fertility outcomes in couples experiencing RPL.

Data availability statement

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

Ethics statement

This study was approved by the Ethical Committee of The First Affiliated Hospital of USTC (No. 2023-RE-196). The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because this study was a retrospective data analysis, the written informed consent was waived.

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Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This study was supported by the National Natural Science Foundation of China (81901543).

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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RECEIVED 29 September 2024

ACCEPTED 09 December 2024

PUBLISHED 09 January 2025

CITATION

Saleh R, Sallam H, Elsuity MA, Dutta S,
Sengupta P and Nasr A (2025) Antioxidant
therapy for infertile couples: a
comprehensive review of the current status
and consideration of future prospects.
Front. Endocrinol. 15:1503905.
doi: 10.3389/fendo.2024.1503905

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Antioxidant therapy for infertile couples: a comprehensive review of the current status and consideration of future prospects

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Oxidative stress (OS) is established as a key factor in the etiology of both male and female infertility, arising from an imbalance between reactive oxygen species (ROS) production and the endogenous antioxidant (AOX) defenses. In men, OS adversely affects sperm function by inducing DNA damage, reducing motility, significantly impairing sperm vitality through plasma membrane peroxidation and loss of membrane integrity, and ultimately compromising overall sperm quality. In women, OS is implicated in various reproductive disorders, including polycystic ovary syndrome, endometriosis, and premature ovarian failure, leading to diminished oocyte quality, disrupted folliculogenesis, and poorer reproductive outcomes. Antioxidant therapy represents a promising intervention to mitigate the harmful effects of ROS on reproductive health in additions to its easy accessibility, safety, and low cost. Despite several findings suggesting improvements in fertility potential with AOX therapy, the data remains inconclusive regarding optimal dosage and combination, duration of treatment, and the specific patient populations most likely to benefit. In this review, we discuss the role of AOXs in the management of infertile couples, focusing on their biological mechanisms, potential adverse effects, therapeutic efficacy, and clinical applications in improving reproductive outcomes in both natural conception and medically assisted reproduction. Additionally, we highlight the current practice patterns and recommendations for AOX supplementation during the course of infertility treatment. Further, we provide an overview on the limitations of the current research on the topic and insights for future studies to establish standardized AOX regimens and to assess their long-term impact on key outcomes such as live birth rates and miscarriage rates.

KEYWORDS

antioxidants, female infertility, male infertility, reactive oxygen species, oxidative stress

Introduction

The term oxidative stress (OS) applies when increased amounts of reactive oxygen species (ROS) surpass the antioxidant (AOX) capacity that naturally offers the protective balance (1). While OS plays a major role in the pathogenesis of male infertility (2–4), it is equally important to recognize the critical physiological roles of ROS in normal reproductive functions. ROS are indispensable for processes such as sperm capacitation, hyperactivation, and the acrosome reaction, which are essential for successful fertilization (5, 6). However, an imbalance favoring excessive ROS production can lead to oxidative damage, negatively affecting male fertility potential and reproductive outcomes (7, 8). Also, OS has been linked with many female infertility conditions such as polycystic ovary syndrome (PCOS) (9, 10), endometriosis (11–13), premature ovarian failure (POF) (14), and recurrent miscarriages (RM) (15).

Hence, AOX therapy to enhance fertility by protecting against the harmful effects of ROS is theoretically feasible. AOXs are not regulated as pharmaceutical drugs, thus allowing their utilization without prescription. Indeed, AOXs are extensively consumed due to their easy availability through different retail outlets (16). Also, the relatively low cost and good safety profile of AOX products are appealing to both healthcare providers and infertile couples. However, a significant diversity exists among practitioners in terms of prescribing AOXs for the purpose of infertility treatment (17).

In this review, we comprehensively discussed the types, mechanisms of action and safety profile of AOXs that are commonly prescribed in infertility clinics. We also highlight the attitudes and practice patterns of clinicians towards AOX prescription during the course of infertility treatment, and compare them with current recommendations by professional societies. Additionally, we discuss the impact of AOX therapy on fertility outcomes in men and women under natural and assisted

reproduction conditions. Furthermore, we highlight limitations of the current research on utility of AOXs for treatment of infertile couples and the need for well-designed studies that can bridge the gap between research and practice in this regard.

Types, natural sources and mechanism of action of antioxidants

An AOX is a natural substance that provides protection to the living cell against oxidative damage and deleterious effects of ROS (18). Under normal circumstances, there is a fine balance that enables AOXs to neutralize excess ROS, and maintain a small amount of ROS necessary for normal cell functions. Thus, when ROS production increases to harmful levels, AOXs play a key role in neutralizing them. In the living cell, the AOX defense system is generally classified into enzymatic and non-enzymatic elements.

Enzymatic AOXs include glutathione (GSH) peroxidase, glutathione reductase, catalase (CAT) and superoxide dismutase (SOD) (19). Nonenzymatic AOXs provide protection against ROS by binding to enzymatic AOXs and ions that helps subsequent suppression of enzymes involved in the oxidation process (16). They function by scavenging free radicals, stabilizing enzymatic AOXs, and chelating metal ions, thereby mitigating OS. Prominent non-enzymatic AOXs include vitamins such as Vitamin C and Vitamin E (17, 20, 21), glutathione (22, 23), coenzyme Q10 (24–27), carotenoids (e.g., beta-carotene, lycopene) (28), polyphenols (29), and trace elements like selenium and zinc (24, 30). AOXs are available in the market as individual or combined products. Generally, AOXs utilized in clinical practice are categorized as substances with direct AOX actions or substances with AOX properties (16). Table 1 summarizes the types, natural dietary sources and the mechanism(s) of action of AOXs.

Safety profile of antioxidants

AOXs are generally well tolerated and have a good safety profile. However, the fact that AOXs are largely available over-the-counter with a reasonable price increases the risk that some patients can overmedicate with AOXs leading to detrimental effects. It has been suggested that administration of large amounts or improper combinations of AOXs may lead to shift of the redox balance towards a 'reductive stress' status (31). The term 'antioxidant paradox' highlights the potential negative impact of over-utilization of AOX supplementation (32, 33). Recent reports indicate that the intake of excess AOXs may even cause impairment of male fertility potential due to reductive stress (34, 35). However, there is no clear understanding of the exact mechanism of action of reductive stress on male reproduction, and further research is required. Additionally, high doses of AOXs may be associated with some adverse effects. Furthermore, it is imperative to consider the role of excipients or fillers in commercially available supplements. For instance, titanium dioxide, previously regarded as a safe additive, has recently been implicated in genotoxicity, raising concerns about its safety. These findings underscore the need for a judicious and evidence-

Abbreviations: ALA, Alpha-Lipoic Acid; ALC, Acetyl-L-Carnitine; AOX, Antioxidant; ARG, L-Arginine; ART, Assisted Reproductive Technology; BMI, Body Mass Index; CAT, Catalase; CoQ10, Coenzyme Q10; COVID-19, Coronavirus disease-19; CPR, Clinical Pregnancy Rate; DHA, Docosahexaenoic Acid; DHEAS, Dehydroepiandrosterone Sulphate; ESCs, Ectopic Endometrial Stromal Cells; EPA, Eicosatetraenoic Acid; FSH, Follicle-Stimulating Hormone; GSH, Glutathione; GST, Glutathione-S-Transferase; HMG, Human Menopausal Gonadotropin; HO-1, Heme Oxygenase-1; HOMA, Homeostatic Model Assessment; ICSI, Intracytoplasmic Sperm Injection; IL, Interleukin; IMI, Idiopathic Male Infertility; IUI, Intrauterine Insemination; IVF, *In Vitro* Fertilization; LBR, Live Birth Rate; LDL, Low-Density Lipoprotein; LH, Luteinizing Hormone; MAR, Medically Assisted Reproduction; MDA, Malonaldehyde; MOSI, Male Oxidative Stress Infertility; MR, Miscarriage Rate; NAC, N-Acetyl Cysteine; NOA, Non-Obstructive Azoospermia; OHSS, Ovarian Hyperstimulation Syndrome; OS, Oxidative Stress; PCOS, Polycystic Ovary Syndrome; PG2 α , Prostaglandin F2 α ; POF, Premature Ovarian Failure; RCT, Randomized Controlled Trial; RM, Recurrent Miscarriages; ROS, Reactive Oxygen Species; SDF, Sperm DNA Fragmentation; SOD, Superoxide Dismutase; TAC, Total Antioxidant Capacity; TGF- β , Tumor Growth Factor- β ; TNF- α , Tumor Necrosis Factor- α ; VEGF, Vascular Endothelial Growth Factor; VLDL-C, Very-Low-Density Lipoprotein-C.

TABLE 1 Types, dietary sources and mechanism of action of antioxidants.

Supplement	Mechanism of action	Dietary source
Supplements with direct antioxidant action		
Carnitines	- Carnitine is found in the epididymis, seminal plasma and in spermatozoa, - Assists sperm metabolism by positively affecting sperm motility and maturation	Carnitines are present in meat, fish, poultry and dairy.
Carotenoids	β carotene is a provitamin A, which can directly scavenge ROS, β carotene protects lipid membranes from peroxidation, β carotene improves sperm motility and morphology.	Carotenoids are present in leafy green vegetables, fruits, and some vegetable oils.
Vitamin C	Diminishes DNA damage by scavenging free radicals and decreasing formation of lipid hydroperoxides.	Vitamin C is present in citrus fruits and vegetables.
Vitamin E	Vitamin E is the first defense against oxidant-induced membrane injury.	Vitamin E is present in vegetable oils (like wheat germ, sunflower, and safflower oils), nuts (such as peanuts, hazelnuts, and almonds) and seeds (like sunflower seeds).
Coenzyme Q10 (CoQ10) (ubiquinol)	- CoQ10 is a fat-soluble AOX synthesized endogenously. - An essential component of the mitochondrial energy metabolism.	CoQ10 is present in meat, fish, nuts and some oils.
Arginine/L-arginine	- Arginine is required for normal spermatogenesis. - Arginine directly protects against oxidative damage, - Arginine plays a role in the inflammatory response.	Arginine is present in meat products, dairy, nuts and seeds.
Cysteine and N-acetyl cysteine (NAC)	- Cysteine plays an important role in glutathione synthesis. - NAC is a precursor of the amino acid cysteine and a direct scavenger of ROS.	Cysteine and NAC are present in meat, fish, seafood, chicken or turkey, eggs, whole-grain products such as breads and cereals, broccoli, onions, and legumes.
Folic acid (folate or vitamin B9)	- Important for the synthesis of DNA, transfer RNA and the amino acids cysteine and methionine. - Scavenges oxidizing free radicals and inhibit lipid peroxidation.	Folate is present in green-leafy vegetables, liver, bread, yeast and fruits.
Selenium	- Selenium is involved in the cellular AOX defense by increasing the activity of the AOX enzyme glutathione peroxidase. - Selenium is essential for normal spermatogenesis.	Selenium is available in fish, meat products, and dairy.
Zinc	- Zinc is involved in DNA transcription and protein synthesis, - Zinc has extensive antioxidants properties. - Zinc has an important role in testes development and sperm functions.	Zinc is available in meat products, wheat and seeds.
Supplements with antioxidant properties		
Vitamin B (complex)	- Vitamin B is a water-soluble vitamin and consists of several precursor and coenzymes such as thiamine (B1), riboflavin (B2) and cobalamin (B12). - Vitamin B plays an important role in lowering plasma levels of homocysteine, thus reducing the pro-oxidant effects of the latter.	Vitamin B is mainly found in meat products, other examples of food sources are beans, potatoes, bananas and mushrooms.
Vitamin D	- Vitamin D is a fat-soluble vitamin, with the natural main source being dermal synthesis (sun light). The active form of vitamin D is 1,25-dihydroxyvitamin D. - Vitamin D3 has AOX properties mainly by inducing the antioxidant protein superoxide dismutase	Fish liver oil, oily fish, salmon, sardines, red meat, liver, egg yolk, and dairy products.
Myo-inositol	- Inositol is a polyalcohol, naturally occurring as nine stereoisomers including myo-inositol (MYO). - Myo-inositol, is considered as a “pseudovitamin”. - Myo-inositol is produced by Sertoli cells in response to follicle-stimulating hormone (FSH). - Inositol plays an important role in cell membrane formation and lipid synthesis. - The seminiferous tubules contain the highest concentration of myo-inositol - Myo-inositol is directly involved in regulation of motility, capacitation and acrosome reaction.	

(Continued)

TABLE 1 Continued

Supplement	Mechanism of action	Dietary source
Supplements with antioxidant properties		
	• Myo-inositol acts by increasing endogenous AOX enzymes and directly enhancing the mitochondria membrane potential.	
Polyunsaturated fatty acids (PUFAs)	• Omega 3 might have a free radical-scavenging potential. • PUFAs have a prooxidant rather than a direct antioxidant effects. • PUFAs increase the plasma fluidity of the sperm membrane. This fluidity makes the sperm vulnerable to damage by ROS and lipid peroxidation.	The main sources of PUFAs are vegetable and fish oils. Omega-9 is synthesized by animals, but omegas-3 and -6 need to be supplemented in the diet.
Resveratrol	• Resveratrol is a natural phytoalexin with antioxidant properties. • Resveratrol enhances AOX defenses and improves sperm motility.	Resveratrol is naturally found in grapes, berries, and several nuts.
Lycopene	• Lycopene is an AOX that plays many roles including cell growth regulation, gap junction communication, gene expression modulation, immune response, and protection of lipid peroxidation.	Lycopene belongs to the family of carotenoids found in many bright fruits and vegetables (e.g., tomato) that includes alpha-tocopherol, alpha-carotene, beta-cryptoxanthin, beta-carotene, and lutein, and is the most effective AOX among the carotenoid family.

AOX, Antioxidant; CoQ10, Coenzyme Q10; NAC, N-acetyl cysteine; ROS, Reactive oxygen species.

based approach in the formulation and administration of AOX supplements to ensure both their safety and therapeutic efficacy (36, 37). A summary of potential adverse effects of AOXs frequently used in the treatment of infertile couples is provided in Table 2 (38–54).

Antioxidant therapy of male infertility

Increased public awareness of the positive impact of nutrition on different body functions highlights the significance of dietary modification as an important strategy to enhance fertility potential (Figure 1). A healthy diet or an extra dietary intake of AOXs has been correlated with high sperm quality in healthy individuals (55–58).

Rationale of antioxidant therapy of infertile men: role of oxidative stress

In the male genital tract, seminal ROS are produced by sperm, white blood cells, and as by- products during various metabolic processes. Small amounts of ROS are required for normal sperm functions such as sperm capacitation and acrosome reaction (2, 59). Excess ROS production may occur under different circumstances such as smoking (60), leukocytospermia (61), and varicocele (62). Male Oxidative Stress Infertility (MOSI) defines a group of infertile men with abnormal semen analysis results and excess ROS in their semen (63). This category of patients was previously classified as having idiopathic male infertility (IMI). The high prevalence of IMI highlights the significance of MOSI diagnosis during infertility workup. OS significantly impairs male fertility through disruption of sperm capacitation, induction of DNA damage such as sperm DNA fragmentation (SDF), reduced motility, and diminished sperm membrane fluidity. These detrimental effects are further

exacerbated by a marked decline in sperm vitality, resulting from plasma membrane peroxidation and loss of membrane integrity. Together, these factors compromise overall sperm quality and hinder the fertilization process (64, 65).

AOX supplementation has demonstrated significant potential in managing MOSI, as highlighted by a systematic review encompassing 97 clinical trials. These studies revealed the beneficial effects of AOX supplementation in men with (a) abnormal semen quality, (b) varicocele, and (c) idiopathic or unexplained infertility (66). However, the efficacy of AOX therapy relies heavily on the accurate identification of OS through reliable diagnostic testing. Assessing OS biomarkers, including ROS levels, total antioxidant capacity (TAC), lipid peroxidation products and DNA fragmentation, is critical for detecting redox imbalances. Advanced diagnostic techniques, such as chemiluminescence, ELISA, and sperm-specific OS tests, provide precise evaluations of OS in semen (67–69). By employing this targeted diagnostic approach, AOX therapy can be personalized for individuals with confirmed OS, avoiding the risks associated with empirical or excessive supplementation, such as reductive stress (7). Integrating OS testing into clinical protocols ensures optimized therapeutic outcomes, personalized interventions, and minimizes potential adverse effects related to inappropriate AOX use.

The impact of different AOX supplementations to improve fertility outcomes in infertile men has been the subject of extensive research in the last few decades. Additionally, the growing interest in AOX therapy for male infertility has led to the development of variety of AOX combinations. In the male, AOXs may enhance fertility through preserving sperm DNA integrity and mitochondrial transport (70). The AOXs that are commonly used in published studies include vitamin E, vitamin C, carotenoids, carnitine, cysteine, coenzyme Q10 (CoQ10), selenium, zinc, and folic acid (16, 24–26, 30, 71–90).

TABLE 2 Potential adverse effects of antioxidants.

Supplement	Adverse effects
Carnitines	Administration of daily doses of carnitines above 3 g daily can give gastrointestinal side effects and malodorous effects (38, 39).
Carotenoids	- Excess intake of vitamin A can lead to toxicity (hypervitaminosis A), which is manifested by nausea, irritability, reduced appetite, vomiting, blurry vision, headaches, hair loss, muscle pain, papilledema, hemorrhage, weakness, drowsiness and altered mental status (40). - Excess intake of provitamins such as carotenoids may lead to carotenaemia (yellow-tinged skin) (41).
Vitamin C	In some people, administration of vitamin C may lead to nausea, vomiting, headache, flushing, fatigue and joint pain (42).
Vitamin E	Excess vitamin E may increase bleeding risk (43).
Coenzyme Q10 (CoQ10) (ubiquinol)	CoQ10 may cause mild gastrointestinal symptoms (44).
Arginine/L-arginine	- No significant adverse events have been observed. - Arginine is contraindicated for patients a history of genital or oral herpes, asthma or cancer (45).
Cysteine and N-acetyl cysteine (NAC)	Up to 8000 mg daily oral NAC does not cause significant adverse events (46).
Folic acid (folate or vitamin B9)	- Folic acid doses of 5 mg/day and over can cause abdominal cramps, diarrhea and rash. - Higher doses of folic acid can cause altered sleep patterns, irritability, confusion, exacerbation of seizures and nausea (47).
Selenium	- Excess intake of selenium may lead to a garlic odor in the breath and a metallic taste in the mouth. - Prolonged intake of high selenium may lead to gastrointestinal symptoms, fatigue, hair loss, joint pain and nail problems (48).
Zinc	High intake of zinc may lead to loss of appetite, nausea, vomiting, abdominal pain and diarrhea (49).
Vitamin B (complex)	High doses of vitamin B may lead to gastrointestinal symptoms, skin flushing, skin rash or liver impairment (50).
Vitamin D	High intake of vitamin D may lead to loss of appetite, nausea, vomiting, constipation, dry mouth, arrhythmia, hypotension and weakness (51).
Myo-inositol	High doses of inositol may cause nausea, abdominal pain, diarrhea, headache, dizziness and difficulty sleeping (52).
Polyunsaturated fatty acids (PUFAs)	Intake of PUFAs may cause fishy taste in the mouth and gastrointestinal symptoms such as vomiting, diarrhea and abdominal pain (53).
Resveratrol	Resveratrol may cause some reversible gastrointestinal side effects (54).
Lycopene	High doses may result in gastrointestinal disturbances such as nausea, diarrhea, and potential skin discoloration (90)

Doses given are for adult male. AOX, antioxidant; CoQ10, Coenzyme Q10; NAC, N-acetyl cysteine; PUFAs, Polyunsaturated fatty acids.

Potential indications of antioxidant supplementation to enhance male fertility

Beyond MOSI, several clinical conditions and lifestyle factors warrant consideration for AOX therapy in male infertility treatment. Chronic diseases, specifically diabetes mellitus and obesity, have been associated with increased seminal OS and subsequent fertility impairment (91–93). In diabetic men, hyperglycemia-induced OS leads to increased SDF and reduced sperm motility, making them potential candidates for AOX supplementation (93).

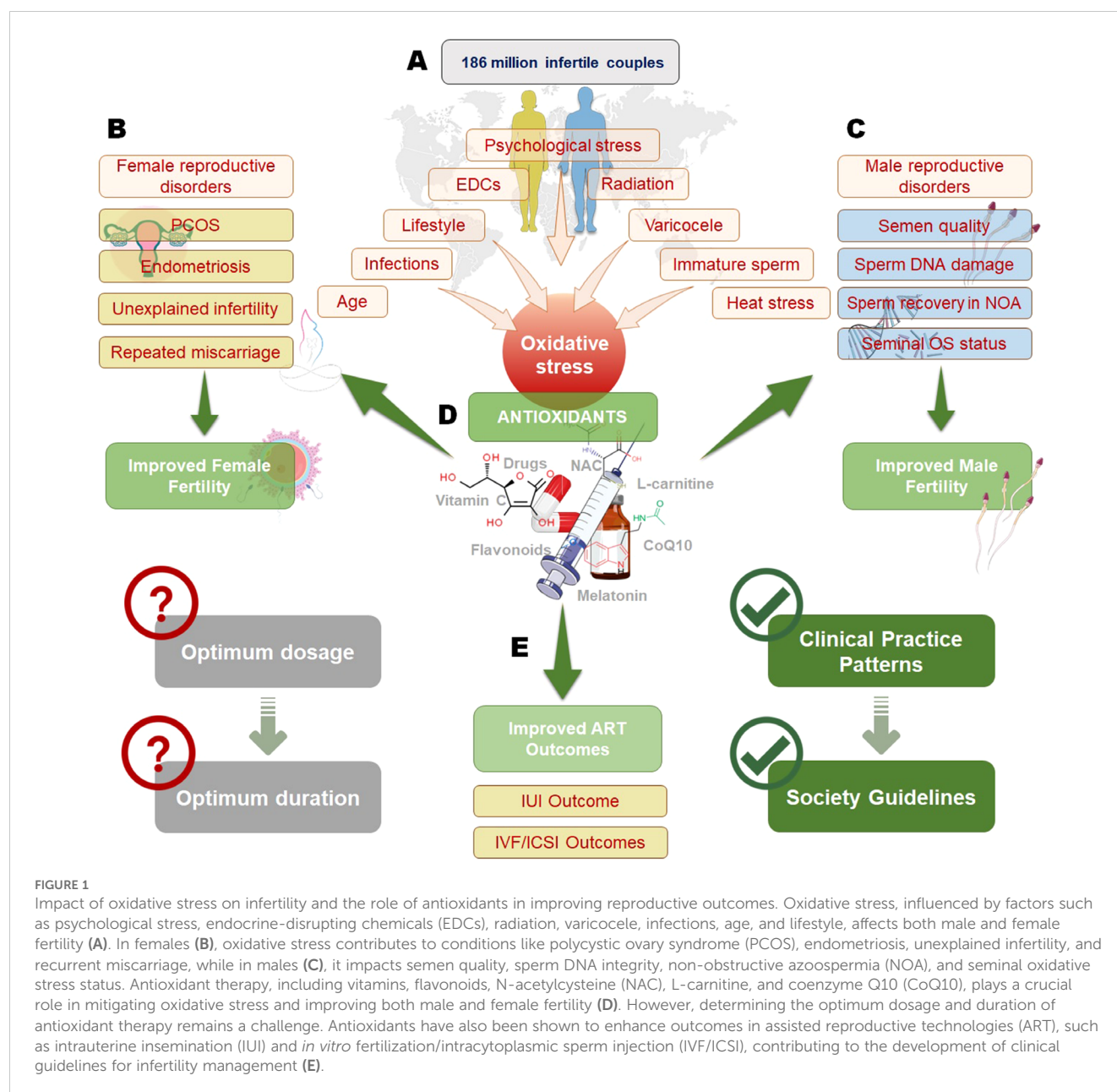
Environmental and occupational exposures represent another significant indication for AOX therapy. Men exposed to heavy metals, pesticides, or electromagnetic radiation often exhibit elevated OS markers in seminal plasma (94). Cancer treatments significantly elevate OS through multiple mechanisms: chemotherapeutic agents, particularly alkylating agents and platinum compounds, generate excessive ROS directly damaging sperm DNA and membranes (87, 88), while radiation therapy induces both acute and chronic OS states in testicular tissue, leading to prolonged spermatogenic dysfunction (95).

Recently, attention has focused on men recovering from Coronavirus disease-19 (COVID-19) infection as potential

candidates for AOX therapy. It has been suggested that COVID-19 infection may impact male reproductive function through OS-mediated mechanisms (96). While more research is needed, AOX supplementation might play a role in reproductive recovery post-infection (97).

Antioxidant therapy of infertile men: recommendations of professional societies

To date, results of studies on AOX therapy of infertile men are controversial, and no firm conclusion could be reached. Therefore, the guidelines of professional societies do not recommend the routine use AOXs in treatment of male infertility, mainly due to the heterogeneity of the available data. This should not be construed as a contraindication to the use of AOXs; rather, it underscores the necessity for more robust, high-quality, and standardized evidence to substantiate their routine application in clinical practice. The latest guidelines by the European Association of Urology state that “no conclusive data are available regarding the beneficial treatment with AOXs in men with idiopathic infertility, although they may improve semen parameters” (98). Similarly, the current American Urological Association and American Society for Reproductive



Medicine guidelines question the benefits of AOXs in treating male infertility, and indicate lack of data to recommend the use of specific agents for this purpose (Moderate Recommendation; Evidence Level: Grade B)” (99).

Antioxidant therapy of infertile men: impact of on fertility outcomes

Basic semen parameters

Results emerging from the review by Agarwal et al. (2022) indicated a significant increase of basic sperm parameters including sperm concentration, total motility, progressive motility and normal forms following AOX therapy versus placebo or no treatment (100). However, these results should be interpreted with caution due to high heterogeneity that was observed among

the studies included in this meta-analysis. Data of another meta-analysis also indicate better semen quality in infertile men who received AOX supplementation after varicocelectomy (101). A previous systematic review also reported that administration of AOXs such as vitamin E, vitamin C, N-acetyl cysteine (NAC), carnitines, CoQ10, lycopene, selenium, and zinc, resulted in significant improvement of sperm concentration, motility, and morphology (102). Further, a recent meta-analysis examined the impact of supplementation with L-Carnitine/ALC or NAC on semen quality and had the same conclusion (81). Further, *in vitro* glycine supplementation has been found to improve the quality of cryopreserved sperms in an animal experiment (103).

According to data from the Cochrane review, administration of carnitines and combined AOX formulations had positive effect of sperm motility, and supplementation with polyunsaturated fats and zinc resulted in increase of sperm concentration (16). Zinc

supplementation plays a pivotal role in enhancing spermatogenesis through its diverse functions in male reproductive physiology. It is indispensable for spermatogenesis, as it supports the functionality of Sertoli cells and maintains the structural integrity of the seminiferous epithelium, which is essential for effective sperm production. Zinc also serves as a cofactor for critical enzymes involved in DNA synthesis, repair, and cellular division, thereby facilitating the proliferation and differentiation of spermatogenic cells. Additionally, its antioxidant properties help counteract OS, shielding germ cells from damage induced by ROS. Moreover, zinc regulates testosterone secretion, a hormone essential for sustaining optimal spermatogenic activity and promoting healthy sperm concentration. Despite abundance of studies reporting positive outcome of AOX therapy on basic sperm parameters, it is difficult to get a firm conclusion due to the high heterogeneity among published studies.

Sperm recovery in patients with non-obstructive azoospermia

A study investigated the outcome of three-month supplementation with multivitamins, micronutrients and CoQ10 in infertile men with non-obstructive azoospermia ($n=24$) as compared to no treatment ($n=11$) (104). Histological evaluation of testicular biopsies of azoospermic patients showed maturation arrest mostly at spermatid level. At monthly follow up visits, the semen analysis of the treated group showed recovery of motile sperm in the ejaculate at concentration of 4.0 million/ml, while the untreated groups remained unchanged. Authors of the latter study reported further increase of sperm density on subsequent visits, and 2 pregnancies within 3 months. However, these results should be taken with caution due to poor study design and high drop-out ratio (105). More importantly, these results were not replicated in further studies.

Sperm DNA fragmentation

Folic acid supplementation resulted in lowering the levels of SDF in carriers of *MTHFR* gene TT polymorphism (106). However, a meta-analysis found no significant difference in SDF levels in infertile men following AOX supplementation as compared to placebo or no treatment (100). Of note, only small number of studies investigated this outcome with heterogeneity in inclusion criteria among study populations, and differences in AOX combinations and SDF assays used. Future studies are warranted to examine the efficacy of AOX therapy in men with high SDF.

Seminal oxidative stress indices

Few studies demonstrated improvement in OS as determined by reduction of levels of malonaldehyde (MDA), a lipid peroxidation byproduct following therapy with Vitamin C, vitamin E, beta-carotene, zinc, selenium, and NAC when used alone or in combination (107–111). A recent meta-analysis indicates a significant increase in seminal AOX and reduction of MDA following AOX therapy although significant inter-study heterogeneity was detected (112). MDA, a byproduct of lipid peroxidation, serves as a key biomarker of OS and exhibits cytotoxic effects on spermatozoa. Elevated MDA levels compromise the structural integrity and fluidity of the sperm membrane, resulting

in diminished motility and reduced viability (113, 114). Furthermore, MDA can directly interact with DNA, causing strand breaks and base modifications, thereby adversely affecting sperm DNA integrity and reducing fertilization potential (115, 116). Consequently, the observed reduction in MDA levels following AOX therapy highlights its therapeutic significance in alleviating OS and improving outcomes in OS-related male infertility.

Clinical pregnancy rate and live-birth rate

Recent years have witnessed a growing interest in the study of the impact of AOX therapy on clinical pregnancy rate (CPR) and live-birth rate (LBR). In a Cochrane review investigated AOX supplementation protocols for male subfertility, focusing on active substances including carnitines, coenzyme Q10, selenium, zinc, vitamins C and E, and carotenoids. Dosages varied according to the specific AOX, with some studies incorporating combinations of more than three AOXs. The duration of supplementation ranged from three to nine months, with outcomes evaluated at defined intervals of three, six, or nine months. The latter Cochrane review reported that AOX supplementation in infertile men resulted in a significant increase of CPR (odds ratio (OR) 2.97, 95% confidence interval (CI) 1.91–4.63) although the level of evidence was found to be low (16). A similar finding was reported by a recent meta-analysis where AOX therapy of infertile men was associated increased spontaneous CPR (OR 1.97, 95% CI: 1.28, 3.04; $p<0.01$) (100). In a third meta-analysis, CPR was significantly higher in infertile men with IMI who received combined L-carnitine and acetyl L-carnitine (ALC) supplementation (OR=3.76, $p=0.002$). The study evaluated the efficacy of combined L-carnitine and ALC supplementation in men diagnosed with idiopathic oligoasthenoteratozoospermia (iOAT). The treatment protocols consisted of LC administered at a dose of 2 g/day and LAC at 1 g/day, with intervention durations spanning 12 to 24 weeks across the included trials. This combination therapy demonstrated potential to improve key semen parameters, particularly forward motility and total motile sperm counts, while also contributing to enhanced pregnancy rates in affected patients (73).

Older reviews have also reported higher CPR in infertile men treated with AOXs whether couples were trying to conceive naturally or through one of the medically assisted reproduction (MAR) procedures (102, 117, 118). However, a study did not find better pregnancy rates in infertile couples in whom the male partner received CoQ10 supplementation, although sperm parameters were found better (26, 27, 119).

On the other hand, the results of systematic reviews by Cochrane (16) and Agarwal et al. (2022) were unable to show improvement of LBR despite higher pregnancy rates possibly due to scarcity of data available on this outcome (100). This may be attributed to methodological difficulties that preclude performing this kind of studies as they require longer durations of follow up.

Miscarriage rate

According to the systematic reviews by Cochrane (16) and Agarwal et al. (2022) (100), AOX therapy of infertile men was not correlated with miscarriage rates mainly due to small number of studies addressing this parameter.

Dose and duration of antioxidant therapy in male infertility

To date, there is no clear guidance as to the optimum dose or duration of AOX therapy for infertile men. The duration reported by different studies examining the effects of AOX therapy in male infertility is variable ranging from 1 month (120) to 12 months (78). A minimum of 26 weeks is recommended by the US Food and Drug Administration to evaluate the therapeutic potential of an intervention for male factor infertility (121). Based on this recommendation, it is assumed that a period of 6.5 months is needed for AOX therapy in infertile men. For instance, L-carnitine, L-acetyl carnitine, NAC, and vitamin E have been reported to require a duration of 6-12 months (52, 56, 57, 62, 63). The dose of AOX supplementation is also variable and is mainly decided by the manufacturer and according to the experience of the individual practitioner (66). A summary of dose schedules of AOXs frequently used in the treatment of infertile men is provided in Table 3 (24–26, 30, 73, 74, 76–89).

Practitioners’ attitude towards antioxidant therapy of male infertility

Reproductive specialists continue to prescribe AOXs in their daily practice of male infertility treatment despite lack of clear guidelines (102). The results of a global survey including 1,327 reproductive physicians indicated that 43.7% of the survey participants prescribe AOXs to all patients and another 41.9% prescribe AOXs for selected categories of patients (66). Interestingly, 80% of the clinicians indicated that they prescribe AOXs for treatment of infertility without testing for seminal OS. The results of the survey showed lack of consensus among practitioners regarding the desired treatment outcome. While 18.8% of clinicians suggested basic semen parameters as their primary outcome measure, an equal number selected LBR, and others selected either SDF or CPR. Additionally, a significant diversity was observed among practitioners regarding the AOXs they prescribe with 36 different AOXs being listed. It may be appropriate to use a combination of AOXs that act by different mechanisms to protect spermatozoa against oxidative damage (66). However, no clear guideline is currently available to recommend the use of specific AOX or AOX combinations for male infertility treatment.

Antioxidant therapy of female infertility

Rationale of antioxidant therapy of role of female infertility: role of oxidative stress

Although the evaluation of OS in male reproduction can be easily done by examining the semen, the same evaluation is more complicated in women as OS indices in the serum do not accurately

TABLE 3 Dose schedule of antioxidants commonly used in treatment of male infertility.

Supplement	Dose schedule*
Supplements with direct AOX action	
Carnitines	• L-carnitine 2 g daily + L-acetyl carnitine 1g daily for 3-6 months (73)
	•
Vitamin C	• 500 mg daily for 3 months (76).
Vitamin E	• 600 mg daily for 3-12 months (77, 78).
Coenzyme Q10 (CoQ10) (ubiquinol)	• 200-400 mg daily for 3 months (24–26).
Arginine/L-arginine	• Arginine-HCL 500 mg daily for 3 months (79).
Cysteine and N-acetyl cysteine (NAC)	• NAC 600 mg daily for 3-6 months (80, 81).
Folic acid (folate or vitamin B9)	• Folic acid 5 mg daily for 6 months (82)
Selenium	• 200 µg daily for 3 months (24, 80)
Zinc	• 66 mg daily for 3 months (30)
Supplements with AOX properties	
Vitamin B (complex)	• Vitamin B12 25 µg daily for 4 months (83). Methylcobalamin 1500-6000 µg daily for 3-6 months (83).
Vitamin D	• 2500 IU daily for 6 months (84), 4000 IU daily for 3 months (85), or 60000 IU weekly for 6 months (86).
Myo-inositol	• 4 gm daily for 3 months (87).
Polyunsaturated fatty acids (PUFAs)	• eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) 1.84 gm daily for 8 months (88).
Resveratrol	• 150 mg twice daily for 3 months (89)
Lycopene	• 4 to 8 mg daily, for 3 to 6 months (74)

*Doses given are for adult male. AOX, antioxidant; CoQ10, Coenzyme Q10; NAC, N-acetyl cysteine; PUFAs, Polyunsaturated fatty acids.

reflect the situation in the female reproductive system. Accordingly, more invasive procedures are needed such as examining the follicular fluid obtained during *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) or examining the peritoneal fluid obtained by laparoscopy. Consequently, much of the information on OS in female reproduction has been obtained from animal studies. Despite this limitation, OS was shown to be involved in many aspects of female reproduction (122–124). ROS in the ovaries is mainly produced in the macrophages, leukocytes, and cytokines present in the follicular fluid. On the other hand, the ovary contains two types of the AOX enzymes; SOD: copper-zinc SOD (Cu/Zn SOD), mainly present in the Graafian follicle and manganese SOD (MnSOD) concentrated mainly in the corpus luteum (124). ROS are involved in many physiological functions of the ovary such as steroidogenesis, oocyte maturation, ovulation, formation of blastocysts, implantation, luteolysis and maintenance of the luteal phase (124, 125). More specifically, in small doses, they are involved in folliculogenesis, dominant follicle selection and

corpus luteum formation in part by regulating angiogenesis (126). In addition, they play a role in dominant follicle selection, as regressing follicles undergo a process of apoptosis which is promoted by ROS. This delicate balance is an interplay between ROS production and the up-regulation of the AOX pathways CAT and GSH which opposes the apoptotic process (124).

ROS are also involved in ovulation, follicular rupture and corpus luteum formation. Besides cytokines, and vascular endothelial growth factor (VEGF), the local production of prostaglandin F2 α (PG2 α) leading to vascular ischemia at the site of follicular rupture (i.e. ovulation) stimulates the production of the ROS (122). On the other hand, following ovulation, the increased activity of Cu/Zn-SOD during the early and mid-luteal phase supports corpus luteum function while its diminished activity during the regression phase is associated with luteolysis and cessation of corpus luteum function, ushering menstruation (127).

OS, resulting from increased ROS production or diminished AOX capacity, can negatively impact female reproductive processes. It can damage oocytes, impair follicular development, reduce endometrial receptivity, and disrupt early embryo development, potentially leading to fertilization failure, and implantation issues. Additionally, OS can cause hormonal imbalances that further compromise fertility, indicating the role of ROS-AOX balance in maintaining reproductive health (128). OS was shown to be associated to a varying extend with many reproductive diseases such as the PCOS (9), endometriosis (11–13), POF (129), unexplained infertility (122) and even RM (15). Consequently, AOXs are used with varying degrees of success in the treatment of these conditions (Figure 1).

Antioxidant therapy of polycystic ovary syndrome

The PCOS is the most common female reproductive disorders and affects 5% to 10% of US women (130). The updated 2018 diagnostic criteria for PCOS require the presence of at least two of the following three features: (1) oligo/anovulation, (2) clinical and/or biochemical hyperandrogenism, and (3) PCO identified by the presence of ≥ 20 antral follicles per ovary on transvaginal ultrasound, with or without increased ovarian volume. These criteria are more stringent than the previous Rotterdam criteria, aiming to identify women with higher metabolic risks associated with PCOS (131).

About two thirds of PCOS patients suffer from insulin resistance often leading to the development of the metabolic syndrome (130). Although many theories have been proposed to explain the pathogenesis of PCOS, recent studies have increasingly linked OS to various aspects of the condition (124, 132). A study demonstrated that hyperglycemia induces excess ROS production from mononuclear cells, which subsequently triggers the release of tumor necrosis factor- α (TNF- α) (105). The resulting inflammatory cascade of events exacerbates OS and insulin resistance. This, in turn, contributes to the formation of multiple ovarian cysts, chronic anovulation, and infertility (133, 134). Another study showed that elevated insulin levels promote the

proliferation of thecal cells and increase the secretion of androgens, including luteinizing hormone (LH), while also upregulating the expression of cytochrome P450 and insulin growth factor-1 (IGF-1) receptors (107). The activity of cytochrome P450 is known to generate ROS, perpetuating a vicious cycle of OS. Additionally, plasma levels of L-carnitine were found to be significantly lower in obese PCOS patients compared to their non-obese counterparts (135). Despite these findings, it remains unclear whether OS is a primary factor in the development of PCOS or a secondary consequence of hyperglycemia, insulin resistance, and associated cardiovascular and necrotic complications in these women (124, 132).

Based on these and other findings, AOXs were proposed as a treatment of PCOS either on their own, as a combination or as adjuvants to other agents for ovulation induction in the infertile PCOS patients. For example, Sahelpour et al. (2019) treated 80 PCOS patients with L-carnitine (3g/day) for 3 months (109). The authors found that insulin sensitivity improved significantly and that the serum levels of low-density lipoprotein (LDL) decreased. Additionally, there was a decrease in the body mass index (BMI) and the patients reported that their menstrual cycles became more regular and that their hirsutism diminished (136). In a randomized controlled trial (RCT), 60 overweight PCOS patients were treated with 250 mg of carnitine supplements for 12 weeks, while the control group received a placebo (110). They found that carnitine administration was associated with a significant reduction in BMI, and waist and hip circumferences compared to placebo. They also found a significant reduction in fasting plasma glucose, serum insulin levels, homoeostasis model of assessment-insulin resistance and dehydroepiandrosterone sulphate (DHEAS) in the treated patients. However, in this latter study, carnitine administration had no effect on lipid profiles or free testosterone. NAC is another popular agent used for the treatment of infertile PCOS patients with encouraging results (137). However, a meta-analysis reported that the use of NAC showed no significant improvement in pregnancy rate, serum LH level, fasting insulin, and LH/follicle-stimulating hormone (FSH) ratio despite that the BMI and total testosterone were significantly reduced (138).

Zinc and selenium were also used in the treatment of PCOS based on the fact that serum zinc and selenium levels in women with PCOS are significantly lower than in healthy controls (139–141). Consequently, PCOS women were treated with the daily administration of 220 mg zinc sulfate (containing 50 mg zinc) for 8 weeks and found that this regimen resulted in the improvement of their metabolic profiles (142). Similarly, the administration of 200 micrograms per day of selenium for 8 weeks in PCOS patients had beneficial effects on their insulin metabolism parameters, triglycerides and very low-density lipoprotein-C (VLDL-C) levels (143).

Combinations of AOXs are now increasingly being recommended for PCOS treatment. For example, a combination of NAC (1200 mg/day) and L-arginine (1600 mg/day) for 6 months to treat 8 oligo-menorrhoeic patients with PCOS helped restoring the menstrual function, as evidenced by the increased number of menstrual cycles, and significantly decreased the homeostatic model assessment (HOMA) index (144).

The potential for AOXs to replace metformin in the treatment of PCOS has also been explored. In a study by Oner and Muderris (2011), NAC was compared with metformin in 100 patients with PCOS (119). Both treatments resulted in significant reductions in BMI, hirsutism scores, fasting insulin levels, HOMA index, free testosterone levels, and menstrual irregularity compared to baseline values, demonstrating equal efficacy. Additionally, NAC significantly lowered both total cholesterol and LDL levels, while metformin only reduced total cholesterol (145).

Antioxidants were also used as adjuvant therapies in combination with ovulation induction agents in infertile patients with PCOS (146). In 2014, a RCT was conducted to evaluate the effectiveness of adding 3g of L-carnitine daily to a regimen of 250 mg clomiphene citrate, administered from day 3 to day 7 of the menstrual cycle, in anovulatory women with PCOS (147). The combination treatment significantly improved both ovulation rates and cumulative pregnancy rates compared to clomiphene citrate alone. Additionally, the L-carnitine group showed significant improvements in patient tolerability, lipid profiles, and BMI. Vitamin D is often used as a supplement in the treatment of PCOS and a meta-analysis showed that this may be beneficial for follicular development and menstrual cycle regulation in the PCOS patients (148). Chen et al. found that using vitamin E (100 mg/day during the follicular and luteal phases of the cycle) did not result in significant improvements in ovulation induction, clinical pregnancy, or ongoing pregnancy rates in infertile women with PCOS (149). In the latter study, a significantly lower dose of human menopausal gonadotropin (HMG) was required in these women, which is expected given that women with PCOS typically have high serum anti-müllerian hormone (AMH) levels and are more prone to developing ovarian hyperstimulation syndrome (OHSS).

In conclusion, while AOXs have shown promising potential in the treatment of PCOS, further research is essential to validate these findings. Additional studies are needed to determine which specific AOX or combination of AOXs offers the most benefit for managing this complex syndrome. New research can also help development of targeted therapies that can effectively address the underlying OS and other related factors in PCOS.

Antioxidant therapy of endometriosis-associated infertility

Endometriosis affects about 10% of reproductive age women (150). Patients usually present with menstrual pain (dysmenorrhea) and/or infertility problems (12). OS has been implicated in the pathogenesis of endometriosis (12, 151–153). In these patients, OS results from excess ROS produced by the erythrocytes and apoptotic endometrial cells present in the endometriosis implants. ROS are also produced by the activated macrophages responsible for phagocytizing these apoptotic cells (11). Cells are damaged by OS-induced lipid peroxidation, which is proportional to the severity of the disease (154). It was also found that the peritoneal fluid of endometriosis patients contains high concentrations of various OS markers such as MDA, pro-inflammatory cytokines (interleukin-6 (IL-6), TNF- α , transforming growth factor- β (TGF-

β) and IL-1 β), angiogenic factors (IL-8 and VEGF), neural growth factor and oxidized LDL (ox-LDL) (155, 156). Besides their role in inflammation, these cytokines are detrimental to the processes of fertilization and embryo development: TNF- α increases the free implants adhesions and their invasiveness through its effect on matrix metallo-proteinases (157) while TGF- β hinders the local immune activity by inhibiting the natural killer cells which in turn impede the development of embryos (158). Further, mitochondria of ectopic endometrial stromal cells (ESCs) was found to generate excess ROS (159). A delicate redox balance between iron and heme oxygenase-1 (HO-1) has been identified, and this balance could be responsible for iron-induced OS in endometriotic cyst fluid (152). Additionally, a genetic role for OS in endometriosis has been proposed (22). A study investigated the genetic polymorphisms of four genes encoding AOX enzymes involved in OS and found that the variant genotypes for the CAT-262C, T polymorphism were significantly more frequent in individuals with endometriosis (160).

Consequently, AOXs have been utilized in the treatment of endometriosis, particularly for managing endometriosis-associated pain, with varying degrees of success (161–163). For instance, daily administration of a combination of vitamin E (1200 IU) and vitamin C (1000 mg) for eight weeks led to significant improvements in chronic pain, dysmenorrhea, and dyspareunia compared to placebo (164). Similarly, the severity of pelvic pain, dysmenorrhea and dyspareunia significantly decreased after 8 weeks of supplementation with vitamin C (1000 mg/day, 2 tablets of 500 mg each) and vitamin E (800 IU/day, 2 tablets of 400 IU each) compared to placebo (165). A recent study observed similar improvements following the administration of vitamin D (50,000 IU) every two weeks for 12 weeks in patients with endometriosis (166). Furthermore, a systematic review concluded that AOXs show promising results in treating endometriosis-related pain (167). However, the authors of the latter study emphasized the need for further studies to draw more definitive conclusions.

Few studies were conducted to evaluate AOX therapy in women with endometriosis-associated infertility (168, 169). Encouraging results were obtained following vitamin C supplementation in a group of women with endometriosis-associated infertility undergoing IVF treatment (170). In addition to human studies, animal research has also shown promise. For instance, studies conducted on rats demonstrated positive effects of AOX therapy on endometriosis-related infertility (146). However, these findings are preliminary and need to be confirmed through rigorous clinical trials in humans.

It is vital to take into account that AOXs should be given in proper dose and duration as excessive intake can lead to reductive stress (35). The delicate oxidant/AOX balance function is a double-edged blade. Although OS is implicated in cell death and apoptosis in endometriosis, upregulation of AOX functions in endometriotic cysts may result in restoration of cell survival (171, 172).

Antioxidant therapy of premature ovarian failure and poor ovarian reserve

POF or premature menopause is the cessation of ovarian function before the age of 40 years. In POF, the ovaries lose their germinative

and hormonal functions because of the exhaustion of the number of ovarian follicles before the typical age for physiological menopause which is around 50 years of age. Women with POF present with amenorrhea, hypergonadotropinism, and hypoestrogenism (173).

OS is increasingly recognized as a key factor influencing reproductive health, with effects that extend beyond fertility into broader physiological systems. Estrogen, owing to its intrinsic AOX properties, plays a critical role in mitigating OS. Its chemical structure enables the direct scavenging of free radicals while simultaneously enhancing the expression and activity of various AOX enzymes, thereby offering substantial protection against oxidative damage (174, 175). Elevated OS has been associated with systemic manifestations, including heightened lipid peroxidation, which correlates with mood disturbances, sleep disorders, and other health concerns (152). Dietary patterns enriched with AOX-rich components, such as the Mediterranean diet, have shown effectiveness in alleviating OS-related symptoms, underscoring the potential of dietary interventions in reducing oxidative damage (176). Similarly, hormone replacement therapy highlights the protective effects of estradiol in combating OS (177). These observations underscore the critical role of AOX-based strategies in advancing reproductive health and improving broader health outcomes, including those related to infertility.

OS has also been implicated in the process of ovarian ageing and an increased incidence of aneuploidy in ageing oocytes. In ageing oocytes, ROS attack the 8th carbon atom of guanine leading to base mutations and mismatches in DNA replication (178). Accordingly, AOXs were explored as a method to delay this ageing process. Studies in mice have shown that peroxiredoxin-4 can be used to protect against ovarian ageing (179, 180). Similar results were reported with quercetin (181, 182). Ochiai et al. used resveratrol as an adjuvant therapy in women with poor ovarian reserve in an attempt to prevent premature menopause (183). Similarly, melatonin was used as an adjuvant for the prevention of POF during chemotherapy (184, 185), given its experimentally proven role in the female reproductive tract (186). Obviously, these promising reports need to be confirmed by prospective randomized trials.

Antioxidant therapy of unexplained infertility

OS has been frequently associated with unexplained infertility. High levels of MDA, a marker of lipid peroxidation, were found in the peritoneal fluid of women with unexplained infertility, suggesting a potential role of OS in these cases (116). More recently, a study identified increased activity of prolyase, a type of matrix metalloprotease, in the plasma of women with unexplained infertility, proposing that this could contribute to implantation defects (187).

Given these findings, several attempts have been made to treat women with unexplained infertility using AOXs. Studies suggest that AOXs, such as CoQ10, vitamins C and E, melatonin, NAC, and alpha-lipoic acid (ALA), may improve oocyte quality, enhance endometrial receptivity, and restore hormonal balance by mitigating OS (188, 189). The evaluation often incorporates

indirect markers such as improved fertilization rates, successful embryo development, and clinical pregnancy rates as proxies for oocyte quality. For example, CoQ10 has been shown to improve oocyte quality by enhancing mitochondrial function (190), while melatonin, may support both oocyte quality and endometrial receptivity (191).

Although some studies have reported positive outcomes when using AOX therapy in conjunction with other fertility treatments, the evidence remains mixed, and large-scale RCTs are needed to confirm its efficacy and safety. A study evaluated the role of adding NAC to clomiphene citrate in treating unexplained infertility but found the combination ineffective (192). Vitamin E supplementation in women with unexplained infertility undergoing controlled ovarian hyperstimulation and intrauterine insemination (IUI) was only effective in increasing the thickness of the endometrium without any effect on the pregnancy rate (193). In a more recent RCT, the addition of daily oral multivitamins and minerals to the stimulation regimen of patients with unexplained infertility treated with IVF/ICSI did not improve oocyte quality (metaphase II oocytes) or pregnancy rates in these women (194). These studies indicate the need for further research to evaluate the efficacy of AOX therapy in unexplained infertility and to determine whether OS is a causative factor or merely an associated finding in this condition.

Antioxidant therapy of repeated miscarriages

RM or recurrent pregnancy loss is defined as the spontaneous loss of two or more pregnancies (195). It affects 1% of couples trying to conceive and in 50% of instances the cause is unknown (196). The relationship between OS and early pregnancy failure was first suggested by Jauniaux et al. (197). Subsequently, OS was explored as a cause of RM (198). Total oxidant level and OS index were significantly increased, and total antioxidant capacity, were significantly decreased in the serum of women with RM (199). Similarly, Ghneim et al. studied 25 women with RM and compared them to non-pregnant women and women with healthy pregnancies (200). They found significant reduction of SOD activities and serum levels of zinc, copper and manganese in healthy pregnant women compared to non-pregnant women with further reduction in RM patients. They suggested that dietary supplementation of Zn, Cu and Mn may have positive impact on these patients pre- and post-conception. The association between OS and RM was also supported by other studies (198, 201, 202). To the contrary, no statistically significant difference was found in the pre-gestational serum levels of OS indices in women with RM compared to controls (203).

Despite these conflicting data, AOXs were used in the treatment of RM with various claims of success. For example, a study compared the effects of administration of NAC 0.6 g + folic acid 500 µg/day in a group of 80 patients with a history of RM with an aged-matched group of 86 patients treated with folic acid 500 µg/day alone (204). They found a significantly increased rate of continuation of a living pregnancy and the take-home baby rate in the NAC supplementation group ($P < 0.047$, RR 1.98, 95% CI 1.3-

4.0). However, a Cochrane review conducted in 2016 showed that AOXs did not help in preventing pregnancy loss (RR 1.12, 95% CI 0.24 to 5.29) but the authors suggested that more studies are needed to further explore this outcome (205).

Antioxidant therapy and the outcome of medically assisted reproduction

OS is an important factor in the success of medically assisted reproduction (IUI, IVF and ICSI) (112, 128, 154). In these techniques, OS can result from endogenous as well as exogenous sources. These factors and the effect of AOXs on ART are discussed in this section (Figure 1).

Impact of antioxidant therapy on intrauterine insemination outcomes

One of the main indications of IUI is the presence of oligozoospermia, asthenozoospermia or teratozoospermia or a combination of the three parameters in the male partner of the infertile couple. As mentioned previously, male partners with these defects exhibit OS in their semen (206–208). In addition, culture media used in the preparation of the semen as well as those used in cell cultures have varying amounts of ROS which adds to the OS burden to which spermatozoa are exposed (209, 210). Moreover, the process of sperm preparation deprives the spermatozoa from the natural AOXs present in the seminal plasma leading to accumulation of ROS. This is even more pronounced when centrifugation is used in the preparation (e.g. Percoll gradient separation). Cryopreservation and thawing are extra sources of OS when frozen semen is used for IUI (211).

Accordingly, AOXs are used to treat the male partners of the infertile couples as mentioned previously (16, 66). In addition, AOX supplementation of the sperm preparation medium for IUI was used in an attempt to counteract the effects of OS. Pentoxifylline exerts several AOX activities, such as the maintenance of GSH levels and mitochondrial viability (212). The addition of pentoxifylline to the sperm preparation medium resulted in improvement of semen characteristics in asthenozoospermic patients (213). Additionally, CPR was significantly increased by adding pentoxifylline to the semen preparation medium for IUI (27.5% versus 11.5% in the control group) (214).

Similarly, the addition of caffeine to the semen preparation medium for IUI was described (215). Finally, adjusting the sperm and embryo cryopreservation media by adding AOXs is used to mitigate the effect of OS (216).

Impact of antioxidant therapy on IVF and ICSI outcomes

The techniques of IVF and ICSI exert a considerable amount of OS on the gametes and embryos. In addition to the endogenous and exogenous sources of OS mentioned previously to which

spermatozoa are subjected, the oocytes and embryos are also subjected to similar OS (217). For example, oocytes lose their AOX protection contained in the cumulus cells during the denudation process. The changes of temperature and the osmotic and pH changes during the oocyte and embryo handling (denudation, pipetting, etc.) are all sources of OS (100). In addition, exogenous sources of OS to which the gametes and embryos are subjected include exposure to the atmospheric O₂, the high oxygen concentration in the incubator (compared to the 2% concentration in the fallopian tube), volatile organic compounds present in the laboratory air or released from the consumables (plastic ware), visible light, humidity in the incubator and the type of mineral oil used. The processes of cryopreservation and thawing are also sources of OS (100). Consequently, many steps are taken in the IVF laboratory in order to counteract or diminish the effect of this OS (128).

Accordingly, men and women undergoing IVF or ICSI can be treated with AOXs in an attempt to improve the clinical results. Treatment of infertile men with the AOX “Menevit” for 3 months prior to IVF resulted in higher viable pregnancy rate (38.5%) compared to the control group (16%). The study enrolled 60 couples undergoing IVF-ICSI treatment, who were randomly assigned into two groups in a 2:1 ratio: one receiving the AOX Menevit and the other a placebo. Male participants were included based on evidence of OS, indicated by impaired sperm parameters such as poor morphology, reduced motility, or compromised membrane integrity, in conjunction with significant SDF (>25% TUNEL positivity). Female partners were excluded if they exhibited diminished ovarian reserve, defined as fewer than five oocytes retrieved in a prior IVF cycle or elevated early follicular phase FSH levels, or were aged over 39 years (218). The Cochrane review published in 2019 showed that although the use of AOXs for men prior to IVF did not improve the CPR (OR = 2.64, 95% CI 0.94 to 7.41), the LBR increased significantly (OR = 3.61, 95% CI 1.27). However, the authors of the review noted that these data were based on 2 RCTs only and that further studies are needed (11).

Women undergoing IVF and ICSI were also treated with AOXs prior to the procedure with variable claims of success. These treatments include NAC, melatonin, coQ10, thiamine, riboflavin, niacin B3, vitamins B6 and B12, folate, vitamins A, C, D and K, calcium, phosphorus, magnesium, sodium, potassium, chloride, iron, zinc, copper, selenium, iodine, vitamin E, vitamin K, L-arginine, inositol, biotin, pantothenic acid, eicosatetraenoic acid (EPA), docosahexaenoic acid (DHA) and various combinations of the above (188). The treatment is usually given for 3 to 6 months prior to the procedure. Pre-treatment of IVF patients with AOXs improved embryo quality, particularly in poor responders (219). Indeed, a significantly lower glutathione-S-transferase (GST) enzyme activity and significantly high MDA levels have been shown in the cumulus cells of poor responders compared to high responders (220). However, the Cochrane review conducted by Showell et al. in 2020 showed no improvement in the CPR (OR = 1.15, 95% CI = 0.95 – 140) or LBR (0.94, 95% CI = 0.41–2.15) when AOXs were given to women treated with IVF or ICSI (188). In this Cochrane review, the LBR was calculated from 2 small RCTs and it is therefore clear that more studies are needed, particularly in view of the multifactorial nature of IVF and ICSI.

Finally, supplementation of embryo culture media with AOXs was shown to improve the quality of the embryos, the implantation rate as

well as the CPR in IVF/ICSI cycles (221, 222). Furthermore, the addition of a combination of AOXs (ALC, NAC and α -lipoic acid) to the cryoprotectant medium of mice embryos resulted in increased blastocyst cryo-survival and viability post-vitrification (216).

The outcome of ICSI is known to be influenced by a myriad of factors. In a recent Cochrane review, it was stated that the chance of live birth with ICSI is between 30% and 41% (223). Consequently, efforts are directed to maximize ICSI outcomes. Increased OS has been identified as an important factor governing success of ICSI (112). Thus, it is plausible to use AOX in an endeavor to optimize the outcome of ICSI. Although the list of publications on the use of AOX in couples undergoing ICSI is steadily growing, knowledge on the relationship between AOX and ICSI outcomes is lacking (224). An RCT including 85 women with different indications for ICSI who have been supplemented with AOX daily from the cycle preceding ICSI cycle, versus 85 women who were not given any AOX acting as controls, concluded that AOX therapy did not change clinical or laboratory outcomes (225). However, A recent systematic review indicated a favorable impact of AOX on pregnancy outcomes following ICSI (224). In a recent systematic review and meta-analysis of RCTs, it was concluded that oral supplementation of CoQ10 may increase clinical pregnancy rates, without an effect on LBR, and MR compared with placebo or no-treatment in women with infertility undergoing ICSI (226). Moreover, a recent meta-analysis concluded that melatonin treatment significantly increases the CPR but not LBR in ART cycles. Melatonin treatment also increased the number of oocytes collected, matured oocytes, and good quality embryos. There was no evidence suggesting that melatonin treatment increases the adverse events in ART cycles. However, the findings of this meta-analysis should be explained cautiously due to remarkable heterogeneity of the included IVF patients (227). AOXs such as inositol, L-carnitine, CoQ10, and ALC may be helpful for women undergoing ICSI or in cases of previous ICSI failure (226). However, the role of AOXs in increasing LBR is limited by low-quality evidence (188). Indeed, conducting large controlled clinical trials using AOXs supplementation is mandatory to evaluate their influence on ICSI outcome.

Future directions

Available research on AOX therapy is surrounded by several limitations and biases, including small sample sizes, heterogeneous patient populations and inclusion criteria, poor study designs, and inconsistent reporting of clinical outcomes. Additionally, the use of a wide range of AOXs (either single or in combinations) and the lack of standardized dosages in different studies preclude the ability to draw a firm conclusion on the efficacy of AOX supplementation in solving infertility problem. This has led to the skepticism of the current guidelines by professional societies regarding AOX therapy for infertility. This may also explain the considerable variability that currently exists in practitioners' attitude towards the prescription of AOX therapy for the purpose of overcoming infertility issues. Such

variability surrounds many aspects of AOX therapy including pre-testing the patient for OS, the choice of a specific AOX or AOX combination, proper dose, optimum duration of therapy and the desired outcome. Therefore, there is a great demand for high-quality research to clarify the exact role of AOXs in the context of infertility treatment.

Future studies should focus on careful diagnostic work-up to assess OS indices as an essential pre-request before starting AOX therapy (206). Such test would be helpful for selecting patients with high levels of OS who are candidates for AOX therapy, and for monitoring the treatment response. This in turn requires the availability of reliable tools for confirming the diagnosis of OS and evaluation its severity. Indeed, this is technically difficult in women. However, assessment of seminal OS in men is feasible using some tools such as the MiOXSYS System that can help assess oxidation-reduction potential (ORP) levels in semen and seminal plasma (207). The test would help personalize treatment for cases with high seminal ORP such as idiopathic infertility, varicocele or those with genital tract infection. The 6th Edition of the World Health Organization (WHO) manual for human semen analysis classifies seminal OS testing as a research tool, emphasizing the lack of robust evidence to confirm its clinical utility (208). Despite this designation, OS testing is extensively utilized in andrology research, clinical laboratories, and ART centers, underscoring its recognized importance in advancing the understanding of male fertility.

Future studies are also warranted to investigate the impact of AOX on LBR, MR, and SDF since only few well-designed studies have analyzed these outcomes in infertile patients following AOX therapy. Additionally, future studies are needed to determine the proper treatment duration, optimum dosage as well as the ideal supplement (single vs. combined AOX) (206). Furthermore, it is essential to ensure that there are no negative consequences of utilizing AOXs as a therapeutic option for infertile men.

Conclusion and take-home message

OS plays a significant role in couples' infertility. The theoretical basis for AOX therapy to counteract the harmful effects of ROS on fertility is feasible. In infertile men, it is hypothesized that cases with elevated seminal OS may benefit from AOX therapy. Hence, several AOXs, including vitamins E and C, carotenoids, carnitine, cysteine, CoQ10, selenium, zinc, and folate, have been employed as potential therapeutic interventions for male infertility. However, results of clinical trials remain controversial, and current guidelines from major professional societies offer no definitive recommendations regarding AOX use in male infertility.

AOXs have shown promise in the management of PCOS, endometriosis, poor ovarian reserve and unexplained infertility. Also, AOX supplements such as inositol, L-carnitine, ALC and CoQ10 have shown potential for women undergoing ICSI, particularly those with previous ICSI failure. However, current evidence supporting AOX in improving important reproductive outcomes such as LBR remains low, necessitating further studies. Additionally, further research is needed to

evaluate the exact role of AOX therapy and to identify the most effective single AOX or AOX combinations in these conditions.

Author contributions

RS: Writing – original draft, Writing – review & editing. HS: Writing – original draft, Writing – review & editing. ME: Writing – original draft, Writing – review & editing. SD: Writing – original draft, Writing – review & editing. PS: Writing – original draft, Writing – review & editing. AN: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

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OPEN ACCESS

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RECEIVED 31 October 2024

ACCEPTED 16 January 2025

PUBLISHED 05 February 2025

CITATION

Wang Y, Fu X and Li H (2025) Mechanisms of
oxidative stress-induced sperm dysfunction.
Front. Endocrinol. 16:1520835.
doi: 10.3389/fendo.2025.1520835

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Mechanisms of oxidative stress-induced sperm dysfunction

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Oxidative stress plays a pivotal role in male infertility by impairing sperm function through various molecular mechanisms. This review explores the impact of excessive reactive oxygen species (ROS) on spermatozoa, particularly focusing on lipid peroxidation, DNA fragmentation, and protein oxidation. Lipid peroxidation damages sperm membranes, reducing fluidity and motility. ROS-induced DNA fragmentation compromises genetic integrity, potentially leading to infertility and adverse offspring outcomes. Protein oxidation alters key structural proteins, impairing sperm motility and the ability to fertilize an egg. The primary sources of oxidative stress in sperm include leukocyte activity, mitochondrial dysfunction, and environmental factors such as smoking and pollution. Despite the presence of natural antioxidant defenses, spermatozoa are particularly vulnerable due to limited repair mechanisms. The review highlights the importance of early intervention through antioxidant therapies and lifestyle changes to mitigate the detrimental effects of oxidative stress on male fertility. Further research is essential to enhance therapeutic approaches and improve reproductive outcomes.

KEYWORDS

oxidative stress, sperm dysfunction, reactive oxygen species, DNA fragmentation and male infertility, male infertility

1 Introduction

Male infertility is a significant global health concern, affecting approximately 8-12% of couples of childbearing age (1–3). Of these, male factor infertility contributes to nearly 50% of cases, underscoring the need for a deeper understanding of its underlying causes. Infertility in men is often associated with abnormalities in sperm quality, including reduced sperm count, motility, and morphology (4, 5). Among the various etiological factors contributing to male infertility, oxidative stress has emerged as a major player, profoundly impacting sperm function and overall reproductive outcomes (6–8). This review aims to explore the critical role of oxidative stress in male infertility, with a focus on the molecular mechanisms through which oxidative stress induces sperm dysfunction.

Male infertility specifically refers to any condition in which a male's reproductive system impairs the ability to achieve a successful pregnancy. The causes of male infertility are multifaceted and include genetic, environmental, lifestyle, and physiological factors (9–11). While genetic abnormalities, such as chromosomal defects or mutations, play a role in some

cases, environmental and lifestyle factors, such as smoking, alcohol consumption, and exposure to environmental toxins, are also significant contributors. Among the physiological factors, oxidative stress is a key determinant of sperm dysfunction (12, 13). Sperm cells are particularly vulnerable to oxidative stress due to their unique characteristics, including high polyunsaturated fatty acid (PUFA) content in their membranes, limited cytoplasmic volume, and minimal antioxidant defenses (14–16). These factors, combined with the fact that spermatozoa cannot repair their DNA as effectively as somatic cells, make them highly susceptible to oxidative damage.

Oxidative stress occurs when there is an imbalance between the production of ROS and the body's ability to neutralize or detoxify these harmful molecules using its antioxidant defenses. ROS are highly reactive and include free radicals like superoxide anion (O_2^-) and hydroxyl radical ($\bullet OH$), as well as non-radical molecules such as hydrogen peroxide (H_2O_2) (17–19). Although low levels of ROS are essential for normal physiological processes, such as sperm capacitation, hyperactivation, and the acrosome reaction, excessive ROS production can be harmful to sperm cells, causing oxidative stress. This imbalance negatively affects sperm function and overall fertility (20–22). In the context of male reproductive health, oxidative stress is a well-established cause of sperm dysfunction. Excessive ROS production damages the sperm membrane, proteins, and DNA, impairing sperm motility, viability, and the ability to fertilize an oocyte. Furthermore, oxidative stress is linked to DNA fragmentation, which has been associated with a higher incidence of infertility, poor pregnancy outcomes, and increased risk of genetic abnormalities in offspring (23, 24). The significance of oxidative stress in male infertility lies not only in its direct impact on sperm quality but also in its potential to induce long-term reproductive and genetic consequences.

Under physiological conditions, a small amount of ROS is essential for sperm functions, including the initiation of capacitation, a process required for sperm to acquire the ability to fertilize an egg. Capacitation involves the hyperactivation of sperm motility and the preparation for the acrosome reaction, a critical step in penetrating the oocyte. ROS participate in this process by modulating signal transduction pathways that promote tyrosine phosphorylation, a key event in sperm activation (25–27). The sperm membrane, rich in PUFAs, is particularly susceptible to lipid peroxidation, a chain reaction that leads to the formation of toxic byproducts such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (28, 29). These byproducts further compromise the sperm membrane's integrity, affecting its fluidity and permeability, which are essential for maintaining sperm motility and enabling fusion with the oocyte during fertilization.

In addition to lipid peroxidation, oxidative stress induces protein oxidation, which affects the function of key proteins involved in sperm motility and structure (30–32). For example, oxidative modification of actin and tubulin, the cytoskeletal proteins that maintain the shape and motility of sperm, can lead to a loss of motility and abnormal morphology. Protein oxidation also affects enzymes that regulate sperm metabolism and energy production, further compromising sperm viability. Sperm DNA is highly compacted and protected by protamines, which replace

histones during spermatogenesis to ensure tight packaging of the paternal genome. However, the DNA within sperm cells is not completely immune to oxidative damage. ROS can induce single-strand and double-strand breaks in sperm DNA, as well as the formation of oxidative base lesions such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) (33, 34). DNA fragmentation resulting from oxidative stress has been strongly correlated with male infertility and poor reproductive outcomes, including recurrent pregnancy loss and an increased risk of congenital anomalies in offspring.

Oxidative stress in spermatozoa can originate from both internal and external sources. Internally, sperm mitochondria are a major source of ROS. The accumulation of mitochondrial ROS not only impairs ATP production, which is essential for sperm motility but also damages mitochondrial DNA, which lacks the robust repair mechanisms present in nuclear DNA. Mitochondrial dysfunction, therefore, contributes to both reduced motility and compromised sperm DNA integrity (35–37). Externally, leukocytes found in seminal plasma are another major source of ROS. Infections or inflammation in the male reproductive system can activate these leukocytes, causing them to produce high levels of ROS as part of the immune response. Although this process helps eliminate pathogens, the excessive ROS produced near sperm cells can result in oxidative damage, impairing sperm function. Leukocytospermia, a condition characterized by elevated levels of leukocytes in semen, has been associated with increased ROS levels and a higher incidence of sperm DNA fragmentation (38, 39). For example, smoking introduces numerous toxic substances, including free radicals, into the body, leading to increased oxidative damage to sperm DNA, proteins, and lipids.

Given the central role of oxidative stress in male infertility, this review aims to provide a detailed exploration of the molecular mechanisms by which oxidative stress induces sperm dysfunction. Each of these mechanisms will be examined in the context of their impact on sperm function and fertility outcomes. Additionally, the review will discuss the primary sources of oxidative stress in spermatozoa, including mitochondrial dysfunction, leukocyte activation, and environmental/lifestyle factors. Understanding the molecular underpinnings of oxidative stress-induced sperm damage is essential for developing effective therapeutic interventions. By elucidating these mechanisms, this review seeks to highlight potential targets for antioxidant therapies and lifestyle modifications that could mitigate oxidative stress and improve reproductive outcomes in men with infertility. Furthermore, the review will address current gaps in research and propose future directions for studying oxidative stress and its role in male reproductive health.

2 Overview of oxidative stress and sperm function

Oxidative stress has become a central theme in understanding male infertility, particularly its connection with sperm dysfunction. The underlying biological phenomenon involves ROS, which play a complex dual role in sperm physiology and pathology. In controlled

amounts, ROS are essential for various sperm functions, such as motility, capacitation, and fertilization (Figure 1). However, when the balance between ROS production and the body's antioxidant defense systems is disrupted, ROS levels rise, resulting in oxidative stress (40, 41). This imbalance leads to structural and functional damage to spermatozoa, significantly reducing male fertility. Understanding how ROS affect sperm function and how oxidative stress leads to male infertility provides critical insights into potential therapeutic approaches. In healthy sperm, ROS serve vital physiological roles that are crucial for reproductive success. One of the primary functions of ROS is their involvement in sperm capacitation, a biochemical process that enables sperm to fertilize an egg. Capacitation is essential for the sperm to undergo hyperactivation, which is a type of vigorous motility required to navigate through the female reproductive tract and ultimately fertilize the oocyte. During capacitation, ROS facilitate the activation of key signal transduction pathways that regulate tyrosine phosphorylation, an essential process for sperm maturation. These pathways lead to alterations in the sperm membrane that increase its fluidity, thereby preparing the sperm for the acrosome reaction and fertilization. Another important function of ROS during capacitation is their role in cholesterol efflux from the sperm membrane (42). Cholesterol removal is

necessary to increase membrane permeability and prepare the sperm for the acrosome reaction, which allows it to penetrate the egg's outer layer (43, 44).

ROS are also involved in sperm motility, which is a critical determinant of male fertility. Motility is an energy-intensive process that requires the coordinated activity of several signaling pathways, particularly those involving calcium ion fluxes that regulate flagellar beating. ROS are known to modulate these pathways, ensuring that the sperm maintain their motility long enough to reach the oocyte. This balance between ROS levels and sperm motility is essential for successful fertilization. Physiological levels of ROS are also required for the acrosome reaction, which is the process by which sperm release enzymes that allow them to penetrate the zona pellucida, the protective layer surrounding the oocyte (45, 46). Without ROS, the acrosome reaction would not be triggered, and the sperm would be unable to fertilize the egg. The benefits of ROS in sperm function are clear under physiological conditions. However, their highly reactive nature means that even slight increases in ROS levels can have detrimental effects on sperm cells. This is where the importance of a tightly regulated balance between ROS production and antioxidant defenses becomes evident. Spermatozoa are highly specialized cells that are particularly vulnerable to oxidative damage due to their unique structural and functional characteristics. One of these

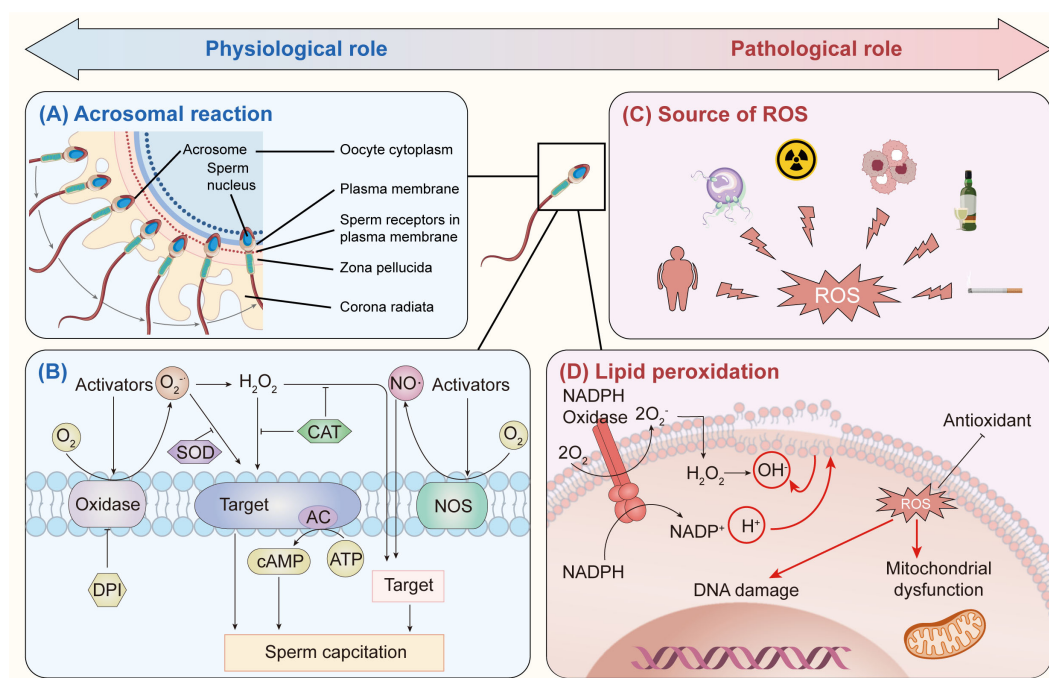


FIGURE 1

Reactive oxygen species (ROS) play both beneficial and harmful roles in the male reproductive system. (A) In small amounts, ROS are crucial for the acrosomal reaction and enable sperm to penetrate the zona pellucida through mild lipid peroxidation. (B) ROS are also involved in the early stages of sperm capacitation, with an oxidase producing extracellular superoxide ($O_2^{\cdot-}$), which aids capacitation. Diphenyliodonium (DPI), an oxidase inhibitor, can block $O_2^{\cdot-}$ production under oxidative stress, and SOD neutralizes excess $O_2^{\cdot-}$. Additionally, hydrogen peroxide (H_2O_2) interacts with specific plasma membrane targets to support capacitation, while catalase prevents H_2O_2 overproduction. Nitric oxide (NO), generated by nitric oxide synthase (NOS), also promotes capacitation, with adenylyl cyclase (AC) as a possible ROS target, producing cAMP. (C) ROS sources in male infertility include obesity, immature sperm, radiation, leukocytosis, alcohol, and smoking. (D) NADPH oxidase in sperm membranes generates $O_2^{\cdot-}$, which is converted to H_2O_2 in the cytosol, producing hydroxyl radicals ($OH^{\cdot}$). These radicals trigger lipid peroxidation, causing mitochondrial dysfunction and DNA damage in sperm.

characteristics is the high content of polyunsaturated fatty acids (PUFAs) in the sperm membrane (47, 48). PUFAs are particularly susceptible to oxidative attack by ROS, which can initiate a chain reaction of lipid peroxidation. This process not only damages the membrane's structural integrity but also impairs its functionality, reducing the sperm's ability to maintain motility and fertilize the egg.

The body's antioxidant defense systems play a crucial role in preventing the harmful effects of excessive ROS. These systems include enzymatic antioxidants such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, as well as non-enzymatic antioxidants like vitamins C and E, glutathione, and albumin (49, 50). Enzymatic antioxidants neutralize ROS by converting them into less reactive species. For instance, SOD converts superoxide anions into hydrogen peroxide, which is then broken down into water and oxygen by catalase and glutathione peroxidase (51, 52). Non-enzymatic antioxidants, on the other hand, act as scavengers that neutralize free radicals before they can cause cellular damage. In the male reproductive system, seminal plasma, the fluid that surrounds sperm, is rich in non-enzymatic antioxidants that protect sperm from oxidative damage. These antioxidants work in tandem with intracellular enzymatic defenses to maintain the fine balance between ROS and antioxidants, ensuring that ROS levels remain within a range that supports normal sperm function without causing oxidative stress.

When this balance is disrupted, however, oxidative stress occurs, leading to significant sperm dysfunction. The pathological effects of oxidative stress on spermatozoa can be grouped into three main categories: lipid peroxidation, DNA fragmentation, and protein oxidation. Each of these mechanisms contributes to reduced sperm quality and male infertility. Lipid peroxidation is one of the most damaging consequences of oxidative stress on sperm. As mentioned earlier, sperm membranes are rich in PUFAs, which are particularly prone to oxidation by ROS. When ROS levels rise, they initiate a chain reaction that leads to the formation of lipid peroxides. These peroxides, in turn, generate toxic byproducts such as MDA and 4-HNE, which further damage the sperm membrane (53–55). Lipid peroxidation reduces membrane fluidity, making it more difficult for sperm to swim effectively. It also impairs the integrity of the acrosomal membrane, which is necessary for the acrosome reaction and subsequent fertilization. Thus, lipid peroxidation has a direct impact on both sperm motility and the ability to fertilize the oocyte.

Another significant consequence of oxidative stress is DNA fragmentation. ROS can cause breaks in the DNA strands within sperm, leading to single-strand and double-strand breaks. These breaks often occur in regions of the sperm genome where chromatin is loosely packed, making them more susceptible to oxidative attack. DNA fragmentation is a major cause of male infertility, as it compromises the integrity of the paternal genome, leading to poor fertilization outcomes and an increased risk of genetic abnormalities in the offspring. Unlike somatic cells, spermatozoa have limited DNA repair mechanisms due to the replacement of histones with protamines during spermatogenesis. This limits their ability to repair oxidative damage, making DNA fragmentation particularly harmful. The use of assisted reproductive technologies (ART) such as *in vitro* fertilization

(IVF) and intracytoplasmic sperm injection (ICSI) can bypass some of the natural selection mechanisms that would prevent sperm with damaged DNA from fertilizing an egg, potentially leading to long-term consequences for the offspring.

Protein oxidation is another detrimental effect of elevated ROS levels. ROS can modify key proteins involved in sperm motility, such as actin and tubulin, through the formation of carbonyl groups. These oxidative modifications can lead to the aggregation and dysfunction of these proteins, compromising the structural integrity of the sperm's cytoskeleton and impairing motility. Protein oxidation can also affect enzymes that regulate sperm metabolism, reducing the availability of ATP, which is required for motility and fertilization. Moreover, the oxidation of surface proteins on the sperm membrane can impair the sperm's ability to recognize and bind to the oocyte, further reducing the likelihood of successful fertilization. The sources of oxidative stress in sperm can be both intrinsic and extrinsic. Intrinsic sources include the mitochondria, which are a major source of ROS during the process of oxidative phosphorylation. Mitochondria are responsible for producing the energy required for sperm motility, but during this process, some electrons leak from the electron transport chain and react with oxygen to form superoxide anions (56, 57). This mitochondrial ROS production is a natural part of cellular metabolism, but when ROS levels are excessive, they can damage the mitochondria themselves, leading to a reduction in ATP production and further impairing sperm motility. Moreover, mitochondrial DNA, which lacks the protective histones found in nuclear DNA, is particularly vulnerable to oxidative damage. Mutations in mitochondrial DNA caused by ROS can exacerbate mitochondrial dysfunction, creating a vicious cycle of oxidative stress and impaired sperm function.

Extrinsic sources of oxidative stress include environmental and lifestyle factors. Exposure to environmental toxins such as heavy metals (e.g., cadmium, lead) and endocrine-disrupting chemicals (e.g., bisphenol A, phthalates) can increase ROS production in spermatozoa. Lifestyle factors such as smoking, excessive alcohol consumption, poor diet, and obesity are also major contributors to oxidative stress (58, 59). Smoking, for instance, introduces numerous free radicals into the body, which increase ROS levels and cause oxidative damage to sperm DNA, proteins, and lipids (60, 61). Infections and inflammation in the male reproductive tract can also lead to elevated ROS levels due to the activation of leukocytes, which produce ROS as part of the body's immune response. This condition, known as leukocytospermia, has been associated with increased oxidative stress and sperm DNA fragmentation.

In conclusion, ROS play a dual role in sperm function, being essential for processes such as capacitation, motility, and fertilization at physiological levels, but harmful when produced in excess. The balance between ROS production and antioxidant defenses is crucial for maintaining sperm health and function. When this balance is disrupted, oxidative stress occurs, leading to lipid peroxidation, DNA fragmentation, and protein oxidation, all of which contribute to sperm dysfunction and male infertility. The pathological effects of oxidative stress underscore the importance of developing therapeutic strategies aimed at reducing ROS levels and enhancing antioxidant defenses to improve male fertility outcomes.

3 Molecular mechanisms of oxidative stress-induced sperm damage

Oxidative stress has been extensively recognized as a significant contributor to male infertility, particularly through its damaging effects on spermatozoa. As one of the most vulnerable cell types in the human body, spermatozoa possess unique structural and functional characteristics that make them highly susceptible to oxidative damage. This vulnerability is primarily due to the high content of PUFAs in their membranes, the limited availability of antioxidant enzymes, and the lack of effective DNA repair mechanisms. When ROS levels exceed the antioxidant defenses, spermatozoa are subjected to oxidative stress, leading to detrimental molecular changes, including lipid peroxidation, DNA fragmentation, and protein oxidation. These molecular alterations compromise sperm function, reducing their motility, fertilization capacity, and genetic integrity. This section delves into the specific molecular mechanisms by which oxidative stress induces sperm damage, focusing on lipid peroxidation, DNA fragmentation, and protein oxidation.

3.1 Lipid peroxidation

One of the earliest and most significant effects of oxidative stress on spermatozoa is lipid peroxidation (62, 63). This process involves the oxidative degradation of lipids, specifically PUFAs present in the sperm membrane. PUFAs, with their multiple double bonds, are highly susceptible to attack by ROS, making spermatozoa particularly vulnerable to oxidative stress. Lipid peroxidation results in the formation of lipid peroxides and various toxic byproducts, which lead to a cascade of cellular damage, ultimately impairing sperm function. Lipid peroxidation occurs when ROS, particularly hydroxyl radicals ($\bullet\text{OH}$) and superoxide anions (O_2^-), interact with the PUFAs in sperm membranes. The process is initiated when ROS abstract a hydrogen atom from the methylene group of a PUFA, creating a lipid radical. This lipid radical reacts with molecular oxygen to form lipid peroxy radicals, which perpetuate a chain reaction of oxidative damage. As lipid peroxy radicals propagate the reaction, they generate lipid hydroperoxides and highly toxic byproducts such as MDA and 4-HNE (54, 64). These byproducts further damage membrane lipids, proteins, and even DNA, amplifying the effects of oxidative stress.

The impact of lipid peroxidation on sperm function is profound. The sperm membrane plays a crucial role in maintaining the structural integrity and functionality of the cell. The lipid bilayer, which is rich in PUFAs, ensures membrane fluidity, which is essential for sperm motility and the ability to undergo capacitation and the acrosome reaction—two key processes required for successful fertilization. Lipid peroxidation compromises membrane fluidity, leading to a reduction in sperm motility. This occurs because the lipid peroxides generated during the process disrupt the normal lipid packing in the membrane, making it more rigid and less flexible. As a result, the sperm's ability to propel itself through the female reproductive tract is impaired,

significantly reducing the chances of reaching and fertilizing the oocyte. Furthermore, lipid peroxidation damages the sperm's ability to fuse with the oocyte. The acrosomal membrane, located at the head of the sperm, is responsible for releasing hydrolytic enzymes during the acrosome reaction, which allows the sperm to penetrate the zona pellucida, the outer layer of the egg. When lipid peroxidation affects the acrosomal membrane, the acrosome reaction is disrupted, preventing the sperm from binding to and fertilizing the egg. Clinically, lipid peroxidation serves as a biomarker of oxidative stress in spermatozoa. Elevated levels of MDA and 4-HNE in semen samples are often correlated with reduced sperm quality and increased infertility rates. These markers are used in diagnostic assessments to evaluate the extent of oxidative damage and to guide antioxidant therapy aimed at reducing lipid peroxidation and improving sperm function.

3.2 DNA fragmentation

In addition to lipid peroxidation, oxidative stress induces significant damage to sperm DNA, leading to DNA fragmentation (Figure 2). Spermatozoa are unique in that their DNA is highly compacted and tightly packaged within protamines, which replace histones during spermatogenesis. This compaction provides a degree of protection against external damage; however, sperm DNA remains vulnerable to oxidative stress due to the limited availability of DNA repair mechanisms and the loose packaging of DNA at certain sites, particularly those involved in early embryonic development. Oxidative damage to sperm DNA occurs through the formation of single-strand and double-strand breaks, as well as the generation of oxidative base adducts. ROS, particularly hydroxyl radicals, interact with DNA bases, leading to base modifications, strand breaks, and the formation of oxidative lesions. One of the most well-documented oxidative DNA adducts is 8-OHdG, which results from the oxidation of guanine (65, 66). 8-OHdG serves as a biomarker of oxidative stress in spermatozoa and has been linked to chromosomal instability and mutagenesis (67, 68).

The process of DNA fragmentation begins when ROS attack the deoxyribose backbone of DNA, leading to the cleavage of phosphodiester bonds and the formation of strand breaks (69, 70). These breaks can occur as single-strand or double-strand breaks, both of which compromise the integrity of the sperm genome. Single-strand breaks may be repaired by the oocyte after fertilization, but double-strand breaks are more difficult to repair and are often associated with severe chromosomal damage. DNA fragmentation has a direct impact on sperm function and male fertility. Spermatozoa with fragmented DNA exhibit reduced fertilization capacity because the damaged DNA impairs the sperm's ability to effectively deliver the paternal genome to the oocyte (71, 72). Even if fertilization occurs, the presence of fragmented DNA increases the likelihood of poor reproductive outcomes, including failed implantation, miscarriage, and the development of genetic abnormalities in the offspring. Studies have shown that sperm DNA fragmentation is strongly correlated with recurrent pregnancy loss, particularly in couples undergoing ART such as IVF and ICSI.

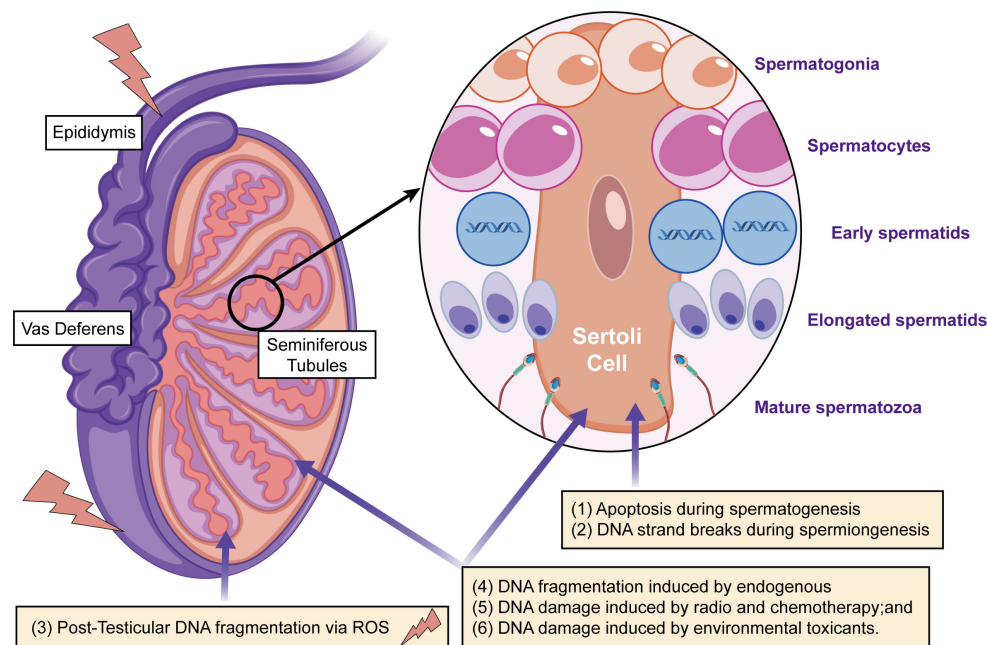


FIGURE 2

The main mechanisms that cause DNA damage in sperm during production or transport include: (i) apoptosis occurring during spermatogenesis; (ii) DNA strand breaks during chromatin remodeling in spermiogenesis; (iii) post-testicular DNA fragmentation, primarily due to oxidative stress, during sperm transit through the seminiferous tubules and epididymis; (iv) DNA fragmentation caused by internal enzymes like caspases and endonucleases; (v) DNA damage from radiotherapy and chemotherapy; and (vi) DNA damage resulting from exposure to environmental toxins.

One of the major challenges in addressing oxidative DNA damage in sperm is the limited DNA repair capacity of spermatozoa. Unlike somatic cells, which possess a variety of DNA repair pathways to correct oxidative damage, spermatozoa have only one active DNA repair enzyme, 8-oxoguanine DNA glycosylase (OGG1), which is responsible for repairing oxidative base lesions (73, 74). The limited repair capacity of sperm makes them highly susceptible to accumulating DNA damage, particularly in the context of oxidative stress. While the oocyte possesses more robust DNA repair mechanisms, its ability to repair sperm DNA damage is finite and decreases with maternal age. Therefore, sperm DNA fragmentation represents a critical barrier to successful reproduction, particularly in older couples or those experiencing male factor infertility. The clinical implications of sperm DNA fragmentation are significant. Sperm DNA fragmentation testing is commonly used to assess male fertility potential, particularly in cases of unexplained infertility, recurrent pregnancy loss, and poor ART outcomes. Therapeutic strategies to reduce sperm DNA fragmentation typically focus on antioxidant supplementation, which aims to neutralize ROS and reduce oxidative damage to DNA. However, given the limited DNA repair capacity of sperm, early intervention is crucial to prevent irreversible damage.

3.3 Protein oxidation

Protein oxidation represents another key mechanism by which oxidative stress impairs sperm function. ROS can modify sperm proteins through the oxidation of amino acid side chains, leading to

the formation of carbonyl groups and disulfide bonds. These oxidative modifications result in the aggregation and dysfunction of key proteins involved in sperm motility, structure, and fertilization, further contributing to male infertility. The process of protein oxidation begins when ROS interact with specific amino acids, such as cysteine, methionine, and tyrosine, which contain sulfur or aromatic groups that are highly susceptible to oxidation. This interaction leads to the formation of protein carbonyls and disulfide bonds, which alter the protein's structure and function. In spermatozoa, the proteins most affected by oxidative stress are those involved in maintaining the cytoskeleton, motility, and the acrosome reaction.

One of the primary targets of protein oxidation in sperm is the cytoskeletal proteins actin and tubulin (75). These proteins form the structural framework of the sperm flagellum, which is responsible for motility. Oxidation of actin and tubulin disrupts the integrity of the cytoskeleton, leading to impaired flagellar movement and reduced sperm motility. Sperm motility is essential for navigating the female reproductive tract and reaching the oocyte for fertilization. When motility is compromised due to protein oxidation, the sperm's ability to reach and fertilize the egg is significantly diminished. In addition to cytoskeletal proteins, enzymes involved in sperm metabolism and energy production are also affected by oxidative stress (75, 76). These enzymes, such as creatine kinase and adenylate kinase, play critical roles in generating the ATP required for sperm motility and the acrosome reaction (77, 78). Oxidative damage to these enzymes reduces their activity, leading to decreased ATP production and impaired sperm function. Without sufficient energy, spermatozoa cannot sustain the vigorous motility required for successful fertilization.

Protein oxidation also affects the acrosome reaction, which is essential for sperm-egg binding and fertilization. The acrosome is a cap-like structure located at the head of the sperm that contains hydrolytic enzymes necessary for penetrating the zona pellucida. Oxidative damage to proteins involved in the acrosome reaction, such as acrosin and hyaluronidase, impairs the sperm's ability to release these enzymes, preventing successful penetration of the oocyte (79, 80). As a result, the sperm's fertilization capacity is significantly reduced. Proteomics approaches have been employed to detect oxidative modifications in sperm proteins. These techniques involve the identification and quantification of protein carbonyls and other oxidative markers, providing valuable insights into the extent of oxidative damage in spermatozoa. By identifying specific proteins that are oxidized in response to oxidative stress, researchers can better understand the molecular mechanisms underlying sperm dysfunction and male infertility. The clinical implications of protein oxidation in male infertility are significant. Oxidative damage to sperm proteins not only impairs motility and fertilization but also contributes to the overall decline in sperm quality observed in infertile men. Therapeutic interventions aimed at reducing protein oxidation typically focus on antioxidant supplementation, which has been shown to improve sperm motility and reduce oxidative damage in clinical studies.

4 Sources of oxidative stress in sperm

Oxidative stress in spermatozoa occurs when there is a disproportion between the generation of ROS and the capacity of the available antioxidant defenses to neutralize them. While controlled ROS levels are essential for normal sperm processes—such as capacitation, motility, and the fertilization mechanism—excessive ROS accumulation leads to oxidative damage, impairing sperm function and ultimately contributing to male infertility. The origins of oxidative stress in sperm are both internal and external, with various physiological, pathological, and environmental influences promoting elevated ROS production. In this section, we will examine the key contributors to oxidative stress in sperm, concentrating on ROS produced by leukocytes, mitochondrial dysfunction, and environmental factors.

4.1 Leukocyte-derived ROS

Leukocytes, or white blood cells, are an important component of the immune system and are often present in semen, particularly in response to infections or inflammatory conditions in the male reproductive tract (81, 82). While leukocytes are essential for immune defense, their activation in the reproductive environment can lead to excessive production of ROS, which in turn can damage spermatozoa. This oxidative stress from leukocyte-derived ROS is a well-established factor in male infertility, especially in cases associated with leukocytospermia, a condition characterized by elevated leukocyte counts in semen. When infections or inflammatory responses occur within the male reproductive

system, leukocytes—particularly neutrophils and macrophages—become activated and initiate a process known as the respiratory burst (83, 84). During this process, activated leukocytes produce large amounts of ROS as part of their mechanism to destroy invading pathogens. Hypochlorous acid is a potent oxidant used by leukocytes to kill bacteria, but in the context of the reproductive environment, it can have deleterious effects on sperm cells.

The ROS generated by leukocytes are not restricted to attacking pathogens; they can also interact with nearby spermatozoa, leading to oxidative damage. ROS from leukocytes can induce lipid peroxidation in sperm membranes, leading to decreased membrane fluidity, impaired motility, and compromised sperm-oocyte fusion. Additionally, ROS can cause DNA fragmentation in sperm, reducing genetic integrity and fertilization potential. Leukocytospermia, characterized by the presence of more than one million leukocytes per milliliter of semen, is a well-established contributor to male infertility. In such cases, the excessive production of ROS by leukocytes surpasses the capacity of the semen's antioxidant defenses, leading to pronounced oxidative stress. Research has demonstrated that men with leukocytospermia exhibit higher levels of sperm DNA fragmentation, diminished sperm motility, and reduced fertilization rates, all of which are significant factors contributing to infertility (85, 86). This imbalance between ROS production and antioxidant capacity in the presence of leukocytes underscores the critical role of managing oxidative stress in treating male infertility (87, 88).

To mitigate leukocyte-induced oxidative stress in sperm, several strategies have been proposed. Anti-inflammatory treatments, such as the use of nonsteroidal anti-inflammatory drugs (NSAIDs) or corticosteroids, can help reduce leukocyte activation and ROS production. In cases where infections are the underlying cause of leukocytospermia, appropriate antimicrobial therapy can reduce leukocyte infiltration and ROS generation. Antioxidant supplementation is another approach used to counteract the effects of leukocyte-derived ROS. Vitamins C and E, glutathione, and coenzyme Q10 are examples of antioxidants that can neutralize ROS and reduce oxidative damage to sperm. Additionally, lifestyle modifications—such as reducing exposure to environmental toxins and managing stress—can help lower inflammation and reduce leukocyte activation.

4.2 Mitochondrial dysfunction

Mitochondria are another important internal source of ROS in sperm cells. As the cell's energy producers, mitochondria create ATP through a process called oxidative phosphorylation, which uses the electron transport chain (ETC) (41, 89). However, during this process, some electrons can escape from the ETC and react with oxygen, forming superoxide anions (O_2^-), a type of ROS. Although mitochondria have antioxidant defenses like SOD, which helps convert superoxide anions into less harmful molecules, too much mitochondrial ROS can cause oxidative stress and harm both the mitochondria and the sperm cell. Mitochondrial problems in sperm are a significant factor in male infertility, as they interfere with

energy production and the balance needed for sperm function. Located in the midpiece of the sperm, mitochondria are essential for providing the energy that enables sperm movement. When ROS levels in mitochondria are elevated due to electron leakage from the ETC, mitochondrial superoxide is generated, initiating a cascade of oxidative damage (35, 90). This oxidative stress can impair mitochondrial function, leading to reduced ATP production, which is necessary for sperm motility. As a result, sperm with dysfunctional mitochondria exhibit decreased motility and an inability to reach the oocyte, significantly reducing fertility potential.

In addition to impaired energy production, mitochondrial dysfunction can lead to direct damage to mitochondrial DNA (mtDNA) (91, 92). Furthermore, the limited DNA repair capacity in mitochondria makes it difficult for mtDNA to recover from oxidative damage. Mutations in mtDNA caused by ROS can further exacerbate mitochondrial dysfunction, creating a vicious cycle of oxidative stress and energy deficits (40, 41). This mitochondrial damage has been strongly associated with asthenozoospermia, a condition characterized by reduced sperm motility, which is a common cause of male infertility. The link between mitochondrial dysfunction and male infertility has been well-established in both clinical and experimental studies. Men with infertility often exhibit higher levels of mitochondrial ROS and lower levels of mitochondrial membrane potential, which correlates with poor sperm motility and reduced fertilization rates. Addressing mitochondrial dysfunction and reducing mitochondrial ROS production are important therapeutic goals for improving sperm quality and male fertility. Antioxidant therapies that specifically target mitochondria, such as coenzyme Q10 and mitochondrial-targeted antioxidants (e.g., mitoquinone), have shown promise in reducing mitochondrial oxidative stress and improving sperm motility (93). These antioxidants help restore the redox balance within mitochondria, reduce oxidative damage to mtDNA, and enhance ATP production, ultimately improving sperm function and fertility outcomes.

4.3 External and environmental sources

In addition to endogenous sources of oxidative stress, spermatozoa are also exposed to a variety of external and environmental factors that contribute to elevated ROS levels. These factors include pollution, exposure to toxins, lifestyle choices, and dietary habits, all of which have been shown to significantly impact sperm quality and fertility. Environmental pollution is a major contributor to oxidative stress in sperm. Airborne pollutants like particulate matter (PM), polycyclic aromatic hydrocarbons (PAHs), and heavy metals (such as cadmium and lead) can trigger ROS production when they are inhaled or absorbed into the body. These pollutants can harm sperm cells directly or cause systemic inflammation and oxidative stress in the male reproductive system. For example, exposure to PAHs and heavy metals has been linked to higher ROS levels, lower sperm motility, and increased DNA fragmentation in sperm. Similarly, men who are exposed to chemicals, pesticides, or

solvents at work have been found to experience greater oxidative damage in their sperm, leading to reduced fertility.

Lifestyle factors, particularly smoking and excessive alcohol consumption, are also significant contributors to oxidative stress in spermatozoa. Cigarette smoke contains a variety of toxic substances, including free radicals and heavy metals, that generate ROS both directly in the male reproductive tract and indirectly through systemic oxidative stress. Studies have consistently shown that smokers have higher levels of sperm DNA fragmentation, reduced sperm motility, and lower antioxidant capacity compared to non-smokers. Alcohol consumption exacerbates oxidative stress by depleting antioxidant reserves, such as glutathione, and increasing the production of ROS through the metabolism of ethanol. Diet and nutrition also play a key role in modulating oxidative stress in sperm. Diets high in saturated fats and processed foods can lead to increased inflammation and oxidative stress, while diets rich in antioxidants like vitamins C and E, zinc, selenium, and omega-3 fatty acids help protect sperm from oxidative damage. Studies show that consuming antioxidant-rich foods and supplements can improve sperm motility, reduce DNA fragmentation, and boost overall sperm quality in men with infertility. On the other hand, unhealthy eating habits that contribute to obesity and metabolic syndrome are linked to higher oxidative stress, more inflammation, and decreased sperm function (94).

Another significant source of external oxidative stress is exposure to heat and radiation. Elevated scrotal temperature, caused by factors such as prolonged sitting, tight clothing, or the use of electronic devices, can increase ROS production in spermatozoa and reduce sperm motility. Similarly, exposure to ionizing radiation, such as from medical imaging or environmental sources, can generate ROS and induce oxidative DNA damage in sperm, leading to reduced fertility. Mitigating the effects of external and environmental sources of oxidative stress on sperm requires a multifaceted approach. Reducing exposure to environmental pollutants and toxins, adopting healthy lifestyle habits, and improving dietary choices can significantly reduce ROS levels and improve sperm quality.

Oxidative stress in spermatozoa arises from a combination of endogenous and exogenous sources, with leukocyte-derived ROS, mitochondrial dysfunction, and external environmental factors all playing significant roles in generating ROS and damaging sperm cells. Leukocyte-derived ROS, particularly in the context of infections and inflammation, can induce lipid peroxidation, DNA fragmentation, and protein oxidation, all of which contribute to reduced sperm quality and male infertility. Mitochondrial dysfunction further exacerbates oxidative stress by impairing ATP production and damaging mitochondrial DNA, leading to compromised sperm motility and energy deficits. External factors, including pollution, toxins, lifestyle choices, and dietary habits, also contribute to oxidative stress and can significantly impact sperm quality and fertility outcomes. Understanding these sources of oxidative stress is essential for developing targeted therapeutic interventions, including antioxidant supplementation, anti-inflammatory treatments, and lifestyle modifications, to improve sperm function and enhance male fertility (95).

5 Antioxidant defense mechanisms in sperm

Spermatozoa are uniquely specialized cells essential for the process of fertilization, yet they exhibit a pronounced susceptibility to oxidative stress due to their distinct biological properties. Their membranes are rich in polyunsaturated fatty acids (PUFAs), which are crucial for preserving membrane fluidity and functionality. However, these PUFAs are particularly prone to oxidative damage from ROS. Compounding this vulnerability, sperm cells contain minimal cytoplasm, thereby limiting their capacity to store antioxidant enzymes and molecules that could neutralize ROS. Consequently, spermatozoa rely extensively on both enzymatic and non-enzymatic antioxidant defense systems to mitigate oxidative damage. This section will delve into the available antioxidant defenses in sperm cells, their inherent limitations, and the therapeutic approaches designed to bolster these protective mechanisms.

5.1 Natural antioxidant defenses in sperm

The body inherently generates antioxidant enzymes and non-enzymatic molecules to neutralize ROS and shield cells from oxidative damage. In spermatozoa, several key antioxidant enzymes are present, including SOD, glutathione peroxidase (GPx), and catalase. These enzymes are essential for scavenging ROS and mitigating the harmful effects of oxidative stress. Superoxide dismutase catalyzes the conversion of superoxide radicals into hydrogen peroxide, which is then broken down by catalase and GPx into water and oxygen, thereby preventing the accumulation of toxic byproducts and preserving the structural and functional integrity of sperm cells. These antioxidant defenses are vital for protecting spermatozoa from the detrimental consequences of oxidative stress, which can impair motility, fertilization potential, and DNA integrity (52, 96). These antioxidant enzymes work in concert to detoxify ROS, thereby reducing their harmful impact on sperm cells. Superoxide dismutase is one of the most important enzymatic antioxidants present in sperm. It catalyzes the conversion of superoxide anions (O_2^-), which are highly reactive, into hydrogen peroxide (H_2O_2), which is less harmful. This process is crucial because superoxide anions are among the first ROS to be produced during oxidative stress, particularly in mitochondria, which are key sources of ROS in sperm cells. By converting superoxide anions into hydrogen peroxide, SOD prevents them from reacting with other molecules and causing further damage (97, 98).

Glutathione peroxidase (GPx) serves as another essential antioxidant enzyme in sperm, playing a pivotal role in neutralizing hydrogen peroxide and lipid hydroperoxides, which arise when ROS target polyunsaturated fatty acids (PUFAs) in the sperm membrane (99, 100). GPx relies on glutathione (GSH), a tripeptide that donates electrons to reduce peroxides and counteract ROS, effectively preventing oxidative damage. By inhibiting lipid peroxidation, GPx safeguards the structural integrity of the sperm membrane, thereby maintaining motility and preserving the

sperm's fertilization potential. Catalase also plays a crucial part in the sperm's antioxidant defense system. Similar to GPx, catalase converts hydrogen peroxide into water and oxygen, thus preventing the buildup of hydrogen peroxide and inhibiting the formation of highly reactive hydroxyl radicals ($\bullet OH$) via the Fenton reaction (101, 102). Hydroxyl radicals, among the most destructive ROS, can inflict severe oxidative damage on DNA, proteins, and lipids within sperm cells. By detoxifying hydrogen peroxide, catalase helps maintain sperm functionality and protects against oxidative stress-induced damage, further reinforcing the cell's defense mechanisms.

In addition to these enzymatic antioxidants, sperm and seminal plasma contain several non-enzymatic antioxidants that provide further protection against oxidative stress. These include vitamins C and E, glutathione, coenzyme Q10, selenium, and zinc. Vitamin C (ascorbic acid) is a water-soluble antioxidant that acts as a primary line of defense against ROS in the seminal plasma. It scavenges free radicals and helps regenerate vitamin E, which is a lipid-soluble antioxidant that protects the sperm membrane from lipid peroxidation. Vitamin E, in particular, is essential for maintaining the integrity of the PUFAs in the sperm membrane, which are highly susceptible to oxidative attack. Glutathione, which works in tandem with GPx, plays a critical role in reducing lipid hydroperoxides and protecting sperm DNA from oxidative damage. Selenium and zinc support the activity of key antioxidant enzymes, including GPx and SOD, helping to enhance sperm antioxidant defenses. Coenzyme Q10, which is involved in mitochondrial energy production, also functions as an antioxidant by reducing mitochondrial ROS production and protecting sperm from oxidative stress. These antioxidants play a vital role in mitigating the oxidative stress induced by the freezing-thawing process, improving sperm viability post-thaw, and reducing DNA fragmentation. For instance, adding antioxidants to cryopreservative media has been shown to enhance sperm motility and fertilizing capacity after thawing, contributing to higher pregnancy rates in ART. Additionally, antioxidants can protect sperm DNA integrity, reducing fragmentation caused by ROS during sperm processing, which is crucial for successful fertilization and embryo development.

5.2 Limitations of sperm's antioxidant capacity

Antioxidants play a critical role in protecting cells from oxidative damage, which can severely affect key cellular components like the plasma membrane, proteins, and DNA. Various techniques are employed to detect ROS, each offering unique insights into their impact on cellular structures. For example, fluorescent probes such as DCFDA and DHE are commonly used to visualize ROS generation in live cells. These probes emit fluorescence upon oxidation, allowing real-time monitoring of ROS levels in various cellular compartments. Another powerful technique is electron spin resonance (ESR), which directly detects free radicals and is often used in combination with spin-trapping agents to enhance the sensitivity

of ROS detection. Chemiluminescence assays can also quantify ROS levels in biological samples, providing a sensitive method for assessing oxidative stress. Moreover, HPLC-MS/MS techniques allow for the precise identification and quantification of ROS byproducts, providing detailed information on the types of oxidative damage occurring within the cell. The damage caused by ROS extends to the plasma membrane, where lipid peroxidation is a primary consequence. Lipid peroxidation can be measured by the TBARS assay or through the detection of MDA, a byproduct of membrane lipid oxidation. This damage compromises membrane integrity, affecting cellular signaling and transport functions. At the protein level, ROS can induce oxidation of amino acids, particularly cysteine and methionine residues. Techniques such as Western blotting and mass spectrometry are often used to identify and quantify protein carbonylation, a hallmark of oxidative protein damage. Finally, DNA is highly susceptible to ROS-induced damage, which can result in strand breaks, base modifications, and crosslinking. The Comet assay is a widely used technique to assess DNA strand breaks at the single-cell level, providing valuable insight into the extent of genotoxic damage caused by ROS. Additionally, the use of immunofluorescence assays to detect γ H2AX foci offers a powerful approach for identifying double-strand breaks in DNA, a direct consequence of ROS-mediated damage.

Despite having these natural antioxidant defenses, spermatozoa are highly susceptible to oxidative stress due to several inherent limitations in their ability to counteract excessive ROS. One of the primary limitations is the reduced cytoplasmic volume in sperm cells, which limits the storage of antioxidant enzymes and molecules. During spermatogenesis, sperm lose most of their cytoplasm to achieve a streamlined shape that facilitates motility. However, this reduction in cytoplasm also reduces the capacity of sperm to harbor antioxidant enzymes, making them more vulnerable to oxidative stress. Compared to somatic cells, sperm cells have fewer resources to neutralize ROS, leaving them more exposed to oxidative damage.

Another limitation is the high content of PUFAs in sperm membranes. While these fatty acids are essential for maintaining membrane fluidity and ensuring that sperm can navigate the female reproductive tract and fertilize the oocyte, they are also highly prone to oxidation. When ROS levels rise, PUFAs in the sperm membrane are oxidized in a process known as lipid peroxidation, which generates toxic byproducts such as MDA and 4-HNE. These byproducts can damage the sperm membrane, reducing its fluidity and impairing sperm motility and function. Once lipid peroxidation is initiated, it can propagate rapidly, causing widespread membrane damage that significantly impairs sperm's ability to fertilize the egg. In addition to limited antioxidant reserves and the vulnerability of PUFAs, sperm mitochondria are another source of ROS that contributes to oxidative stress. Mitochondria are responsible for producing the energy (in the form of ATP) required for sperm motility through the process of oxidative phosphorylation. While mitochondria have their own antioxidant defenses, including mitochondrial SOD, these defenses are often insufficient to neutralize the high levels of ROS produced during oxidative phosphorylation, especially if mitochondrial function is

compromised. The continuous production of ROS by mitochondria can lead to mitochondrial dysfunction, impaired ATP production, and reduced sperm motility.

Another major limitation of spermatozoa is their inability to effectively repair DNA damage. During spermatogenesis, sperm DNA is highly compacted and packaged with protamines, replacing histones to create a more condensed structure. While this packaging protects the DNA from some forms of damage, it also limits the sperm cell's ability to repair oxidative damage, such as DNA fragmentation caused by ROS.

5.3 Therapeutic strategies to enhance antioxidant defense

Given the limitations of sperm's natural antioxidant defenses, therapeutic strategies aimed at enhancing antioxidant capacity are essential for protecting sperm from oxidative stress and improving male fertility (103). Antioxidant supplementation is one of the most widely studied and applied approaches to enhancing sperm antioxidant defenses. A variety of antioxidant supplements, including vitamins C, glutathione, selenium, and zinc, have been shown to improve sperm quality by reducing ROS levels and preventing oxidative damage (104, 105). These antioxidants work by scavenging ROS, reducing lipid peroxidation, and protecting DNA and proteins from oxidative stress-induced damage. Antioxidants, including vitamin C, vitamin E, and Coenzyme Q10, play a critical role in protecting cells from oxidative stress (106, 107). In fertility treatments, antioxidants help to safeguard the DNA integrity of sperm and eggs, which is essential for successful conception. Research has shown that antioxidants can enhance egg quality in women undergoing IVF treatments and improve sperm motility in men, offering a practical solution for couples struggling with infertility (108, 109). For instance, CoQ10 supplementation has been linked to improved egg quality and better outcomes in IVF patients (110, 111). Furthermore, the use of antioxidants in animal conservation efforts, such as enhancing reproductive health in endangered species, is gaining traction. By mitigating oxidative damage in animals exposed to captivity stress or environmental toxins, antioxidants have been used to improve breeding success rates, exemplified by their use in endangered amphibian programs. These practical applications highlight the growing significance of antioxidants not only in human fertility treatments but also in global biodiversity conservation efforts.

In addition to supplementation, dietary and lifestyle modifications can also help enhance sperm antioxidant defenses. A diet rich in antioxidant-rich foods, such as fruits, vegetables, nuts, and seeds, provides a wide range of vitamins, minerals, and polyphenols that help neutralize ROS and protect sperm from oxidative damage. Lifestyle changes, such as quitting smoking, reducing alcohol intake, and avoiding environmental toxins, can also reduce ROS production and improve sperm quality. Smoking and alcohol consumption are known to increase oxidative stress, deplete antioxidant reserves, and impair sperm function. By making these lifestyle changes, men can significantly reduce their oxidative stress levels and improve their reproductive health.

In conclusion, while sperm possess natural antioxidant defense mechanisms, these defenses are limited by the unique characteristics of spermatozoa, including their reduced cytoplasmic volume, high content of PUFAs, and mitochondrial ROS production. Enhancing antioxidant defenses through supplementation, dietary modifications, and lifestyle changes is a promising approach to reducing oxidative stress, protecting sperm from damage, and improving male fertility outcomes. Understanding the limitations of sperm's antioxidant capacity and implementing strategies to boost these defenses is essential for addressing oxidative stress-related male infertility.

6 Conclusion

Oxidative stress is broadly acknowledged as a principal contributor to male infertility, exerting a substantial influence on spermatozoal dysfunction. As emphasized in the discourse, this stress emerges from a disproportion between the generation of ROS and the organism's capacity to counteract these reactive entities via its antioxidant mechanisms. Sperm cells, in particular, are highly susceptible to oxidative stress because of their membranes' rich PUFA content, coupled with a scarcity of antioxidant protections and a restricted ability for DNA repair. The molecular mechanisms by which oxidative stress induces sperm damage are diverse and have profound implications for male fertility. These mechanisms include lipid peroxidation, DNA fragmentation, and protein oxidation, all of which lead to compromised sperm function, reduced motility, impaired fertilization capability, and genetic abnormalities that may affect offspring. Lipid peroxidation stands out as one of the most critical outcomes of oxidative stress affecting sperm function. This process entails the oxidation of polyunsaturated fatty acids (PUFAs) within the sperm membrane, giving rise to harmful byproducts such as MDA and 4-HNE. These toxic compounds compromise the fluidity and integrity of the membrane, which are essential for preserving sperm motility and facilitating key processes like capacitation and the acrosome reaction, both of which are vital for successful fertilization. Moreover, lipid peroxidation disrupts the acrosomal membrane, diminishing the sperm's capacity to fuse with the oocyte. Consequently, oxidative damage induced by lipid peroxidation directly impairs sperm motility and fertilization potential, thereby contributing to infertility.

Oxidative stress also induces DNA fragmentation in spermatozoa, leading to the formation of single-strand and double-strand breaks in sperm DNA. This damage is exacerbated by the limited capacity of sperm to repair oxidative damage due to the compact packaging of DNA with protamines and the minimal availability of DNA repair mechanisms. DNA fragmentation reduces the genetic integrity of sperm, impairing fertilization and increasing the risk of poor reproductive outcomes, such as miscarriage, failed implantation, and the transmission of genetic defects to offspring. In cases where ART are used, the presence of fragmented sperm DNA can further complicate successful fertilization and increase the risk of developmental abnormalities in embryos. Protein oxidation is another critical molecular

mechanism by which oxidative stress impairs sperm function. ROS can modify sperm proteins, leading to structural changes that impair motility, metabolic processes, and the sperm's ability to bind to the oocyte. Oxidation of cytoskeletal proteins, such as actin and tubulin, compromises the structural integrity of the sperm's flagellum, reducing motility and the sperm's ability to reach the oocyte. Additionally, oxidative damage to metabolic enzymes reduces ATP production, further impairing sperm motility and energy availability. Protein oxidation also affects surface proteins involved in sperm-egg recognition, further diminishing fertilization potential.

The substantial impact of oxidative stress on sperm functionality and male fertility underscores the necessity for early detection and proactive management in infertile men. Prompt diagnosis of oxidative stress in seminal fluid is feasible through evaluating biomarkers such as levels of ROS, lipid peroxidation byproducts like MDA, and DNA fragmentation indices. Systematic screening for oxidative stress should be prioritized for men exhibiting infertility, especially those with contributing risk factors such as smoking, obesity, or exposure to environmental toxins. Such early identification is crucial for directing timely interventions that can alleviate the harmful effects of oxidative stress on spermatozoa and enhance fertility prospects. Management strategies for oxidative stress-induced sperm damage predominantly concentrate on boosting antioxidant defenses via dietary supplements and lifestyle adjustments. Antioxidant treatment, extensively examined for its potential to decrease ROS levels and shield sperm against oxidative harm, includes the use of supplements like vitamins C and E, coenzyme Q10, glutathione, selenium, and zinc. These antioxidants not only neutralize ROS and diminish lipid peroxidation but also safeguard sperm DNA from oxidative injuries. Furthermore, they bolster mitochondrial function, curb the generation of mitochondrial ROS, and augment sperm motility. Clinical research has validated the effectiveness of antioxidant supplements in mitigating oxidative stress, enhancing sperm quality, and elevating pregnancy rates among couples grappling with infertility.

In addition to antioxidant supplementation, lifestyle modifications are crucial in reducing oxidative stress and improving sperm health. Men are encouraged to adopt healthier habits, such as quitting smoking, reducing alcohol consumption, managing stress, and improving their diet. Smoking and excessive alcohol intake are well-known contributors to oxidative stress, depleting antioxidant reserves and increasing ROS production. Adopting a diet rich in antioxidants—such as fruits, vegetables, nuts, seeds, and omega-3 fatty acids—can provide additional protection against oxidative damage and enhance the body's natural antioxidant defenses. Regular physical activity and stress management techniques, such as meditation and yoga, can also reduce oxidative stress and improve overall reproductive health. Despite the progress made in understanding and managing oxidative stress in male infertility, there remain several areas for future research. One of the primary challenges is optimizing antioxidant therapies. While antioxidant supplementation has shown promise, there is still a need to determine the optimal types, doses, and combinations of antioxidants that are most

effective for reducing oxidative stress and improving fertility outcomes. Research should focus on personalized approaches to antioxidant therapy, taking into account individual variations in ROS levels, antioxidant capacity, and underlying health conditions that may influence the effectiveness of treatment.

Another area for future research is the development of novel diagnostic tools for detecting oxidative stress in semen. While current methods focus on assessing ROS levels, lipid peroxidation products, and DNA fragmentation, more advanced techniques are needed to provide a comprehensive assessment of oxidative damage in spermatozoa. Proteomics and metabolomics approaches could be used to identify specific oxidative modifications in sperm proteins and metabolites, providing more detailed insights into the molecular mechanisms of oxidative stress-induced sperm damage. These tools could also help identify new therapeutic targets for reducing oxidative stress and improving sperm function. Furthermore, research should explore the long-term impact of oxidative stress on male fertility and offspring health. While it is clear that oxidative stress impairs sperm function and increases the risk of infertility, there is growing evidence to suggest that oxidative damage to sperm DNA may have long-term consequences for offspring, including increased susceptibility to genetic disorders and developmental abnormalities. Investigating the mechanisms by which oxidative stress affects epigenetic modifications in sperm and how these modifications may influence embryonic development and offspring health is a promising area of research that could have significant implications for reproductive medicine.

In summary, oxidative stress plays a pivotal role in male infertility, primarily through its deleterious effects on spermatozoa, which manifest as diminished motility, reduced fertilization capabilities, and compromised genetic integrity. Prompt detection and proactive intervention are imperative for the effective management of oxidative stress in men suffering from infertility, with antioxidant supplementation and lifestyle modifications serving as the cornerstone of treatment strategies. Nonetheless, there remains a critical need for further research to refine antioxidant therapies, develop more sophisticated diagnostic tools, and elucidate the long-term effects of oxidative stress on male fertility and the health of subsequent generations. By surmounting these challenges, we can enhance the management of oxidative stress-induced male infertility and improve reproductive outcomes for affected couples.

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YW: Conceptualization, Methodology, Writing – original draft. XF: Conceptualization, Writing – original draft. HL: Conceptualization, Project administration, Supervision, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors would like to thank the biological Science Department of Peking ChipStone for helpful on the figure conduction related to this work

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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OPEN ACCESS

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RECEIVED 13 June 2024

ACCEPTED 05 February 2025

PUBLISHED 25 February 2025

CITATION

Chen P, Ni S and Ou-Yang L (2025) Causal inference of inflammatory proteins in infertility: a Mendelian randomization study. *Front. Endocrinol.* 16:1448530. doi: 10.3389/fendo.2025.1448530

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Causal inference of inflammatory proteins in infertility: a Mendelian randomization study

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Background: Infertility affects 8-12% of couples globally, manifesting as a complex reproductive disorder with varied causes, negatively impacting emotional, physical, and social well-being. Inflammation is implicated in many diseases, including male and female infertility.

Methods: This study employed Mendelian randomization (MR) with two-sample, bidirectional, and mediation approaches to explore the relationship between circulating inflammatory proteins and infertility. Causal analysis was conducted using inverse variance-weighted (IVW) and MR-Egger regression, supplemented by enrichment analysis, protein-protein interaction (PPI) network exploration, and drug signature analysis.

Results: Our findings identified a significant positive correlation between C-X-C motif chemokine 6 (CXCL6) and male infertility, positioning CXCL6 as a potential therapeutic target or biomarker. No causal links were detected between circulating inflammatory proteins and female infertility post-FDR adjustment. Minor mediation effects were observed for metabolites such as androstenediol monosulfate, arachidonoylcholine, and serum phosphate to glycerol ratio. Cytokine-related pathways emerged as significant in both male and female infertility. Gene-drug interaction analysis highlighted the need for further investigation of pioglitazone in treating female infertility.

Conclusion: This study establishes a potentially causal relationship between CXCL6 and male infertility, suggesting its potential as a drug target or molecular biomarker. The integrative approach combining causal inference with molecular pathway and drug interaction analysis opens new avenues for understanding and treating infertility.

KEYWORDS

infertility, Mendelian randomization, drug target, molecular biomarker, CXCL6

Introduction

Infertility is a worldwide human health problem that affects approximately over 186 million individuals globally (1, 2). The causes of infertility vary, with female factors accounting for about 70% of infertility cases, with common causes including ovulatory disorders and fallopian tube damage (3). Male factors contribute to the remaining roughly 30% of infertility cases due to causes such as abnormal sperm count, morphology, or lifespan (4). Independent of etiology, infertility has a profound emotional, physical, and social impact on couples and results in a significant psychological burden (5). Thus, understanding the causes of infertility and exploring a possible causal link between risk factors and infertility are crucial for affected couples and healthcare professionals.

Inflammation is a physiologic host response to infection or injury. However, aberrant inflammatory responses result in tissue damage and are central to the pathogenesis of multiple diseases, including reproductive diseases (6). Inflammation can have significant effects on both male and female infertility. For example, untreated sexually transmitted diseases in females can lead to tubal factor infertility due to tubal inflammation, damage, and scarring (7). Mutations in TNFAIP3 have been linked to both infertility and central nervous system inflammation, highlighting the role of inflammation in female infertility (8). In males, inflammation can lead to leukocytospermia, due to the production of reactive oxygen free radicals, and chronic testicular inflammation, such as orchitis, is also known to cause male infertility (9, 10). The role of inflammation and oxidative stress in male infertility is well-documented, with mechanistic pathways linking causative factors of male reproductive tract inflammation, oxidative stress induction, and oxidant-sensitive cellular cascades leading to male infertility (11, 12). The presence of inflammatory states and the identification of semen markers of inflammation are crucial for the diagnostic-therapeutic management of male infertility (13). These results emphasize the critical role of inflammation in both male and female infertility, highlighting the need for further research and therapeutic interventions targeting inflammatory pathways to address infertility.

Inflammatory responses are orchestrated by a complex network of cells and inflammatory mediators, such as circulating inflammatory proteins (CIPs). For instance, high levels of CIPs, like growth-regulated oncogene- α (GRO α) are related to increased risks of female infertility (14). CXCL6 plays a key role in recruiting neutrophils to facilitate non-specific immunity during the process of inflammation (15). Additionally, GCP-2/CXCL6 exhibits antibacterial activity and plays key roles in host defense of the male urogenital tract as well as during fertilization (16). Furthermore, CIPs are vulnerable to the direct effects of conventional small molecules or biologics, making them attractive potential drug targets (17). Therefore, the discovery of genetic determinants affecting CIP levels may offer important clues about the etiology of multiple diseases, such as infertility.

Randomized controlled trials (RCTs) are a gold standard means to test hypotheses at a population level (18, 19). However, RCTs require intensive human resources, cost, and time, and some interventional strategies are not suitable for RCT-dependent assessments. Recently,

Mendelian randomization (MR) has gained attention as a complementary method of exploring the relationship between the treatment and outcome in patients, based on the frequency of single nucleotide polymorphisms (SNPs) as the instrumental variable (IV). This approach might avoid the confounding bias of conventional epidemiological investigations (20). Here, we performed a comprehensive two-sample MR analysis to determine the potential causal association between CIPs and infertility.

Materials and methods

Study design

This epidemiologic MR study was carried out by adhering to the STROBE guidelines (21). The reliable implementation of MR is based on three major assumptions: (i) relevance (genetic variants are strongly linked to the exposure), (ii) independence (variants are not correlated with confounders), and (iii) absence of horizontal pleiotropy (variants affect the outcome only via the exposure) (22). Here, we utilized bidirectional and 2-sample MR to understand the pathological correlation between CIPs and infertility. Then we used significant positive CIPs to construct a phenome-wide association study (PheWAS) and phenotype scanning analysis. We also used MR to assess for a causal relationship between significant CIP and plasma metabolites to find potential mediators of infertility. A protein-protein interaction (PPI) map was then generated between the positive CIP and infertility, and we performed functional enrichment analysis and drug signature analysis with positive CIPs (Figure 1).

GWAS summary data sources

The genome-wide pQTL (protein quantitative trait loci) mapping for CIP was obtained from studies by Zhao et al. (23). This dataset contained 91 CIP measured using the Olink Target Inflammation panel in 11 cohorts, with a total of 14,824 participants of European ancestry. The plasma metabolite data was obtained from a series of large GWASs by Chen et al. (24). This dataset included 1,091 metabolites and 309 metabolite ratios in 8,299 individuals from the Canadian Longitudinal Study on Aging (CLSA) cohort. We used publicly available GWAS summary datasets for male infertility from the FinnGen cohort (R9 release, total $n = 73479$; case = 680, control = 72799) and female infertility data from the FinnGen cohort (R9 release, total $n = 75450$; case = 6481, control = 68969). FinnGen is a large public-private collaboration that includes genomic data from 500,000 individuals of Finnish individuals who are over 18 years of age (25). These GWAS summary datasets and the GWAS summary datasets data for sensitivity analysis are listed in Table 1.

SNP selection

Exposure-associated independent SNPs ($r^2 = 0.001$, ≥ 10000 kb) were selected according to their genome-wide (GW) significance

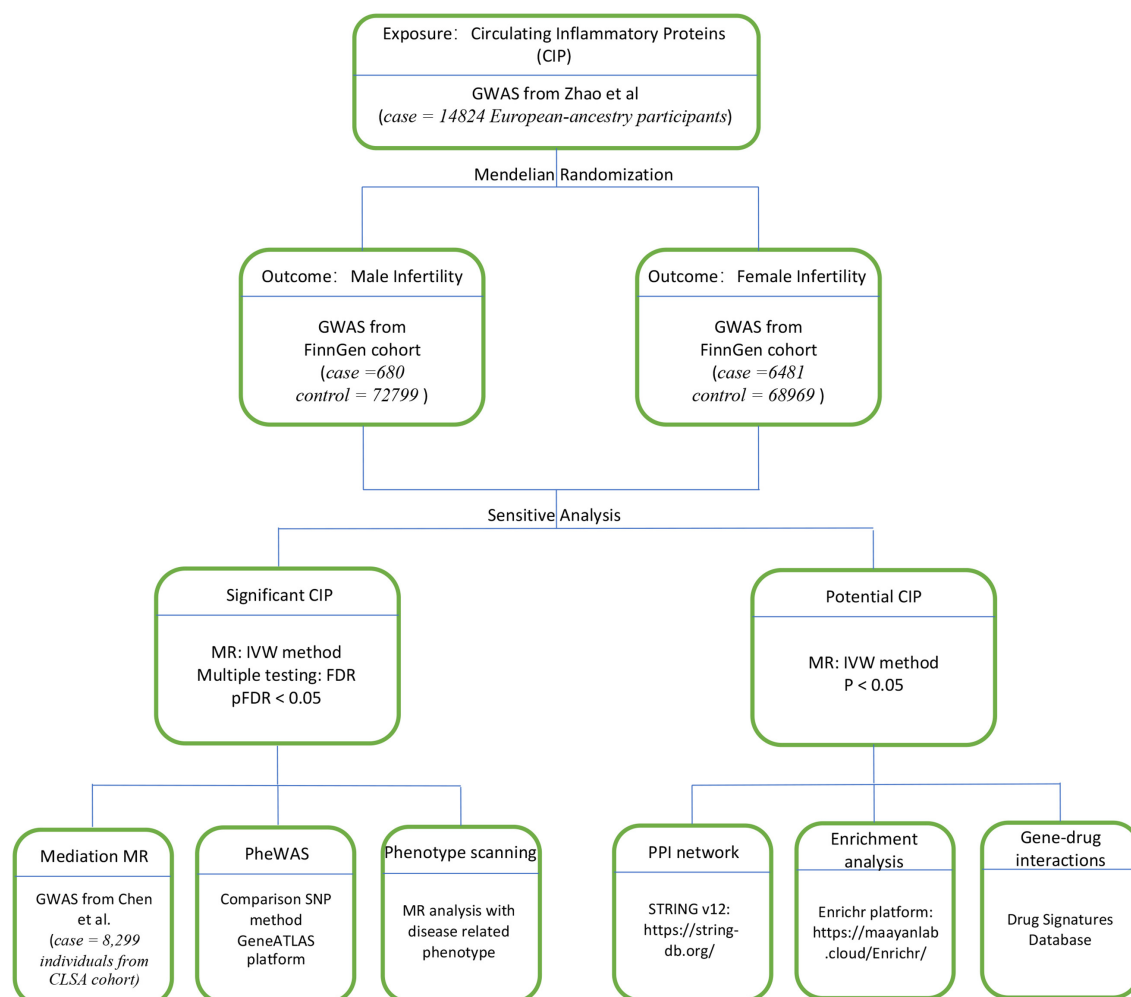


FIGURE 1
Study design. Overview of the MR study design.

($p < 1.0 \times 10^{-5}$) (26). The F-statistic (F-stat) of a phenotype is a measure of instrument strength (IS) and is related to the proportion of variance. An F-stat of at least 10 variables indicates a relatively lower risk of instrument bias (IB) in MR analysis (27). The number of SNPs for each CIP and the corresponding scale unit and F-stats are presented in **Supplementary Table S1**.

MR analysis

First, SNPs were harmonized to the exposure and outcome in an allele-specific manner to ensure proper alignment of effects. Wherever applicable, if any instrumental SNPs for the exposure were absent in the corresponding outcome dataset, a proxy was incorporated using GV in the linkage disequilibrium ($r^2 > 0.8$).

Initially, we employed an inverse variance-weighted (IVW) meta-analysis approach for MR analysis (28), in addition to the weighted median (29) and MR-Egger regression (MR-ER) approach. Multiple testing was conducted based on the false

discovery rate (FDR) method and a $p\text{FDR} < 0.05$ was deemed a significant CIP. P values < 0.05 indicated a potential CIP.

The MR-ER method was further used to assess any potential impacts of the directional pleiotropy (DP) (30) and to perform the MR-pleiotropy residual sum and outlier (MR-PRESSO) method. A P value > 0.05 indicated no horizontal pleiotropy. Data heterogeneity was determined by Cochran's Q test (31), with P > 0.05 indicating no heterogeneity. Leave-one-out (LOO) analysis indicated the influence of a single SNP on overall estimates.

"TwoSampleMR v0.5.8," "MRPRESSO v1.0", and "MendelianRandomization v0.9.0" packages in R v4.3.1 (source codes: <https://github.com/studentyaoshi/MR>) were applied for statistical analyses.

Reverse causality detection

To detect potential RC, genetic instruments for infertility were selected in a similar strategy to CIPs selection from GWAS datasets for

TABLE 1 Description of the contributing GWAS studies.

Traits	PMID	Case size	Sample size	Ancestry
Exposure				
Circulating inflammatory proteins	37563310	NA	14824	European
Outcomes				
Male infertility	FinnGen cohort	680	73479	European
Female infertility	FinnGen cohort	6481	68969	European
Mediator				
Plasma metabolites	36635386	NA	8299	European
Phenotypes				
Myocardial infarction	33959723	11081	484598	European
Allergic disease asthma hay fever or eczema	29083406	180129	360838	European
Celiac disease	22057235	12041	24269	European and Asian
Colorectal cancer	34594039	14886	637693	European and Asian
Coronary artery disease	29212778	122733	547261	European
Diabetes type 1	34012112	18942	501638	European
Glaucoma primary open angle	FinnGen cohort	4433	214634	European
Hypertension	33959723	129909	484598	European
Hypothyroidism	29892013	NA	473703	European
Inflammatory bowel disease	33959723	4101	480497	European
Juvenile idiopathic arthritis including oligoarticular and rheumatoid factor negative polyarticular JIA	23603761	2816	13056	European
Primary biliary cirrhosis	22961000	2861	11375	European
Primary sclerosing cholangitis	27992413	2871	14890	European
Rheumatoid arthritis	33959723	5427	484598	European
Selective immunoglobulin A deficiency IgAD	27723758	1635	6487	European
Self-reported psoriasis	33959723	5427	484598	European
Systemic lupus erythematosus	34594039	964	659165	European and Asian
Vitiligo	FinnGen cohort	131	207613	European

bidirectional MR analysis (32). The effect was estimated by MR-Egger, IVW, and weighted mode. A $P<0.05$ indicated statistical significance.

coefficients” approach to evaluate the indirect effects of significant CIPs on infertility. The delta method was applied to calculate the standard error (SE) for an indirect effect.

Mediation MR analysis

In mediation terms, a two-step MR was applied to distinguish between indirect and direct effects. Significant CIP-related genetic instruments (GIs) were used to assess the causal effects of plasma metabolites. GIs for plasma metabolites were then used to determine the effect of each plasma metabolite on possible CIP-related infertility. Notably, if significant CIP-related infertility was found to influence the mediator, we used the “product of

PheWAS

To evaluate the horizontal pleiotropy of significant CIPs, a PheWAS was performed. We compiled a comparison SNP-set to serve as a control for PheWAS analyses. Four control SNPs were matched to the significant CIP-associated SNPs on: minor allele frequency ($\pm 5\%$), surrounding gene density ($\pm 50\%$), distance to the nearest gene ($\pm 50\%$), and as a proxy for haplotype block size,

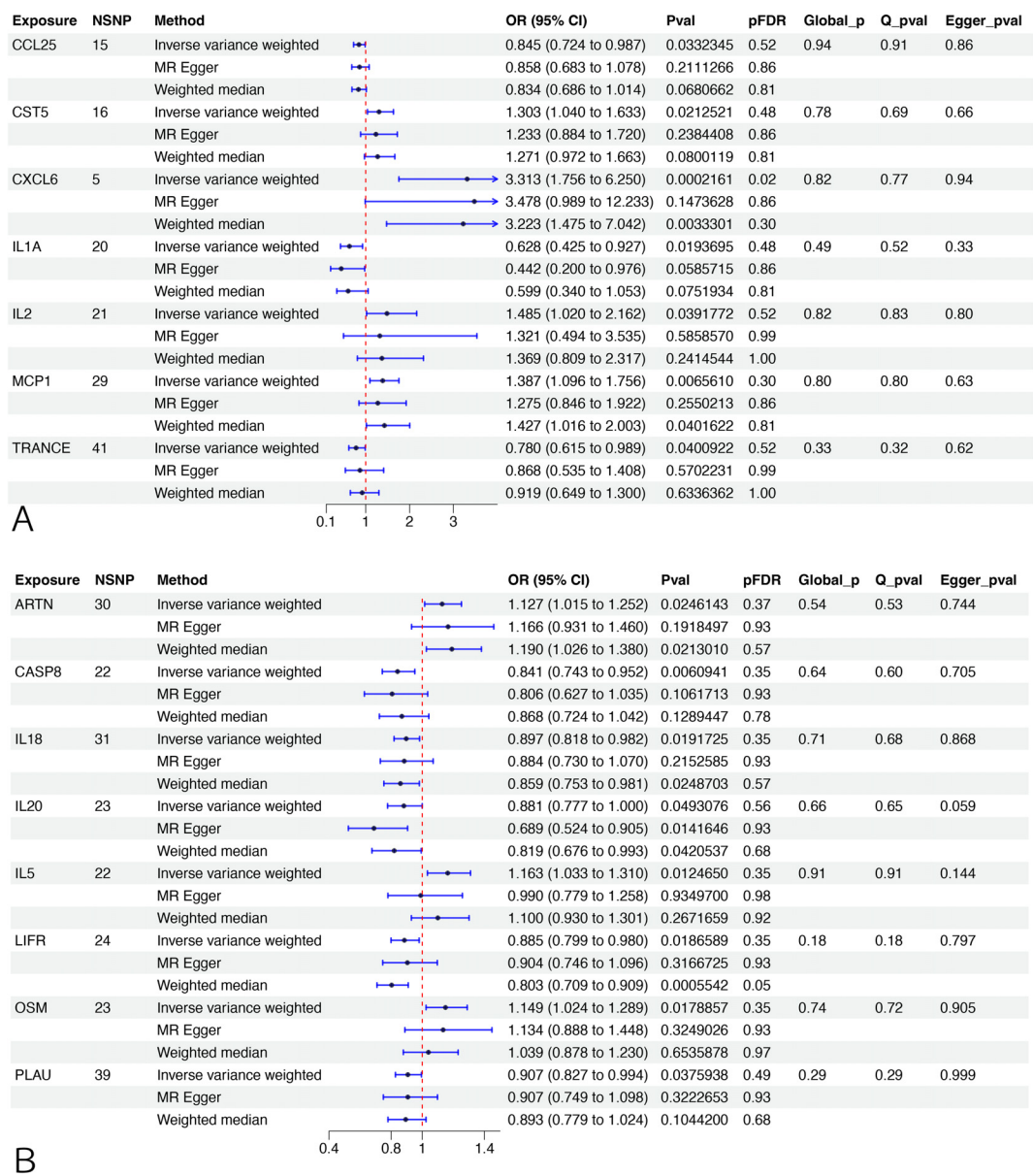


FIGURE 2 MR analysis between CIPs and infertility. **(A)** The association between CIPs trait and male infertility. **(B)** The association between CIPs trait and female infertility. NSNP, number of single-nucleotide polymorphism. OR (95%CI): odds ratio (95% confidence interval). FDR, false discovery rate. A pFDR<0.05 was deemed a significant CIP, and Pval <0.05 indicated a potential CIP. Q_pval, p value of Cochran's Q test. Egger_pval, p value of MR-Egger regression (MR-ER) method. Global_p, p value of MR-PRESSO.

the number of other SNPs in LD at $R^2 \geq 0.50$ ($\pm 50\%$) (33). GeneAtlas is a searchable dataset for 778 traits (118 quantitative, 660 binary) and associations with 9,113,133 genetic variants (genotyped or imputed) and can be queried for genetic or phenotypic data to assess genotype-phenotype associations (34). We queried GeneAtlas for trait associations with significant CIPs. Nominally significant SNP-trait associations ($p < 0.01$) were carried forward to trait-enrichment analyses, as previously described (35). PheWAS associations of the significant CIP SNPs with 778 phenotypic traits were compared to PheWAS results for the control SNP-set. We used Fisher's exact tests to compare the frequency at which individual traits were associated with

significant CIPs SNPs versus control SNPs to determine if traits were enriched for association with significant CIP risk variants. Fisher's exact p-values for trait enrichment underwent FDR correction to adjust for multiple testing.

Phenotype scanning

Phenotype scanning was carried out by searching the previous GWAS via a 'phenoscanner' to reveal the associations of an identified CIPs with other traits (36). A pleiotropic SNP was considered to possess: (i) a significant genome-wide association ($P < 5 \times 10^{-8}$), (ii) a

relationship with European descendants in GWAS, and (iii) associations with any known etiological risk factors, including protein, metabolic, and clinical traits. Additionally, the LD r^2 among identified CIPs revealed their potential linkages with potential infertility drug targets.

PPI network

The PPI network analysis (STRING v12; <https://string-db.org/>; minimum interaction score = 0.4) was used to detect any associations between positive CIPs.

Functional enrichment analysis of positive CIPs and gene-drug interactions

Enrichr platform (<https://maayanlab.cloud/Enrichr/>), an independent web server for gene set enrichment analysis (37), was used to perform functional enrichment analysis on positive CIPs. Drug Signatures Database (DSigDB) was used to identify drug molecules that interacted with positive CIPs through the Enrichr platform.

Data availability

All relevant data for this study are included in the article or uploaded as online supplemental information. Full GWAS

summary statistics for the exposure and outcome data used in the manuscript can be found at <https://www.ebi.ac.uk/gwas>, <https://gwas.mrcieu.ac.uk/>, or <https://r9.risteyns.finnngen.fi>.

Results

Associations between CIPs and male infertility

Two-sample MR and IVW analyses were performed to reveal any causal effects of CIPs on male infertility. After FDR adjustment, only C-X-C motif chemokine 6 (CXCL6) was found to have a positive relationship with male infertility (95% CI: 1.7564 to 6.2502, $pFDR=0.01966$) and considered a significant CIP. Another 6 CIPs were identified as having potentially significant relationships impacting male infertility, including 3 beneficial: Monocyte chemoattractant protein-1 (MCP1) (95% CI: 1.0956 to 1.7563, $p=0.0066$), Cystatin D (CST5) (95% CI: 1.0403 to 1.6323, $p=0.0213$), and Interleukin-2 (IL2) (95% CI: 1.0198 to 2.1619, $p=0.0392$) and 3 deleterious: Interleukin-1-alpha (IL1A) (95% CI: 0.4252 to 0.9275, $p=0.0194$), C-C motif chemokine 25 (CCL25) (95% CI: 0.7241 to 0.9867, $p=0.0332$), and TNF-related activation-induced cytokine (TRANCE) (95% CI: 0.6154 to 0.9888, $p=0.0401$). These results were further validated by LOO testing. Sensitivity analysis confirmed heterogeneity in the results without evidence of pleiotropy (Figure 2, Supplementary Table S2). MR-PRESSO analysis showed no horizontal pleiotropy and identified outliers (Supplementary Table S2), and bidirectional MR analyses did not

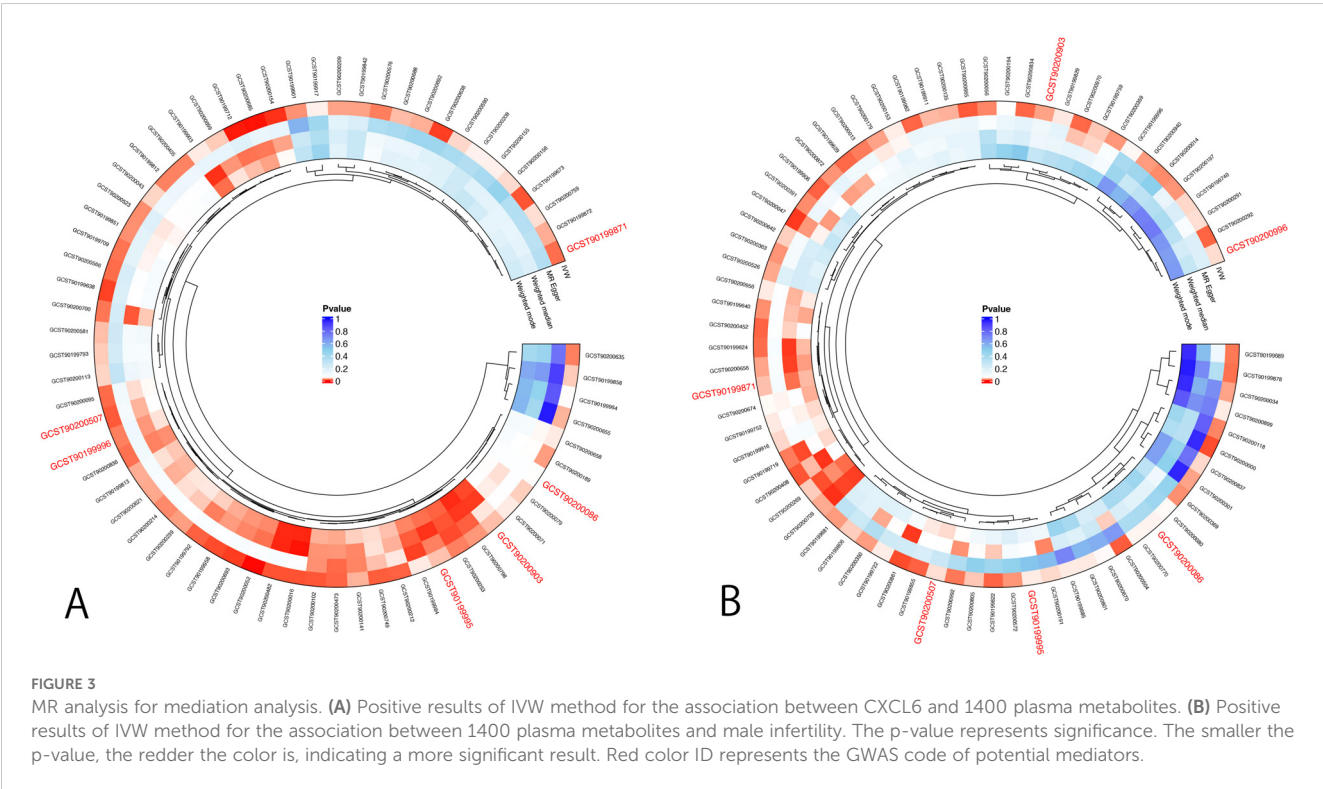


TABLE 2 The mediation effect of CXCL6 on male infertility via 1400 plasma metabolites.

Exposure	Mediator	Outcome	Total effect		Direct effect A		Direct effect B		Mediation effect		p	Mediation proportion (%) (95%CI) (Suppressing effect)
			b	se	b	se	b	se	b	se		
CXCL6	Androstenediol (3beta,17beta) monosulfate (2) levels	Male Infertility	1.1979	0.3238	-0.0787	0.0321	-0.2124	0.1002	0.0167	0.0109	0.1253	1.4 (-0.39 to 3.18)
CXCL6	Sphingomyelin (d18:1/20:2, d18:2/20:1, d16:1/22:2) levels	Male Infertility	1.1979	0.3238	0.0640	0.0302	-0.1803	0.0899	-0.0115	0.0084	0.1684	-0.96 (-2.33 to 0.41)
CXCL6	Sphingomyelin (d18:1/20:1, d18:2/20:0) levels	Male Infertility	1.1979	0.3238	0.0724	0.0305	-0.1873	0.0947	-0.0136	0.0094	0.1483	-1.13 (-2.67 to 0.4)
CXCL6	Arachidonoylcholine levels	Male Infertility	1.1979	0.3238	0.0646	0.0329	0.2228	0.1121	0.0144	0.0109	0.1885	1.2 (-0.59 to 2.99)
CXCL6	X-12822 levels	Male Infertility	1.1979	0.3238	-0.0847	0.0328	0.2171	0.1066	-0.0184	0.0120	0.1262	-1.53 (-3.5 to 0.43)
CXCL6	Phosphate to glycerol ratio	Male Infertility	1.1979	0.3238	-0.0644	0.0302	-0.3449	0.1549	0.0222	0.0152	0.1432	1.85 (-0.63 to 4.33)

‘Total effect’ indicates the effect of CXCL6 on male infertility.
‘Direct effect A’ indicates the effect of CXCL6 on plasma metabolites.
‘Direct effect B’ indicates the effect of plasma metabolites on male infertility and ‘mediation effect’ indicates the effect of CXCL6 on male infertility through mediators.
Total effect, direct effect A and direct effect B were derived by IVW; mediation effect was derived by using the delta method.
All statistical tests were two-sided. P < 0.05 was considered significant.

identify significant causal relationships between male infertility and 7 positive CIPs (Supplementary Table S3).

Associations between CIPs and female infertility

After FDR adjustment, no CIPs were identified as having potential casual relationships with female infertility compared to non-FDR adjusted data, which identified 8 potentially significant CIPs. Of these, 3 had a beneficial impact on female infertility: Interleukin-5 (IL5) (95% CI: 1.0332 to 1.3101, p=0.0125), Oncostatin-M (OSM) (95% CI: 1.0242 to 1.2889, p=0.0179), and Artemin (ARTN) (95% CI: 1.0155 to 1.2518, p=0.0246), and 5 had a deleterious effect: Caspase 8 (CASP8) (95% CI: 0.7427 to 0.9517, p=0.0061), Leukemia inhibitory factor receptor (LIFR) (95% CI: 0.799 to 0.9798, p=0.0187), Interleukin-18 (IL18) (95% CI: 0.8182 to 0.9823, p=0.0192), Urokinase-type plasminogen activator (PLAU) (95% CI: 0.8273 to 0.9944,p=0.0376), and Interleukin-20 (IL20) (95% CI: 0.7768 to 0.9996, p=0.0493). Sensitivity analysis confirmed heterogeneity in the results without pleiotropy (Figure 2, Supplementary Table S2). MR-PRESSO analysis showed no horizontal pleiotropy and identified outliers (Supplementary Table S2). Bidirectional MR analyses also did not find evidence of significant causal relationships between female infertility and the 8 significant CIPs (Supplementary Table S3).

Mediation analysis

Given that CIPs may influence plasma metabolites, we used 1400 plasma metabolites from a prior study by Chen et al. (24) for mediation analysis.

First, IVs for CXCL6 were used to evaluate the potential causal effects of CXCL6 exposure on the plasma metabolites. Using IVW analysis, we found potential causal effects of CXCL6 on 57 of the 1400 tested plasma metabolites (Figure 3, Supplementary Table S4). Next, we assessed the potential associations of these plasma metabolites with male infertility. Sixty-one plasma metabolites were identified as having causal effects on male infertility via IVW analyses (Figure 3, Supplementary Table S4). Of the total 118 plasma metabolites associated with CXCL6 or male infertility, androstenediol (3beta,17beta) monosulfate (2) levels, sphingomyelin (d18:1/20:2, d18:2/20:1, d16:1/22:2) levels, sphingomyelin (d18:1/20:1, d18:2/20:0) levels, arachidonoylcholine levels, X-12822 levels, and phosphate to glycerol ratio appeared in both groups.

We estimated the indirect effect of CXCL6 on male infertility via 6 potential mediators (Table 2), however, the results were not significant. Androstenediol (3beta,17beta) monosulfate (2) levels, arachidonoylcholine (AA-CHOL) levels, and phosphate to glycerol ratio had 1.4%, 1.2%, and 1.85% mediation effect, respectively. While sphingomyelin (d18:1/20:2, d18:2/20:1, d16:1/22:2) levels, sphingomyelin (d18:1/20:1, d18:2/20:0) levels and X-12822 levels indicated suppressing effects instead of any mediating effects (38). Additionally, the bidirectional MR analyses didn’t find any evidence



FIGURE 4 Bar graphs of GO enrichment analysis and pathway enrichment analysis for potential causal CIPs. (A) Top 10 terms of male infertility in BP. (B) Top 10 terms of male infertility in MF. (C) WikiPathway of male infertility. (D) KEGG Pathway of male infertility. (E) Reactome Pathway of male infertility. (F) BioCarta Pathway of male infertility. (G) Top 10 terms of female infertility in BP. (H) Top 10 terms of female infertility in MF. (I) Top 10 terms of female infertility in CC. (J) WikiPathway of female infertility. (K) KEGG Pathway of female infertility. (L) Reactome Pathway of female infertility. (M) BioCarta Pathway of female infertility. BP, Biological Process; MF, Molecular Function; CC, Cellular Component.

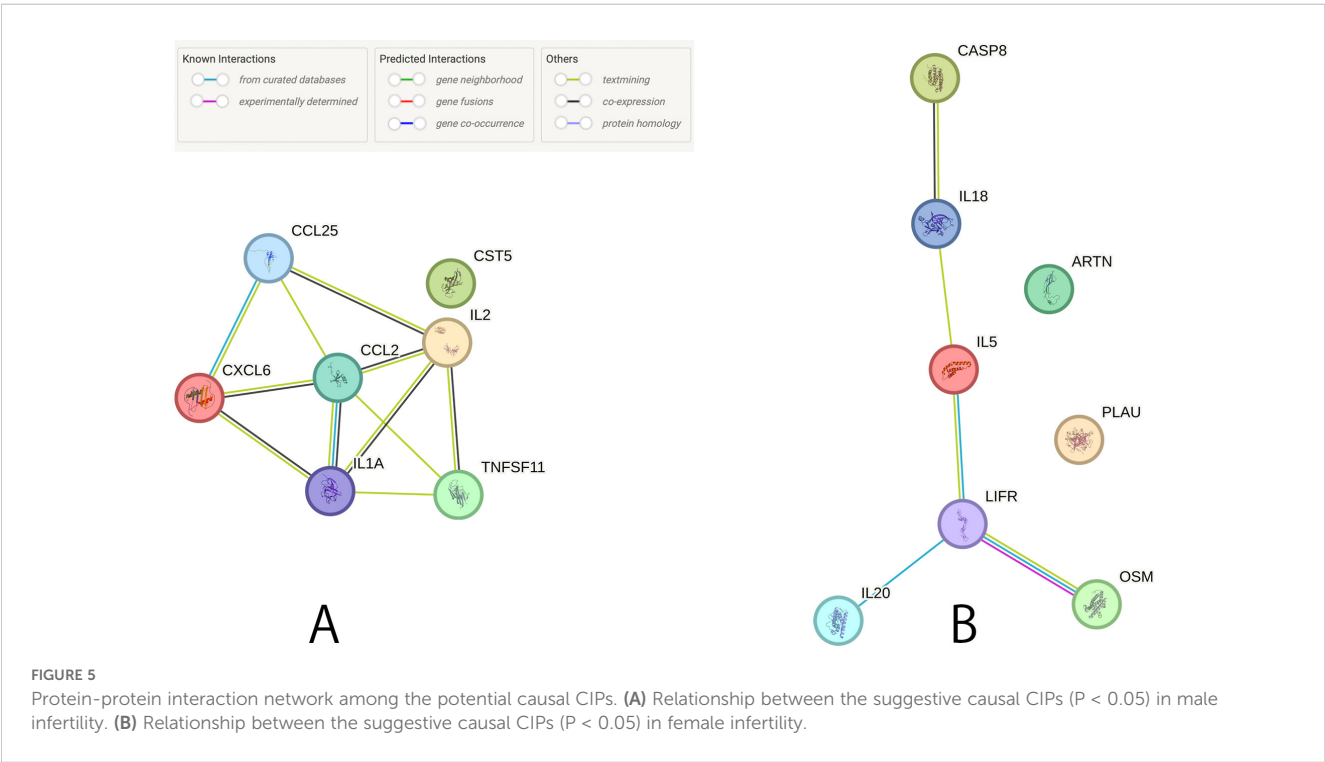


FIGURE 5 Protein-protein interaction network among the potential causal CIPs. (A) Relationship between the suggestive causal CIPs ($P < 0.05$) in male infertility. (B) Relationship between the suggestive causal CIPs ($P < 0.05$) in female infertility.

TABLE 3 Suggested top 10 drugs for male infertility.

Drugs predict for male infertility positive related CIPs						Drugs predict for male infertility negative related CIPs					
Term	P-value	Adjusted P-value	Odds Ratio	Combined Score	Genes	Term	P-value	Adjusted P-value	Odds Ratio	Combined Score	Genes
MANGANESE CTD 00006240	0.0007	0.0476	93.7678	685.5760	CXCL6; IL2	Leupeptin CTD 00001574	0.0016	0.0251	999.3500	6403.3348	IL1A
BETAMETHASONE VALERATE CTD 00005505	0.0022	0.0476	666.2000	4077.1879	IL2	6401-97-4 CTD 00000925	0.0016	0.0251	999.3500	6403.3348	IL1A
NICKEL CHLORIDE CTD 00001064	0.0023	0.0476	49.3678	299.4843	CXCL6; IL2	BENZOYL PEROXIDE CTD 00005495	0.0016	0.0251	999.3500	6403.3348	IL1A
buprenorphine CTD 00005539	0.0024	0.0476	605.6061	3653.6995	IL2	urethane CTD 00006966	0.0021	0.0251	768.6154	4739.6562	IL1A
Modrasone CTD 00001031	0.0024	0.0476	605.6061	3653.6995	IL2	POVPC CTD 00003737	0.0022	0.0251	713.6786	4351.6858	IL1A
methanol CTD 00005338	0.0028	0.0476	512.3846	3012.3745	IL2	Estrone sulfate CTD 00000891	0.0024	0.0251	666.0667	4018.4162	IL1A
urethane CTD 00006966	0.0028	0.0476	512.3846	3012.3745	IL2	sodium chloride CTD 00006751	0.0024	0.0251	666.0667	4018.4162	IL1A
propanil CTD 00004257	0.0030	0.0476	475.7619	2764.2762	IL2	nefazodone CTD 00002219	0.0024	0.0251	666.0667	4018.4162	IL1A
KN-62 CTD 00002580	0.0032	0.0476	444.0222	2551.2387	IL2	AC1L1WKQ CTD 00007050	0.0027	0.0251	587.6471	3476.1500	IL1A
Phorbol 12-myristate 13-acetate CTD 00006852	0.0034	0.0476	40.5717	230.8336	CXCL6; IL2	Nebivolol CTD 00002249	0.0027	0.0251	587.6471	3476.1500	IL1A

TABLE 4 Suggested top 10 drugs for female infertility.

Drugs predict for female infertility positive related CIPs						Drugs predict for female infertility negative related CIPs					
Term	P-value	Adjusted P-value	Odds Ratio	Combined Score	Genes	Term	P-value	Adjusted P-value	Odds Ratio	Combined Score	Genes
pioglitazone PC3 DOWN	1.1527E-05	0.0022	665.8333	7571.0827	PLAU; IL18	histamine BOSS	0.0002	0.0163	222.6854	1855.8501	IL5; ARTN
(-)-Epigallocatechin gallate BOSS	1.1560E-05	0.0022	142.0048	1614.3065	CASP8; PLAU; IL18	CORTICOSTERONE BOSS	0.0002	0.0163	222.6854	1855.8501	IL5; ARTN
gemcitabine CTD 00002382	1.5740E-05	0.0022	127.7780	1413.1374	CASP8; PLAU; LIFR	PIPERONYL BUTOXIDE BOSS	0.0003	0.0163	206.3021	1688.4212	IL5; ARTN
15442-64-5 CTD 00000915	1.8851E-05	0.0022	512.0256	5570.3046	CASP8; IL18	BETAMETHASONE VALERATE CTD 00005505	0.0016	0.0313	999.3500	6403.3348	IL5
Honokiol CTD 00000234	2.0245E-05	0.0022	493.0370	5328.5455	CASP8; IL18	Beclomethasone 17-monopropionate CTD 00002649	0.0016	0.0313	999.3500	6403.3348	IL5
Destroxin B CTD 00001859	3.1393E-05	0.0022	391.3922	4058.3164	CASP8; PLAU	Modrasone CTD 00001031	0.0018	0.0313	908.4545	5741.9213	IL5
Luronit CTD 00006106	3.1393E-05	0.0022	391.3922	4058.3164	CASP8; PLAU	Dimaprit CTD 00007150	0.0022	0.0313	713.6786	4351.6858	IL5
2-Naphthoxyacetic acid BOSS	3.5024E-05	0.0022	369.6111	3792.0209	CASP8; PLAU	Desloratadine CTD 00003720	0.0024	0.0313	666.0667	4018.4162	IL5
mitomycin C CTD 00007136	3.5349E-05	0.0022	96.8361	992.5936	CASP8; PLAU; IL18	terbinafine CTD 00001890	0.0024	0.0313	666.0667	4018.4162	IL5
AMILORIDE CTD 00005369	0.0001	0.0030	295.5556	2905.3408	CASP8; PLAU	HEXACHLOROBENZENE CTD 00006091	0.0025	0.0313	624.4063	3729.2533	IL5
Melitten CTD 00006261	0.0001	0.0035	260.7059	2499.6356	CASP8; IL18	leukotriene D4 CTD 00007224	0.0025	0.0313	624.4063	3729.2533	IL5

of significant causal relationships between 6 potential mediators and CXCL6 ([Supplementary Table S5](#)), suggesting that CXCL6 cannot serve as a mediator as well.

PheWAS and phenotype scanning analysis

PheWAS results can be interpreted as the association of genetically determined protein expression with specific diseases or traits. We first identified 7 CXCL6-associated SNPs from previous MR analyses, 4 of which successfully passed clumping and matched to 4 control SNPs each ([Supplementary Table S6](#)). PheWAS analyses were then performed with the UK Biobank GeneAtlas database to test each CXCL6-associated variant and control variant for association with 778 traits. A total of 6 traits were more likely to be associated with CXCL6 SNPs than with control SNPs at statistical significance ($p < 0.05$), including neutrophil count, lymphocyte percentage, neutrophil percentage, white blood cell (leukocyte) count, monocyte count, and basophil percentage ([Supplementary Table S7](#)).

In phenotype scanning, CXCL6 (rs12075, rs13148728, rs597808, rs74361503) expression was found to have associations with 138 other traits ([Supplementary Table S8](#)), 18 of which were related to disease. We then used MR to check the causal relationship between CXCL6 and 18 related diseases, but no causal relationship was identified ([Supplementary Table S9](#)). Collectively, these data suggest that CXCL6 may serve as a plasma molecular biomarker for male infertility.

Functional enrichment analysis of positively associated CIPs

GO analyses in male infertility identified potential CIPs chiefly involved in cytokine pathways with effects on cytokine activity ([Figure 4](#)), however in female infertility, potential CIPs had effects on cytokine receptor binding, serine protease inhibitor complex, or pro-inflammatory and profibrotic mediators ([Figure 4](#)).

The pathways with the strongest implications for potential CIPs were captured among four global bases, including WikiPathway, KEGG, Reactome, and BioCarta. These results identified pro-inflammatory and profibrotic mediators and cytokine-cytokine receptor interaction as the strongest pathways in male and female infertility in WikiPathway and KEGG, respectively. Inflammatory Bowel Disease Signaling also appeared in 4 pathway results ([Figure 4](#)).

PPI network construction

We generated a PPI network of potential CIPs in male and female infertility ([Figure 5](#)). Of the potential CIPs, IL2 and IL1A had the highest combined score (0.997) in male infertility, and OSM and LIFR had the highest combined score (0.999) in female infertility ([Supplementary Table S10](#)).

Gene-drug interactions

We then used Enrichr to analyze the positive or negative relationships between potential CIPs infertility and identify drugs that may modulate these CIPs using the DSigDB database ([Tables 3, 4](#)). For positively related CIPs indicated that manganese and pioglitazone are the top drug candidates for male and female infertility, respectively. With respect to negatively related CIPs, leupeptin and histamine emerged as the top drug candidates for treating male and female infertility, respectively.

Discussion

Infertility is a complex problem affecting both males and females through varied processes. Male infertility etiologies include genetic mutations (39), autoimmune responses (40), oxidative stress (41), and inflammatory indicators (42). Female infertility has also been linked to oxidative stress (43) and inflammatory mechanisms (44). Here, we use two-sample Mendelian Randomization to investigate infertility-associated CIPs and plasma metabolites linked to infertility. We identified CXCL6 as a promising therapeutic target and possible plasma biomarker for male infertility. Using the infertility-associated CIPs, we also explored potential infertility mechanisms using enrichment analyses, Protein-Protein Interaction (PPI), and Gene-drug interaction studies. We found some potential drugs like leupeptin or pioglitazone which are already used for some diseases, thereby advancing the understanding of both male and female infertility.

CXCL6 is a chemokine involved in neutrophil trafficking and activation that has been implicated in the pathogenesis of several diseases and has been shown to promote cancer cell metastasis, fibrosis, and inflammation (45–47). Moreover, studies have suggested that CXCL6 can be regulated by specific cell signaling pathways, such as the HIF-1 α pathway in hepatocellular carcinoma and the IL4 pathway through the JAK-STAT mechanism in atopic dermatitis (48, 49). The diverse roles of CXCL6 in various diseases and its involvement in cellular processes and the tumor microenvironment make it a promising drug target for therapeutic intervention in multiple pathological conditions. Our findings align with existing research identifying CXCL6 as a potential plasma biomarker in many conditions (50–52). Significantly, our phenotype analysis and PheWAS results indicate that CXCL6 is not associated with other diseases. This lack of association with additional conditions enhances its suitability as a specific plasma biomarker for male infertility, offering a more targeted and potentially accurate diagnostic tool in this context. Our findings propose that CXCL6 not only warrants in-depth study but also presents potential as both a drug target and a plasma molecular biomarker for male infertility.

In our study, we analyzed potentially causal links between 1,400 plasma metabolites and male infertility using the Inverse Variance Weighted (IVW) methodology. This approach led to the identification of 66 metabolites positively correlated with male infertility. Notably, we

observed that levels of androstenediol (3 β ,17 β) monosulfate (2), AA-CHOL, and the phosphate-to-glycerol ratio were associated with both CXCL6 and male infertility. Androstenediol (3 β ,17 β) monosulfate (2) is a metabolite involved in various physiological functions. Matsui et al. (53) noted its role in testosterone sulfate metabolism in rats, while other studies have identified it as a precursor in testosterone synthesis from DHEA (54). Additionally, the anabolic steroid 19-nor-4-androstenediol-3 β and one of its derivatives 17 β -diol (3 β ,19-NA), have been hypothesized to increase muscle and bone mass with minimal prostate stimulation (55). AA-CHOL acts as an endogenous modulator of acetylcholine signaling, found in atherosclerotic plaques and the arterial intima (56), and is known to regulate cholinesterases (57). Acetylcholine is involved in penile erection and relaxation as well as the central control of ejaculation. Acetylcholinesterase is found to play a role in the pathogenesis of male infertility via modulating inflammatory pathways (58). These data suggested that AA-CHOL might affect male fertility via modulating acetylcholine and acetylcholinesterase activities. Furthermore, phosphate is crucial for male fertility and affects sperm motility and function, as well as semen liquefaction (59, 60). However, its role in correcting infertility is unclear, as simple dietary supplementation cannot reverse infertility in mice (61). Glycerol plays a critical role in lipid metabolism and sperm motility but has both beneficial and harmful effects on sperm health and viability (62–64). Exactly how the phosphate-to-glycerol ratio affects male infertility merits further research.

Our enrichment analysis underscored the importance of cytokine-related pathways in infertility, aligning with existing research that implicates a deleterious role of cytokines in both male and female infertility. Cytokines are signaling molecules that play crucial roles in testicular function and male infertility. Cytokines like interleukin 1 β (IL-1 β), IL-6, IL-10, IL-18, tumor necrosis factor α (TNF- α), interferon γ (IFN- γ), and transforming growth factor β 1 (TGF- β 1) are notably elevated in idiopathically infertile males and those with varicocele (65, 66). Attia et al. (67) also highlighted the significant role of pro-inflammatory cytokines and microRNAs in male infertility. Cytokines in the male gonad primarily originate from testicular macrophages, with Leydig and Sertoli cells also contributing. Additionally, cytokines, such as IL2 and IL1A had the highest combined score in the PPI network associated with male infertility. IL-2 plays a crucial role in T-cell mediated immune responses. IL-2 levels in seminal plasma are found to be related to sperm count, motility, and morphology, and may be a potential marker in male infertility (68). IL-2 concentrations are closely associated with Testosterone hormone levels in COVID-19 infertile patients (69). IL-1 is primarily secreted by macrophages and monocytes in reaction to foreign antigens and inflammatory stimuli. A previous study has demonstrated that IL1A is involved in testicular paracrine/autocrine regulation, potentially influencing spermatogenesis, spermiogenesis, and male fertility (70). In female infertility, it has been shown that cytokine profiles are differentially expressed in the peritoneal fluid of patients with and without endometriosis being evaluated for infertility (71). Notably, the levels of CXCL6 are influenced by other cytokines and reproductive hormones, further supporting its function in both normal and abnormal human reproduction (72). A deeper

understanding of cytokines' roles in infertility could lead to the development of novel therapeutic approaches for those facing conception challenges.

Our gene-drug interaction analysis for positively related CIPs indicated that manganese and pioglitazone are the top drug candidates for male and female infertility, respectively. Manganese is necessary for metabolism, growth, and reproduction (73), but excess levels have been linked to male infertility (74) via mechanisms that include increased spontaneous abortion rates in partners, impotence, and infertility (75). Pioglitazone already shows potential in treating female infertility, particularly in polycystic ovary syndrome (PCOS). Nagao et al. (76) found that pioglitazone can suppress excessive ovarian follicle development in mice, suggesting a mechanism explaining its impact on reproductive health. With respect to negatively related CIPs, leupeptin and histamine emerged as the top drug candidates for treating male and female infertility, respectively. Leupeptin is a protease inhibitor used in the treatment of AIDS, hepatitis, pancreatitis, and cancer (77) with high antiviral activity against influenza and coronavirus, notably binding strongly with the TMPRSS2 protease in COVID-19 (78). Leupeptin also acts as an autophagy-blocking agent, which is implicated in its mechanism of action in the treatment of HIV-1-associated neurological disorders (79), yet a role for it in the treatment of male infertility is unknown. Histamine has a regulatory effect on the female reproductive system and is known to influence the electrophysiology of the human oviduct, affecting fertility (80, 81). Together, these two molecules merit further study in the treatment of female infertility.

Limitations of this study included the analysis of disease-causing effects identified in different studies, and inconsistent measurements across studies might have introduced biases in results. Additionally, only one target CXCL6 passed our initial FDR correction, so we performed other analyses on potential associated targets. Moreover, the CXCL6 detection results was not validated in clinical male infertility patients, and further studies are required to confirm the diagnostic value of CXCL6 and its practical applicability as a biomarker in male infertility. Furthermore, the study population and datasets used here primarily comprise patients of European ancestry, which may limit applicability with respect to other racial and ethnic groups. Therefore, caution is needed when generalizing our results to more diverse global populations. Further research in ethnically diverse cohorts is essential to validate our results and to assess whether similar genetic associations hold true across different racial and ethnic groups. Lastly, we did not analyze the correlation between CXCL6 and ART outcomes. Moreover studies are necessary to explore this relationship and its potential role in reproductive success.

Conclusions

Using several methods of genetic analyses and Mendelian randomization, we found that circulating CXCL6 may be causally associated with male infertility and have applications as a potential drug target or plasma molecular biomarker in male infertility.

Cytokine-related pathways were also highly associated with the development and progression of infertility, and gene-drug interaction analysis suggested pioglitazone may help treat female infertility. Further studies are warranted to explore the potential of CXCL6 as a biomarker for early screening and diagnosis of male infertility, evaluate its role in therapeutic development, and assess the clinical feasibility of related drugs such as pioglitazone in improving fertility outcomes of patients struggling with infertility.

Data availability statement

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

Ethics statement

This study used only publicly available, deidentified summary statistics, and specific ethical review and informed consent had been obtained in all the original studies. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

PC: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. SN:

Conceptualization, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. LO: Conceptualization, Supervision, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

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.2025.1448530/full#supplementary-material>

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OPEN ACCESS

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RECEIVED 11 November 2024

ACCEPTED 13 February 2025

PUBLISHED 10 March 2025

CITATION

Zhou Y, Wang G, Liu L, Yu J and Ju S (2025)
Impact of dual residual risk of cholesterol and
inflammation on adult male sex hormones: a
cross-sectional study from NHANES.
Front. Endocrinol. 16:1526056.
doi: 10.3389/fendo.2025.1526056

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Impact of dual residual risk of cholesterol and inflammation on adult male sex hormones: a cross-sectional study from NHANES

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Purpose: Sex hormones are closely linked to inflammation and lipid metabolism. This study explores the correlation of residual cholesterol risk and residual inflammation risk with sex hormones.

Materials and methods: Logistic regression and dose-response curve analyses were conducted to examine the associations of total testosterone (TT), Sex Hormone Binding Protein (SHBG), Estradiol (E2), and Free testosterone (FT) with low density lipoprotein cholesterol (LDL-C) and high sensitive c-reactive protein (hs-CRP). Testosterone deficiency, defined as TT below 300 ng/dL, was analyzed across various subgroups based on LDL-C and hs-CRP levels. Grouped by LDL-C and hs-CRP: normal, LDL-C < 2.6 mmol/L, hs-CRP < 3mg/L, residual cholesterol risk only (RCR): LDL-C ≥ 2.6 mmol/L, hs-CRP < 3mg/L, residual inflammation risk only (RIR): LDL-C < 2.6 mmol/L, hs-CRP ≥ 3mg/L, both risk (BR): LDL-C ≥ 2.6 mmol/L, hs-CRP ≥ 3mg/L.

Results: The results indicated a negative association between hs-CRP and TT ($\beta = -1.98$, 95% CI [-3.54, -0.42], $p = 0.013$), as well as FT ($\beta = -0.04$, 95% CI [-0.07, -0.02], $p = 0.0002$). Similar trends were observed for the relationship between hs-CRP and SHBG ($\beta = -3.61$, 95% CI [-5.33, -1.90], $p = 0.0003$). In the presence of both risk factors (BR), TT decreased most significantly ($\beta = -79.37$, 95% CI [-112.74, -46.00], $p < 0.0001$), as did FT in the same subgroup ($\beta = -1.00$, 95% CI [-1.61, -0.40], $p = 0.0012$). Notably, hs-CRP exhibited a non-linear correlation with TT, SHBG, and FT, with distinct inflection points. Furthermore, in diabetic patients, hs-CRP was positively linked to E2 ($\beta = 0.39$, 95% CI [0.03, 0.74], $p = 0.0328$).

Conclusions: LDL-C was independently correlated with SHBG, hs-CRP with TT and FT, and the BR population had a higher risk of testosterone deficiency. Special populations with diabetes and hypertension need to be concerned about residual cholesterol risk and inflammatory risk.

KEYWORDS

testosterone, inflammation, residual cholesterol, residual risk, testosterone deficiency

1 Introduction

Testosterone is a hormone that plays a key role in the development and maintenance of male reproductive tissues, such as the testes and prostate, and secondary sexual characteristics, such as muscle mass, bone density, and body hair. Testosterone is synthesized in the Leydig cells of the testes and is transported in the bloodstream in combination with a carrier protein called sex hormone binding globulin (SHBG) (1).

SHBG is produced in the liver and binds highly affinity to testosterone and other sex hormones such as estradiol. By binding to these hormones, SHBG regulates their bioavailability and distribution in the body, which can affect their biological activity and influence a wide range of physiological processes (2). Studies have also shown that abnormal testosterone levels may be associated with a number of non-reproductive disorders, such as cardiovascular disease, metabolic disorders and cognitive impairment (3). For example, low testosterone levels are associated with an increased risk of developing type 2 diabetes, obesity and metabolic syndrome, while high levels of SHBG are associated with a reduced risk of developing cardiovascular disease, and a low-fat diet also increases testosterone levels (4, 5).

Residual cholesterol represented by low density lipoprotein cholesterol (LDL-C) and residual inflammatory markers by high sensitive-c-reactive-protein (hs-CRP) have been shown to be high risk factors for cardiovascular events, and even after receiving aggressive therapeutic medications, there is still an increased risk of cardiovascular disease recurrence with elevated LDL-C. Sex hormones are strongly associated with lipid metabolism and inflammation, with one study showing a significant negative correlation between LDL-C and testosterone and SHBG (6), and that androgen therapy also significantly improves lipid levels and increases LDL-C (7). The effect of hs-CRP and sex hormones was demonstrated in a survey of 12-16 year old adolescents in which hs-CRP was negatively correlated with testosterone (8) and SHBG was independently and negatively correlated with hs-CRP concentrations in males (9).

Both LDL-C and hs-CRP are strongly associated with sex hormones, then more studies are needed to demonstrate the relationship between these two residual risks and sex hormones and the extent to which these two wind residual risks jointly affect sex hormones.

In the current study, we analyzed the relationship between sex hormones and dual residual risk of cholesterol and inflammation in adult men using a population from the NHANES database.

2 Materials and methods

2.1 Study population

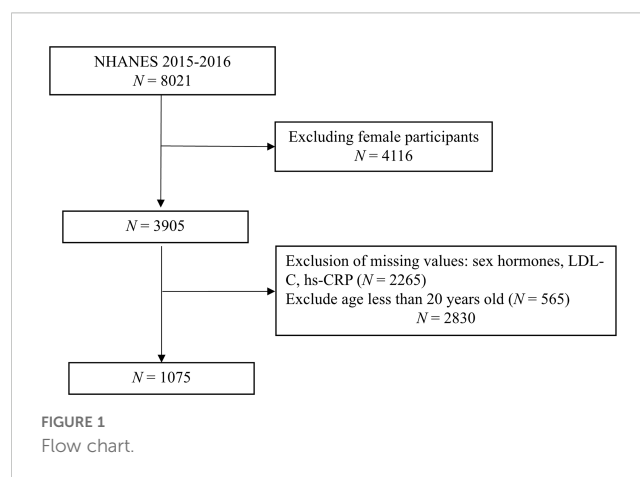
We conducted an analysis of the physical status of the US population using data from the National Health and Nutrition Examination Survey (NHANES), conducted by the Centers for Disease Control and Prevention (CDC). The survey used a

multistage, stratified, whole group survey design to ensure representation of the non-institutionalized population in the US. All NHANES participants gave written informed consent and the study was approved by the NCHS Institutional Review Board. For this cross-sectional study, we included men aged 20 years and older from the 2015-2016 survey cycle and calculated survey weights. We removed extreme values and covariates with more than 10% missing data, resulting in the inclusion of 1075 participants after excluding individuals with missing data for sex hormone-related indicators (Figure 1).

2.2 Definition of exposure

The NHANES database contains serum samples for total testosterone (TT) and estradiol (E2) measured by isotope dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) at the University of Minnesota. Sex hormone-binding globulin was measured using immuno-188 antibody reactions and chemiluminescence of reaction products. Free testosterone is testosterone that is not bound to albumin and SHBG. The NHANES Laboratory Procedures Manual (LPM) provides comprehensive guidelines for sample collection and handling. Testosterone deficiency was defined as a total testosterone level of less than 300 ng/dL (10). High-sensitivity C-reactive protein measurement is based on a highly sensitive Near Infrared Particle Immunoassay rate method in which anti-CRP antibody-coated particles bind to CRP in the patient sample, resulting in the formation of insoluble aggregates that cause turbidity. Participant serum specimens were transferred to Ottumwa, Iowa for analysis at -30°C. For more specific information on the analyzers and methods used, a document entitled Laboratory Procedures is available on the NHANES Web site.

Based on previous groupings (11), we divided participants into no residual risk group: LDL-C < 2.6 mmol/L, hs-CRP < 3mg/L, residual cholesterol risk only (RCR): LDL-C ≥ 2.6 mmol/L, hs-CRP < 3mg/L, residual inflammation risk only (RIR): LDL-C < 2.6 mmol/L, hs-CRP ≥ 3mg/L, both risk (BR): LDL-C ≥ 2.6 mmol/L, hs-CRP ≥ 3mg/L.



2.3 Covariates

Sociodemographic characteristics such as age, education, race/ethnicity, household income, and BMI were collected using the Department of Health and Human Services poverty criteria. Smoking was categorized on the basis of the self-administered questionnaire into three categories: current smoker (smoked more than 100 cigarettes in life and smoke some days or every day), former smoker (smoked more than 100 cigarettes in life and smoke not at all now), and never smoker (smoked less than 100 cigarettes in life), and alcohol consumption into never, moderate, mild and heavy. We also collected a variety of laboratory data, including uric acid, creatinine, total protein, HDL-C, LDL-C, and total cholesterol. Blood glucose >6.11 mmol/L to 7.1 mmol/L, or oral glucose tolerance test >11.1 mmol/L, or self-reported and physician-diagnosed diabetes was defined as fasting glucose >7.1 mg/dL, or use of insulin was considered a diabetic indicator and self-report taking glucose-lowering medication. Mean blood pressure ≥ 130 mmHg (systolic) or ≥ 90 mmHg (diastolic) or use of antihypertensive medication, self-reported and physician-diagnosed hypertension. Participants were taking lipid-lowering drugs, mainly including metabolic agents, antihyperlipidemic agents, hmg-coa reductase inhibitors (statins).

2.4 Statistical methods

Each participant's data was subjected to acceptable statistical analyses, according to NHANES' complex multistage cluster survey design, which incorporated sample weights and statistical reporting guidelines. For continuous and categorical variables, we reported the mean $\pm$ standard deviation (SD) and percentages, respectively. Chi-square tests were employed to detect differences between categorical variables, whereas analysis of variance (ANOVA) was used to analyze continuous data. We examined the independent associations between LDL-C and hs-CRP and sex hormones using multivariate logistic regression, reported as beta values, and risk values between the four groups and testosterone deficiency were expressed as OR. To assess the potential impact of this association, we used a weighted corrected model to adjust for different covariates, with statistically significant differences defined as $P < 0.05$. We also performed subgroup analysis to identify specific populations. Interaction and stratified analyses were used to evaluate subgroup heterogeneity; $P < 0.05$ indicates significant interaction term heterogeneity. In addition, we apply the model to investigate whether there is a critical point or not. Based on the model Based on the maximum likelihood value given by the model, the inflection point was determined using a two-step recursive method. Smooth curve fitting and two-segment linear regression modeling were used to determine the association between sex hormones and LDL-C and hs-CRP.

We used R version 4.2.0 (<http://www.R-project.org>, R Foundation) and EmpowerStats software (www.empowerstats.com; X & Y solutions, Inc., Boston MA) for all analyses.

3 Results

1075 people were finally included in the analysis through screening (Figure 1). The RIR population was the oldest but the RCR population was the largest, diabetics had a higher risk of inflammation, hypertensives had a higher risk of residual cholesterol, 44.51% of the population was at residual risk even after taking lipid-lowering medications, and had a higher risk of inflammation. The dual-risk group had the highest BMI, protein intake, and uric acid, and the lowest energy intake. For sex hormones, total and free testosterone were low in those at risk for inflammation, sex hormone binding protein was lowest in those at dual risk, and estrogen was low in those at high residual cholesterol risk. The details are in Table 1.

3.1 Relationship of LDL-C and hs-CRP to sex hormones

Within the comprehensively adjusted framework (Table 2), an intriguing observation unveiled itself. A significant and inverse correlation emerged between the continuous metric of high-sensitivity C-reactive protein and testosterone ($\beta = -1.98$, 95% CI $[-3.54, -0.42]$, $p = 0.013$), alongside free testosterone ($\beta = -0.04$, 95% CI $[-0.07, -0.02]$, $p = 0.0002$). Furthermore, a nuanced linkage was discerned between the continuous variable LDL-C and sex hormone binding protein ($\beta = -3.61$, 95% CI $[-5.33, -1.90]$, $p = 0.0003$).

Delving deeper into our investigation, we initiated sensitivity analyses pertaining to the tertile transitions of hs-CRP and LDL-C. This discerning approach revealed noteworthy revelations. Specifically, within the Hs-CRP tertile, the parameters of testosterone ($\beta = -107.45$, 95% CI $[-138.17, -76.73]$, $p < 0.0001$), SHBG ($\beta = -8.46$, 95% CI $[-5.33, -1.90]$, $p < 0.0001$), and free testosterone exhibited diminishment. This phenomenon was mirrored within the LDL-C tertile, where testosterone ($\beta = -1.50$, 95% CI $[-1.95, -1.05]$, $p < 0.0001$) bore the most pronounced decrease. Intriguingly, testosterone displayed a notable decrease within the dichotomous tertile of LDL-C ($\beta = -31.38$, 95% CI $[-61.37, -1.39]$, $p = 0.0406$), whereas SHBG registered a similar pattern within the tertiary tertile ($\beta = -8.52$, 95% CI $[-12.60, -4.44]$, $p < 0.0001$).

Additionally, an insightful exploration into the realms of high residual cholesterol risk and high inflammation risk groups unfolded (Table 3). The outcomes proved captivating; testosterone exhibited a substantial decline within the high residual cholesterol risk and high inflammation risk groups ($\beta = -79.37$, 95% CI $[-112.74, -46.00]$, $p < 0.0001$), alongside a marked decrease within the high inflammation group ($\beta = 66.50$, 95% CI $[-107.92, -25.09]$, $p < 0.0001$). Amidst this landscape, SHBG displayed a parallel descent, registering significant reductions within both the BR ($\beta = -8.29$, 95% CI $[-12.86, -3.72]$, $p = 0.0004$) and RCR ($\beta = -5.48$, 95% CI $[-9.35, -1.62]$, $p = 0.0055$) populations. Conversely, estrogen remained devoid of significant alterations.

A pivotal synthesis of our discoveries unveiled substantial risk disparities among men grappling with testosterone deficiency

TABLE 1 The baseline characteristics of participants, grouped by risk, have been weighted.

	BR	RCR	RIR	Normal	P-value
Age (year)	51.13 ± 14.31	49.12 ± 15.50	48.79 ± 17.64	48.26 ± 19.01	0.0593
Sex					<0.0001
Male	41.63	53.08	35.80	52.22	
Female	58.37	46.92	64.20	47.78	
Race					0.0265
Mexican American	8.36	7.76	8.58	6.42	
Non-Hispanic White	66.00	69.09	64.79	70.37	
Other Hispanic	7.31	5.79	9.43	5.09	
Non-Hispanic Black	12.21	7.43	12.29	9.27	
Other Race - Including Multi-Racial	6.12	9.94	4.91	8.84	
Poverty					0.5029
≤1	12.38	12.68	13.78	15.38	
>1	87.62	87.32	86.22	84.62	
Education					<0.0001
Less than High school	43.74	44.56	49.61	39.36	
High school	29.35	22.02	21.21	18.76	
More than high school	26.91	33.42	29.18	41.88	
Smoke					0.0124
Never	53.23	54.32	47.08	55.77	
Former	20.12	18.59	13.15	17.20	
Now	26.65	27.09	39.77	27.03	
Alcohol user					0.0916
Never	28.13	28.17	36.92	29.14	
Moderate	15.13	19.54	17.04	17.57	
Mild	39.61	34.33	26.97	37.14	
Heavy	17.13	17.96	19.08	16.15	
Diabetes					<0.0001
No	77.89	87.63	69.87	79.62	
Yes	22.11	12.37	30.13	20.38	
Hypertension					<0.0001
No	50.68	33.15	52.41	34.78	
Yes	49.32	66.85	47.59	65.22	
Take drug					<0.0001
No	31.68	48.50	24.61	41.76	
Yes	68.32	51.50	75.39	58.24	
BMI (kg/m ²)	33.88 ± 7.73	27.67 ± 5.40	33.36 ± 8.17	27.36 ± 6.15	<0.0001
Total protein (g/L)	71.47 ± 4.40	71.04 ± 4.34	71.22 ± 4.43	70.37 ± 4.04	0.0010
Creatinine (μmol/L)	74.34 ± 34.25	75.28 ± 18.87	73.29 ± 25.23	78.05 ± 39.90	0.1625

(Continued)

TABLE 1 Continued

	BR	RCR	RIR	Normal	<i>P</i> -value
Take drug					<0.0001
Uric acid (μmol/L)	343.85 ± 82.68	318.32 ± 75.43	337.28 ± 90.40	308.94 ± 73.04	<0.0001
TC (mg/dL)	5.53 ± 0.89	5.55 ± 0.86	4.16 ± 0.70	4.02 ± 0.62	<0.0001
HDL-C (mg/dL)	1.33 ± 0.38	1.49 ± 0.44	1.48 ± 0.74	1.55 ± 0.54	<0.0001
LDL-C (mg/dL)	3.51 ± 0.74	3.49 ± 0.69	2.10 ± 0.34	2.01 ± 0.38	<0.0001
Hs-C reactive protein (mg/L)	8.59 ± 9.13	1.24 ± 0.79	9.61 ± 12.47	1.06 ± 0.78	<0.0001
Kcal	1979.28 ± 804.26	2220.03 ± 900.72	2183.32 ± 1015.00	2137.86 ± 840.02	0.0001
Testosterone (ng/dl)	182.05 ± 221.68	275.31 ± 276.66	148.53 ± 189.37	262.40 ± 270.56	<0.0001
Sex hormone binding globulin (nmol/L)	51.17 ± 33.45	64.07 ± 44.28	62.20 ± 47.63	64.56 ± 43.04	<0.0001
Estradiol (pg/ml)	40.10 ± 57.73	33.52 ± 51.30	48.10 ± 54.38	43.69 ± 64.10	0.0023
Free testosterone (ng/dL)	3.22 ± 3.84	4.71 ± 4.84	2.52 ± 3.31	4.37 ± 4.77	<0.0001

Data are expressed as weighted proportions for categorical variables (%) and as weighted means ± Standard Error for continuous variables depending on its type. Grouped by LDL-C and hs-CRP. BR, both risk; RCR, residual cholesterol risk; RIR, residual inflammation risk. Statistically significant results are bolded.

TABLE 2 Relationship of LDL-C and hs-CRP to TT, E2, SHBG, and FT.

	Non-adjusted		Adjusted I		Adjusted II	
TT	β	<i>P</i>	β	<i>P</i>	β	<i>P</i>
Hs-CRP	-4.42 (-6.03, -2.81)	<0.0001	-3.67 (-5.27, -2.08)	<0.0001	-1.98 (-3.54, -0.42)	0.0130
T1	Ref				Ref	
T2	-86.82 (-114.95, -58.69)	<0.0001	-76.15 (-104.17, -48.12)	<0.0001	-58.83 (-86.36, -31.30)	<0.0001
T3	-157.06 (-186.91, -127.22)	<0.0001	-142.80 (-172.63, -112.98)	<0.0001	-107.45 (-138.17, -76.73)	<0.0001
LDL-C	-1.09 (-13.84, 11.65)	0.8665	6.75 (-19.25, 5.76)	0.2905	-11.77 (-24.54, 0.99)	0.0710
T1	Ref		Ref		Ref	
T2	-6.41 (-37.70, 24.89)	0.6884	-15.06 (-45.57, 15.46)	0.3337	-31.38 (-61.37, -1.39)	0.0406
T3	3.83 (-26.37, 34.02)	0.8040	-10.21 (-39.86, 19.44)	0.4999	-26.83 (-57.30, 3.65)	0.0848
SHBG						
Hs-CRP	-0.03 (-0.27, 0.21)	0.8004	-0.21 (-0.43, 0.00)	0.0550	0.02 (-0.20, 0.23)	0.8768
T1	Ref				Ref	
T2	-4.63 (-8.92, -0.34)	0.0346	-9.49 (-13.31, -5.67)	<0.0001	-6.98 (-10.74, -3.22)	0.0003
T3	-8.84 (-13.41, -4.27)	0.0002	-13.76 (-17.85, -9.66)	<0.0001	-8.46 (-12.68, -4.23)	<0.0001
LDL-C	-3.93 (-5.78, -2.08)	<0.0001	-2.54 (-4.22, -0.87)	0.0030	-3.61 (-5.33, -1.90)	<0.0001
T1	Ref				Ref	
T2	-5.77 (-10.29, -1.25)	0.0125	-4.24 (-8.32, -0.17)	0.0417	-7.36 (-11.38, -3.34)	0.0003
T3	-9.13 (-13.50, -4.77)	<0.0001	-5.37 (-9.33, -1.41)	0.0080	-8.52 (-12.60, -4.44)	<0.0001
E2						
Hs-CRP	-0.04 (-0.14, 0.05)	0.3648	-0.03 (-0.13, 0.06)	0.4803	-0.05 (-0.15, 0.05)	0.3018
T1	Ref		Ref		Ref	
T2	-0.92 (-2.57, 0.74)	0.2775	-0.32 (-1.99, 1.34)	0.7027	-0.56 (-2.25, 1.13)	0.5176
T3	0.77 (-0.99, 2.53)	0.3913	1.28 (-0.51, 3.07)	0.1606	0.81 (-1.10, 2.71)	0.4064

(Continued)

TABLE 2 Continued

	Non-adjusted		Adjusted I		Adjusted II	
TT	β	P	β	P	β	P
E2						
LDL-C	-0.06 (-0.77, 0.66)	0.873	-0.15 (-0.87, 0.57)	0.6858	-0.48 (-1.25, 0.29)	0.2241
T1	Ref		Ref		Ref	
T2	-0.26 (-2.00, 1.49)	0.7751	-0.29 (-2.04, 1.45)	0.7421	-0.65 (-2.46, 1.16)	0.4847
T3	-0.29 (-1.98, 1.40)	0.7398	-0.47 (-2.16, 1.23)	0.5877	-1.26 (-3.10, 0.58)	0.1791
FT						
Hs-CRP	-0.08 (-0.11, -0.06)	<0.0001	-0.05 (-0.08, -0.03)	<0.0001	-0.04 (-0.07, -0.02)	0.0002
T1	Ref		Ref		Ref	
T2	-1.32 (-1.81, -0.82)	<0.0001	-0.64 (-1.04, -0.25)	0.0015	-0.58 (-0.98, -0.18)	0.0049
T3	-2.34 (-2.87, -1.82)	<0.0001	-1.60 (-2.03, -1.18)	<0.0001	-1.50 (-1.95, -1.05)	<0.0001
LDL-C	0.34 (0.12, 0.56)	0.0025	0.07 (-0.11, 0.24)	0.4446	0.04 (-0.15, 0.22)	0.6877
T1	Ref		Ref		Ref	
T2	0.14 (-0.40, 0.68)	0.6070	-0.12 (-0.55, 0.30)	0.5725	-0.23 (-0.67, 0.21)	0.3036
T3	0.85 (0.33, 1.37)	0.0013	0.19 (-0.22, 0.60)	0.3639	0.07 (-0.37, 0.52)	0.7418

Model I: Age; Race, Education, Poverty.
Model II: Adjust I + BMI; Smoke; Alcohol users; Uric acid; Creatinine; Total protein; Diabetes; Hypertension; Take drugs.
 β , effect size for regression; LDL-C, low density lipoprotein cholesterol; hs-CRP, high sensitive c reactive-protein; TT, total testosterone; SHBG, sex hormone binding globulin; E2, estradiol; FT, free testosterone.
Statistically significant results are bolded.

(Figure 2). Specifically, within the BR context, these individuals were exposed to a formidable 1.81-fold higher risk (OR = 2.81, 95% CI [1.65, 4.81], $p = 0.0002$), and within the RIR landscape, a comparable 1-fold elevated risk (OR = 2.00, 95% CI [1.07, 3.76], $p = 0.03$) came to light when juxtaposed with the normal population.

3.2 LDL-C and hs-CRP dose response and thresholds to sex hormones

The amalgamation of generalized linear models and the finesse of smoothed curve fitting was harnessed to orchestrate a synthesis of the intricate interplay between sex hormones and LDL-C as well as hs-CRP, as elegantly illustrated in Figure 3 and meticulously detailed in Table 4.

Exemplifying the results of this endeavor, we divulge that LDL-C unfolded its linear connectivity with TT, SHBG, E2, and FT underpinned by p -values that surpassed the threshold of significance at > 0.05 . Conversely, a discernibly more complex duality took shape within the interface of HS-CRP and total testosterone. As per the comprehensive bipartite linear regression model, our computation revealed an inflection point at 1. Precisely, at the <1 stratum, the estimation loomed at -69.51 (95% CI: -111.71, -27.31), while at the ≥ 1 stratum, it settled at -1.54 (95% CI: -4.50, 1.42). This phenomenon was endorsed by the log-likelihood ratio test with a consequential P -value of 0.002. The narrative of SHBG, within its own inflection point at 0.8, unfolded as follows: at <0.8 , a pronounced descent of -12.77 (95% CI: -20.69, -4.86) emerged, and

at ≥ 0.8 , a gentle ascent of 0.12 (95% CI: -0.29, 0.54) came to the fore, with a commanding P -value of 0.001. Echoing a similar pattern, the narrative of free testosterone, marked by its inflection point at 9.2, illustrated a notable drop at <9.2 , quantified at -0.13 (95% CI: -0.20, -0.05), and a muted increase at ≥ 9.2 , tabulated at 0.03 (95% CI: -0.06, 0.11), although its significance remained intact with P -values ≥ 0.002 . Not to be overlooked, the relationship between HS-CRP and estradiol presented a linear association, albeit with a p -value of 0.091, evoking a sense of subtlety in the overall context.

3.3 Subgroup analysis

The homeostatic interactions among sex hormones, hs-CRP, and LDL-C were carefully analyzed by subgroup analyses, which revealed some intriguing findings. Only hs-CRP and estradiol were significantly different in the interaction test in diabetic patients ($P = 0.0204$), indicating that the higher the degree of inflammation, the higher the estradiol in diabetic patients. The other subgroups analyzed did not show significant heterogeneity in the results of the interaction effect test, suggesting that there were no significant differences in the associations between the different subgroups and that the results were stable (Table 5).

Within this nuanced exploration, a distinctive pattern emerged wherein the relationship between SHBG and elevated LDL-C displayed a more marked decline, notably evident among individuals who were not utilizing lipid-lowering medications ($\beta = -2.82$, 95% CI [-4.88, -0.76], $p = 0.0075$). Parallel to this, a

TABLE 3 Relationship between TT, E2, SHBG and FT in different risk subgroups.

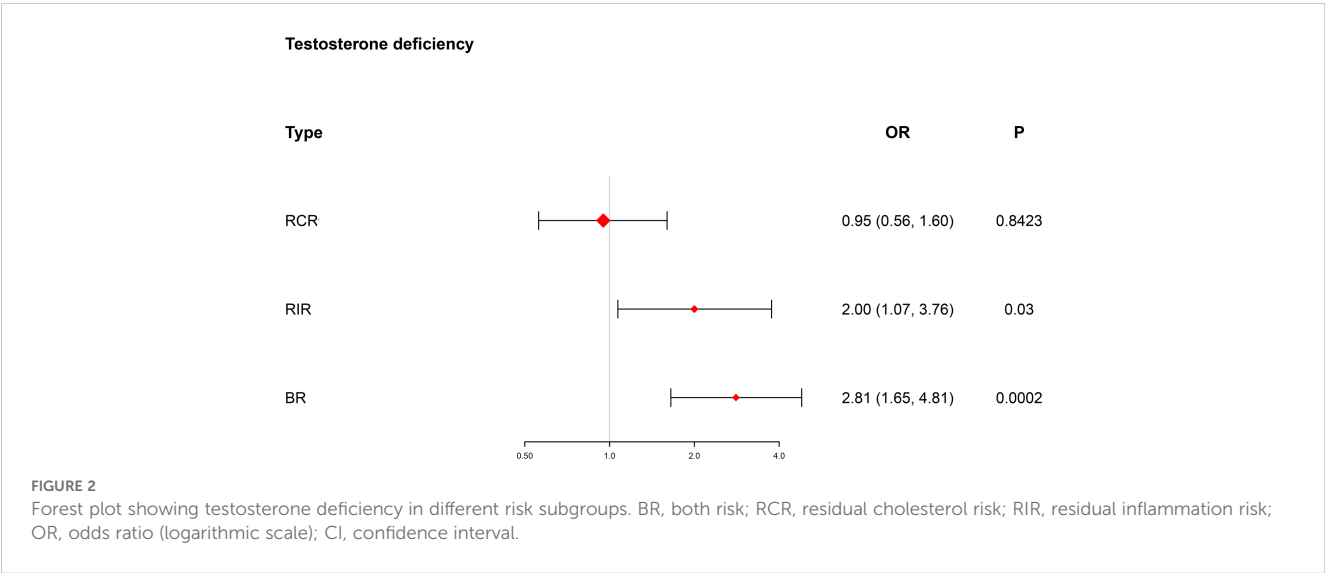
Exposure	β	P
TT		
Normal	Ref	
RCR	-12.23 (-40.58, 16.13)	0.3982
RIR	-66.50 (-107.92, -25.09)	0.0017
BR	-79.37 (-112.74, -46.00)	<0.0001
SHBG		
Normal	Ref	
RCR	-5.48 (-9.35, -1.62)	0.0055
RIR	-4.47 (-10.18, 1.24)	0.1252
BR	-8.29 (-12.86, -3.72)	0.0004
E2		
Normal	Ref	
RCR	-0.86 (-2.60, 0.87)	0.3300
RIR	1.28 (-1.29, 3.84)	0.3287
BR	0.38 (-1.67, 2.44)	0.7141
FT		
Normal	Ref	
RCR	0.22 (-0.20, 0.63)	0.3056
RIR	-1.00 (-1.61, -0.40)	0.0012
BR	-0.97 (-1.46, -0.48)	0.0001

β , effect size for regression; TT, total testosterone; SHBG, sex hormone binding globulin; E2, estradiol; FT, free testosterone; BR, both risk; RCR, residual cholesterol risk; RIR, residual inflammation risk; SHBG, sex hormone binding globulin. Statistically significant results are bolded.

discernible distinction was observed within non-diabetic patients, where hs-CRP exhibited a statistically significant inverse correlation with both total testosterone ($\beta = -4.70$, 95% CI [-7.95, -1.46], $p = 0.0046$) and free testosterone ($\beta = -0.08$, 95% CI [-0.13, -0.03], $p = 0.0033$). Additionally, a positive correlation surfaced between LDL-C and free testosterone ($\beta = -0.23$, 95% CI [0.01, 0.45], $p = 0.0428$) within this same subgroup. The narrative took a different turn for diabetic patients, where LDL-C displayed an adverse relationship with SHBG ($\beta = -4.28$, 95% CI [-7.99, -0.57], $p = 0.025$). As the analysis pivoted towards the realm of hypertension, a notable constancy was witnessed in the relationship between LDL-C and each respective indicator. Yet, a distinctive dynamic emerged in the context of hypertensive patients, where hs-CRP assumed significance by evincing a substantial inverse correlation with both total testosterone ($\beta = -4.69$, 95% CI [-8.45, -0.93], $p = 0.0148$) and free testosterone ($\beta = -0.09$, 95% CI [-0.16, -0.03], $p = 0.0054$).

4 Discussion

Both LDL-C and hs-CRP exhibit robust connections with sex hormone dynamics. Noteworthy alterations in sex hormone concentrations surfaced in individuals confronting cholesterol-driven residual risk and inflammation-associated residual risk. However, within the realm of adult male physiology, inflammation emerged as a more potent influencer of testosterone levels compared to LDL cholesterol. Upon meticulous adjustment and refinement of the model, LDL cholesterol relinquished its autonomous correlation with testosterone, while hs-CRP retained its significant, independent and adverse correlation with both testosterone and free testosterone. The amalgamation of these factors posed a heightened risk for testosterone insufficiency. Furthermore, an autonomous and unfavorable link



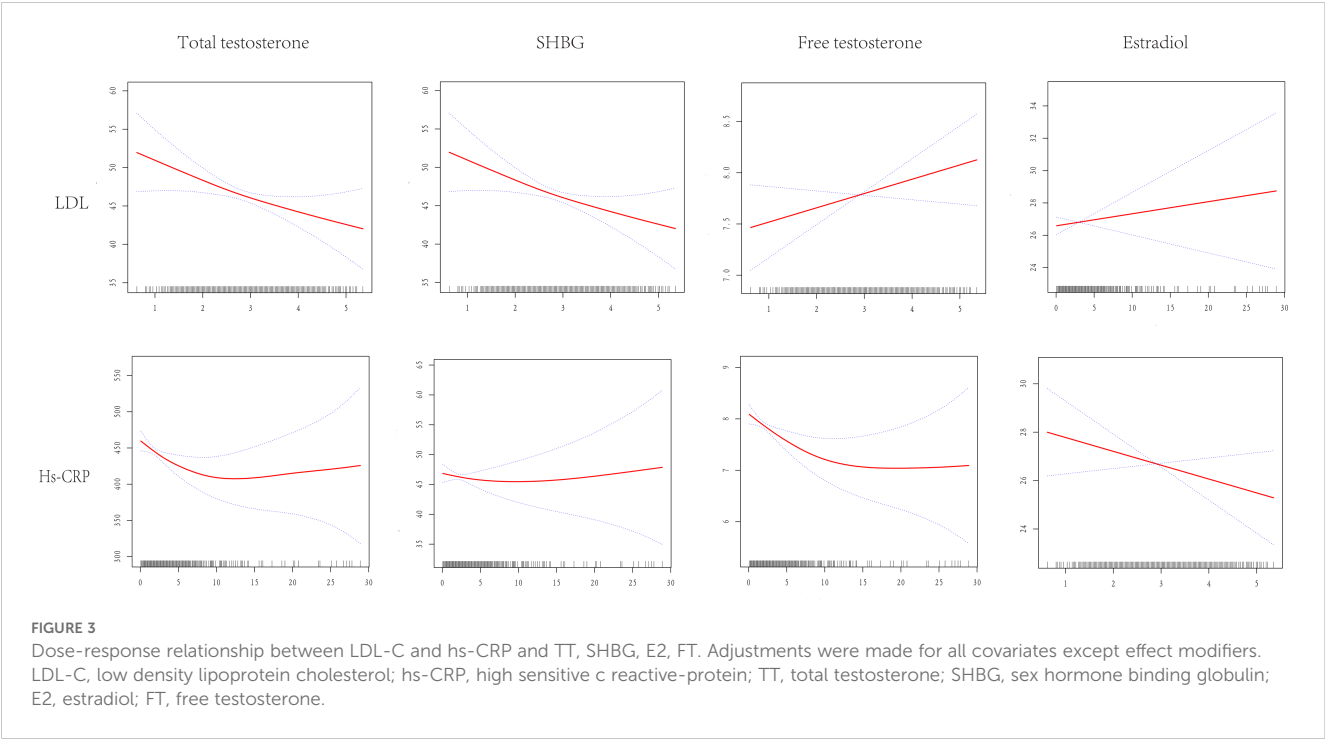


TABLE 4 Two-way linear regression and log-likelihood ratio tests explained the analysis of threshold effects of LDL-C and hs-CRP on TT, SHBG, E2, and FT.

	ULR Test β (95% CI)	PLR Test β (95% CI)	LRT test P-value
LDL-C			
Total testosterone			
<1.89	0.42 (-11.81, 12.65)	-68.84 (-143.97, 6.30)	0.063
≥1.89		6.94 (-7.13, 21.01)	
SHBG			
<2.46	-1.91 (-3.64, -0.18)	-5.79 (-10.95, -0.63)	0.112
≥2.46		-0.49 (-2.97, 2.00)	
Estradiol			
<1.58	-0.53 (-1.32, 0.25)	2.46 (-5.82, 10.74)	0.47
≥1.58		-0.64 (-1.48, 0.20)	
Free testosterone			
<1.89	0.14 (-0.04, 0.32)	-0.72 (-1.83, 0.39)	0.118
≥1.89		0.22 (0.02, 0.43)	
Hs-CRP			
Total testosterone			
<1	-2.80 (-5.67, 0.07)	-69.51 (-111.71, -27.31)	0.002
≥1		-1.54 (-4.50, 1.42)	

(Continued)

TABLE 4 Continued

	ULR Test β (95% CI)	PLR Test β (95% CI)	LRT test P-value
SHBG			
<0.8	-0.03 (-0.43, 0.38)	-12.77 (-20.69, -4.86)	0.001
≥0.8		0.12 (-0.29, 0.54)	
Estradiol			
<0.3	-0.07 (-0.11, 0.26)	-12.90 (-28.20, 2.41)	0.091
≥0.3		0.08 (-0.10, 0.27)	
Free testosterone			
<9.2	-0.06 (-0.10, -0.01)	-0.13 (-0.20, -0.05)	0.022
≥9.2		0.03 (-0.06, 0.11)	

ULR, univariate linear regression; PLR, piecewise linear regression; LRT, logarithmic likelihood ratio test, statistically significant: $p < 0.05$. TT, total testosterone; SHBG, sex hormone binding globulin; E2, estradiol; FT, free testosterone. Statistically significant results are bolded.

between LDL-C and sex hormone binding globulin (SHBG) was distinctly discerned. In the intricate tapestry of male hormonal milieu, estrogen did not manifest any significant associations. Nevertheless, within the context of subgroup analysis, the presence or absence of lipid-lowering medications exerted a discernible influence solely on the interplay between SHBG and free testosterone, along with LDL-C. All other interrelations retained their unaltered constancy. On a divergent note, among diabetic patients, the responsiveness of both risks to the sway of sex hormones seemed comparatively muted when

TABLE 5 Subgroup analysis.

	LDL-C			Hs-CRP		
Testosterone	β (95% CI)	P	P for interaction	β (95% CI)	P	P for interaction
Take drugs			0.1058			0.4029
No	-5.50 (-22.29, 11.30)	0.5213		-3.72 (-8.11, 0.68)	0.0979	
Yes	4.68 (-12.84, 22.21)	0.6007		-2.53 (-5.88, 0.81)	0.1387	
Diabetes			0.4079			0.0755
No	4.80 (-8.83, 18.43)	0.4905		-4.70 (-7.95, -1.46)	0.0046	
Yes	-11.20 (-35.57, 13.16)	0.3686		0.67 (-4.00, 5.34)	0.7781	
Hypertension			0.81			0.2054
No	-0.11 (-19.30, 19.09)	0.9913		-1.79 (-5.70, 2.12)	0.3711	
Yes	4.43 (-10.31, 19.17)	0.5563		-4.69 (-8.45, -0.93)	0.0148	
SHBG						
Take drugs			0.4013			0.7519
No	-2.82 (-4.88, -0.76)	0.0075		-1.29 (-4.05, 1.47)	0.3614	
Yes	-0.15 (-0.70, 0.39)	0.5778		-0.03 (-0.56, 0.50)	0.9022	
Diabetes			0.3085			0.5962
No	-1.70 (-3.52, 0.11)	0.0661		-0.12 (-0.55, 0.32)	0.605	
Yes	-4.28 (-7.99, -0.57)	0.025		0.08 (-0.63, 0.78)	0.8343	
Hypertension			0.9059			0.9772
No	-2.21 (-5.16, 0.73)	0.1416		-0.07 (-0.67, 0.52)	0.8089	
Yes	-1.38 (-3.29, 0.53)	0.158		-0.03 (-0.52, 0.46)	0.8999	
Estradiol						
Take drugs			0.505			0.542
No	-0.48 (-1.49, 0.53)	0.3497		-0.49 (-1.67, 0.69)	0.4128	
Yes	-0.15 (-0.41, 0.12)	0.2725		0.10 (-0.13, 0.32)	0.4131	
Diabetes			0.9209			0.0204
No	-0.35 (-1.16, 0.47)	0.4087		-0.15 (-0.35, 0.05)	0.1318	
Yes	-0.68 (-2.52, 1.16)	0.4715		0.39 (0.03, 0.74)	0.0328	
Hypertension			0.4572			0.1703
No	-0.02 (-1.23, 1.19)	0.977		0.16 (-0.09, 0.41)	0.2003	
Yes	-0.71 (-1.65, 0.24)	0.1427		-0.18 (-0.42, 0.06)	0.1427	
Free testosterone						
Take drugs			0.4874			0.395
No	0.18 (-0.10, 0.46)	0.2179		0.18 (-0.08, 0.44)	0.1685	
Yes	-0.07 (-0.15, -0.00)	0.0493		-0.05 (-0.10, 0.00)	0.0521	
Diabetes			0.7392			0.1395
No	0.23 (0.01, 0.45)	0.0428		-0.08 (-0.13, -0.03)	0.0033	
Yes	0.11 (-0.22, 0.45)	0.5119		-0.01 (-0.07, 0.05)	0.7627	

(Continued)

TABLE 5 Continued

Testosterone	LDL-C			Hs-CRP		
	β (95% CI)	P	P for interaction	β (95% CI)	P	P for interaction
Free testosterone						
Hypertension			0.7343			0.1744
No	0.13 (-0.12, 0.39)	0.3105		-0.03 (-0.08, 0.02)	0.2891	
Yes	0.20 (-0.06, 0.45)	0.1326		-0.09 (-0.16, -0.03)	0.0054	

β , effect size; CI, confidence interval; LDL-C, low density lipoprotein cholesterol; hs-CRP, high sensitive c reactive-protein; TT, total testosterone; SHBG, sex hormone binding globulin; E2, estradiol; FT, free testosterone. All relevant variables were adjusted except for those in the stratified analysis (Model 3). Statistically significant results are bolded.

juxtaposed with normative patients. In the domain of hypertensive patients, the interplay of high inflammation and sex hormones yielded a heightened sensitivity, particularly in relation to testosterone and free testosterone. This phenomenon illuminates the elevated vulnerability of male participants situated within the high-inflammation cohort to testosterone deficiency. Moreover, while the presence of high residual cholesterol in isolation did not distinctly manifest a specific risk profile, participants ensnared in the dual-risk realm exhibited an escalated susceptibility to testosterone deficiency in comparison to their single-risk counterparts.

The heightened state of inflammation is likely an outcome of interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-17 (IL-17), and tumor necrosis factor, which collectively perturb the equilibrium of the hypothalamic-pituitary-gonadal axis, consequently leading to a decrement in testosterone synthesis (12). Further, evidence has indicated that individuals burdened with obesity, due to augmented visceral adiposity, undergo a conversion of testosterone to estrogen within inflamed adipose tissue, thereby contributing to a depletion of testosterone levels within the organism (13, 14). In preclinical studies, androgens have demonstrated anti-inflammatory attributes with regard to inflammation markers, while conversely, inflammation can impede the production of testosterone (15, 16). Investigations have also unveiled an inverse association between the sex hormone testosterone and C-reactive protein (CRP) concentration, as well as a similar inverse correlation between estradiol and CRP. Strikingly, SHBG is independently correlated with white blood cell count, irrespective of CRP, thereby suggesting that both excessive androgen and inadequate estrogen levels can elicit anti-inflammatory effects within the male populace (17). The repercussions of excessive inflammation on cellular functioning are notably profound. The implementation of anti-inflammatory interventions can enhance the abundance of inflammation-sensitive T-cells, thereby affording relief from testosterone insufficiency. Furthermore, empirical data has spotlighted that certain dietary choices exhibit pro-inflammatory tendencies, which, when consumed excessively, can indeed impact sex hormone equilibrium, particularly testosterone. Hence, an awareness of the imperative to adopt a low pro-inflammatory diet is incumbent (18). Sex hormone binding globulin, a hepatically produced protein, aligns itself with levels of both high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C). The principal avenue through which testosterone exerts its influence is facilitated by the conveyance of SHBG within the serum. Diminished free testosterone precipitates an

elevation in SHBG, thereby fostering the liberation of luteinizing hormone, which in turn augments testosterone synthesis. A discernible negative correlation between SHBG and triglycerides (TG) and LDL-C, coupled with a positive correlation with HDL-C, substantiates this phenomenon. The nexus between adipose accumulation and insulin resistance, intricately intertwined with fat metabolism, may underpin the association with reduced SHBG levels (19, 20). Notably, a previous inquiry demonstrated a lack of link between SHBG and LDL-C in males, a variance that might be attributed to dissimilarities in ethnicity and test methodologies. Conversely, SHBG exhibits a robust correlation with HDL-C and serves as a pivotal protein in the orchestration of lipid regulation (21). In normoglycemic men, LDL was independently and significantly linked to both SHBG and total testosterone, with this relationship intensifying in the nonobese subset (22).

Symptoms associated with testosterone deficiency (TD) can exhibit variability contingent upon the extent of the deficiency and the timing of its onset. These manifestations may encompass diminished libido and erectile dysfunction, dwindled muscle mass and strength, fatigue and depleted vitality, alterations in mood accompanied by depression, reduced bone density, and even weight loss. Numerous models have been developed predicated on rudimentary metrics, ostensibly poised to prognosticate the advent of TD, such as the visceral adiposity index (VAI) and the triglyceride-to-HDL cholesterol ratio (TG/HDL-C), yet the prognostic potency of these models remains somewhat insubstantial (23). Metrics such as TyG, LAP, VAI, and HOMA-IR have emerged as adept predictors of TD, characterized by commendable specificity and sensitivity across both diabetic and non-diabetic cohorts (24, 25). Notably, within the realm of participants categorized under the RCR cohort, the significance of TD waned due to the pronounced gravity of the deficiency itself. However, in the RIR and BR cohorts, the risk of TD experienced a marked elevation. This underscores the necessity for individuals, particularly those who are non-diabetic and hypertensive, afflicted by high levels of inflammation, to exercise vigilance towards potential fluctuations in testosterone levels, given the amplified risk entailed in such circumstances.

Nonetheless, our study remains vulnerable to certain limitations which merit scrupulous acknowledgment: 1. There are limitations in the sample size for inclusion; 2. Cross-sectional studies are limited by the simplification of biomarkers, population heterogeneity, and lack of causal evidence, and should be cautious about generalizing to populations with different genetic

backgrounds or environments, and future integration of multi-omic data and cross-ethnic validation are needed to improve the models. Subgroups based on LDL-C and hs-CRP thresholds may not be directly translated into actionable clinical insights; 3. Results from subgroup analyses need to be further explored through multi-omics integration (e.g., lipoprotein subfractions vs. tissue-specific inflammatory markers) and cross-ethnic validation, and hypothesis-generating attributes of the subgroup results should be interpreted with caution to avoid over-interpretation as a basis for clinical decision-making. Initially, our data elucidating the adverse interplay between RCR and SHBG warrants prudent scrutiny. Though the pivotal role of HDL-C in SHBG dynamics is firmly established, the extent to which SHBG is diminished in individuals confronting high residual cholesterol risk has not garnered widespread empirical substantiation. Moreover, while it is undeniable that patients with RCR did indeed exhibit diminished testosterone levels, it is imperative to underscore that while a direct correlation eluded our observation, the revelation of such an association warrants the conduction of an extensive array of investigations for future affirmation. Secondary to this, while our findings allude to the robust concordance between heightened inflammation and testosterone levels, and the linkage between high residual cholesterol risk and SHBG, with potential utility in prognosticating sex hormone perturbations, the inherent constraints of cross-sectional studies preclude us from delineating the underlying mechanistic underpinnings. This necessitates the undertaking of more comprehensive investigations to uncover the intricate mechanisms at play. Furthermore, it is worth noting that our classification of RCR was based upon LDL-C levels. Nonetheless, there remains an intriguing query as to whether other lipid constituents contribute to residual risk, such as triglycerides and the cholesterol content within triglyceride-rich lipoproteins. Lastly, our study was predicated upon the expansive NHANES database, encompassing the demography of the U.S. populace and its ethnic diversity. However, it is incumbent upon us to acknowledge that the generalizability of our findings to other ethnicities and races may be subject to limitation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://wwwn.cdc.gov/nchs/nhanes>.

Ethics statement

The studies involving humans were approved by National Health and Nutrition Examination Survey (NHANES), conducted

by the Centers for Disease Control and Prevention (CDC). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YZ: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. GW: Investigation, Writing – original draft. LL: Methodology, Writing – original draft. JY: Conceptualization, Software, Writing – original draft. SJ: Project administration, Supervision, Writing – review & editing, Writing – original draft.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

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.2025.1526056/full#supplementary-material>

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OPEN ACCESS

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RECEIVED 02 October 2024

ACCEPTED 25 April 2025

PUBLISHED 21 May 2025

CITATION

Wang Z, Li D, Zhou G, Xu Z, Wang X, Tan S,
Li Z, Li X, Song C and Yuan S (2025)
Deciphering the role of reactive oxygen
species in idiopathic asthenozoospermia.
Front. Endocrinol. 16:1505213.
doi: 10.3389/fendo.2025.1505213

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Deciphering the role of reactive oxygen species in idiopathic asthenozoospermia

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Asthenozoospermia is a severe condition characterized by abnormal sperm motility, contributing to 50% of male infertility cases. Idiopathic asthenozoospermia refers to a form of this condition with no identifiable causes through routine clinical examinations, potentially linked to apoptosis and oxidative stress induced by excessive reactive oxygen species (ROS). At low concentrations, ROS positively influence physiological processes, including sperm mature and motility. However, elevated ROS levels can harm human spermatozoa through oxidative stress, primarily due to the absence of effective DNA damage repair mechanisms and inadequate antioxidant defenses. In this review, we summarize the physiological and pathophysiological roles, endogenous and exogenous sources, and therapeutic strategies related to ROS in idiopathic asthenozoospermia. Ultimately, maintaining a proper balance between ROS concentrations and antioxidants is crucial for ensuring male reproductive health.

KEYWORDS

reactive oxygen species, idiopathic asthenozoospermia, oxidative stress, sperm motility, antioxidants, male infertility

1 Introduction

Sperm motility is a crucial capability of human spermatozoa necessary for their journey across the female genital tract post-ejaculation (1), with progressive motility (PR) serving as a key metric (2, 3). In recent years, sperm parameters have witnessed a declining trend, especially with a sharp drop in sperm motility, which, in severe cases, leading to male infertility in severe instances (4). Currently, male infertility is responsible for the 14% of couples experiencing fertility issues (1, 2). Asthenozoospermia (AZS) is a severe condition characterized by abnormal sperm motility, defined by progressive motility of less than 32% (PR<32%) among sperm parameters (2, 3). The majority of patients with male infertility also present with asthenozoospermia. The common causes of AZS include varicocele,

endocrine abnormalities, environmental factors, inflammation, drug-induced injury, and certain underlying diseases (5, 6). Nevertheless, in numerous cases, routine clinical examinations fail to identify clear causes, leading to a classification of idiopathic AZS (iAZS) (5).

The exact pathogenesis of iAZS remains unclear, but it is currently believed that excessive reactive oxygen species (ROS) leading to apoptosis and oxidative stress is a key factor in its development. ROS is a group of highly reactive oxygen-containing molecules that include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^\cdot$), and singlet oxygen (1O_2) (7). Due to their short half-life (8), they cannot be directly detected in human specimens. The $OH^\cdot$ is particularly unstable and rapidly reacts with nearby biomolecules. Furthermore, H_2O_2 is a predominant form of ROS capable of crossing cell membranes to exert effects beyond cellular boundaries (9).

The intracellular levels of ROS are closely regulated by various synthesis and degradation pathways. Maintaining physiological levels of ROS is critical for redox regulation involved in processes such as repair, survival, and differentiation (10). However, when ROS are produced in excess, they can damage sperm cells, leading to impaired motility, DNA fragmentation, and cellular apoptosis, significantly affecting male fertility (11). Additionally, excessive ROS can induce lipid peroxidation in the sperm plasma membrane, which is rich in polyunsaturated fatty acids, disrupting membrane integrity and impairing sperm function and morphology (12). While ROS are often considered detrimental, they also play a vital physiological role in sperm function (13, 14). In spermatozoa, these molecules play essential roles in sperm capacitation, acrosome reaction, and fertilization (15). The challenge lies in the delicate balance between the beneficial and harmful effects of ROS (16). The role of ROS in idiopathic asthenozoospermia remains unclear (17). Furthermore, the mechanisms for maintaining the dynamic balance of ROS in sperm to manage oxidative stress in idiopathic asthenozoospermia require further investigation.

Idiopathic asthenozoospermia (iAZS) may be linked to apoptosis and oxidative stress caused by excessive ROS (18, 19). Nevertheless, the exact pathogenesis of iAZS remains unclear. This review explores the sources of ROS, their physiological and pathological roles in sperm motility, and potential therapeutic strategies targeting ROS in iAZS. By investigating these aspects, we offer new insights for the clinical management of iAZS and provide a comprehensive framework for understanding the complex interplay between ROS and sperm function.

2 Physiological roles of low concentrations of ROS in sperm maturation and motility

At low concentrations, ROS positively influence physiological processes such as spermatogenesis, sperm motility, and fertilization (20, 21). This process might be associated with phosphorylation, the expression of regulatory transcription factor and oxidative effects (Figure 1) (22).

2.1 Phosphorylation

During spermatogenesis, the DNA replication process of meiosis relies on energy supplied through oxygen consumption and ROS generation in mitochondria, which also provide the necessary ATP for sperm motility (23). The proliferation of spermatogonia and the differentiation of spermatocytes into spermatozoa, including the formation of sperm flagella crucial for motility, depend on various internal environmental factors (24). These factors include receptor-tyrosine kinase (RTK) phosphorylation signaling pathways, which are mediated by ROS in some somatic cells (25). Therefore, physiological concentrations of ROS play beneficial roles by modulating phosphatases and facilitating phosphorylation (Figure 1).

2.2 Regulatory transcription factor

ROS at appropriate concentrations play a crucial role in the transcriptional processes of spermatogenesis by functioning as regulatory transcription factors (26). Several sex hormones, including follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are also involved in the mechanism of transcriptional regulation (27). In the testes, Sertoli cells are the primary targets of FSH (28). The cAMP response element-binding protein (CREB), whose receptors are activated by optimal levels of ROS, serves as a pivotal transcription factor within Sertoli cells under the influence of FSH-mediated signaling pathways (29). However, the role of ROS as regulatory transcription factors varies distinctly from that of various sex hormones (Figure 1) (30).

2.3 Oxidative effect

Sperm maturation in the epididymis is influenced by the oxidative effects of ROS (22). Due to the lack of histone packaging, spermatozoa struggle to maintain the integrity of their genetic material through DNA damage repair processes (21). Consequently, the histone-to-protamine replacement during spermiogenesis provides an alternative mechanism for maintaining genetic stability, which results from the oxidation of small nuclear thiol group proteins in protamine (31). Additionally, ROS, as oxidants derived from redox reactions, are implicated in the formation of chromatin packaging and the mitochondrial capsule—a protective cover surrounding the chromatin and mitochondria (32). Improper formation of these structures can compromise both the integrity and energy generation required for sperm motility, leading to functional impairments in spermatozoa (Figure 1) (33, 34).

3 Pathophysiological roles of ROS in idiopathic asthenozoospermia

Sperm motility is an essential capability of human spermatozoa required for their journey through the female genital tract post-

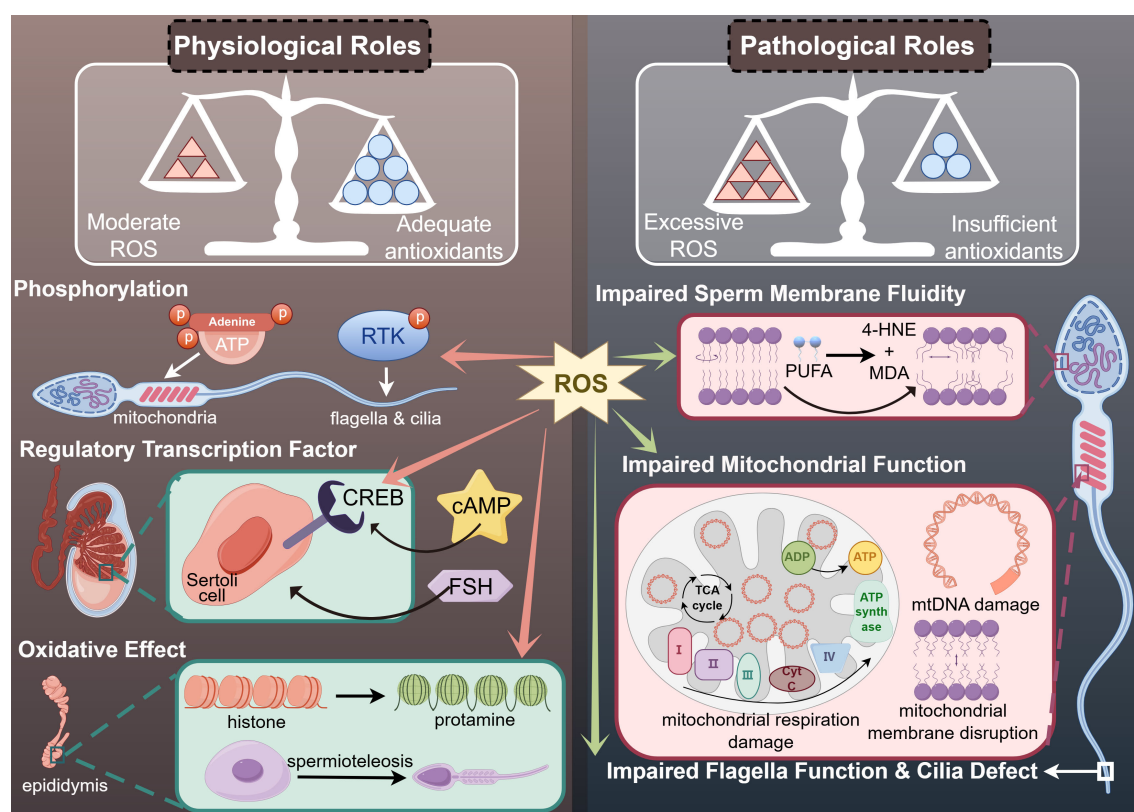


FIGURE 1
The physiological and pathological roles of ROS in human sperm motility.

ejaculation (1). Due to the lack of adequate oxygen radical-scavenging enzymes in their cytoplasm, human spermatozoa are highly vulnerable to oxidative stress induced by reactive oxygen species (ROS), leading to idiopathic asthenozoospermia without a clear etiology (35). ROS produced by mitochondrial complex I can cause mitochondrial dysfunction through peroxidation in the mid-piece of spermatozoa, resulting in a rapid depletion of ATP, which adversely affects sperm motility (36, 37). The primary sites of ATP generation in human spermatozoa are the mitochondria, the central part of the flagella, and the sperm head (38). The key metabolic pathways involved are oxidative phosphorylation and glycolysis (18). ATP production via oxidative phosphorylation primarily occurs in the mitochondrial respiratory chain complexes through respiration, while glycolysis takes place in the central part of the flagella (Figure 1) (39, 40).

3.1 Impaired mitochondrial function

The specific activity of mitochondrial enzymes, which depend on the mitochondrial electron transfer chain complexes (ETCs), can influence sperm motility and potentially lead to idiopathic asthenozoospermia (41). Sperm motility has been shown to correlate with oxygen consumption and the efficiency of mitochondrial respiration. Several inhibitors of ETCs have been observed to impair sperm motility. Complex I in the mitochondria

is particularly sensitive to excessive ROS (42), and this sensitivity arises because ROS generated through unsaturated fatty acids inhibit complex I (42). Meanwhile, the absence of mitochondrial protein OPA1 leads to disorganization of mitochondrial cristae structure and impaired assembly of ETC Complexes I, III, IV, and V, but does not affect the assembly of Complex II. OPA1 plays a role in the accumulation of mitochondrial ROS and lipid ROS induced by cysteine deprivation. Additionally, ferroptosis is a form of iron-dependent non-apoptotic cell death primarily triggered by the accumulation of intracellular iron and lipid peroxidation. Mitochondria-targeted antioxidants such as SkQ1 and redox mediators like methylene blue can inhibit the production of ROS in Complex I of the mitochondrial electron transport chain, preventing mitochondrial lipid peroxidation and ferroptosis (13).

The primary characteristics of non-motile sperm include the disruption of the mitochondrial dysfunction (43–45). Mitochondrial DNA (mtDNA) damage resulting from interactions between nitric oxide (NO) and superoxide (O_2^-) can also affect sperm motility and function (12, 46). And mtDNA repair is inadequate because of the complete absence of nucleotide-excision repair pathways (47, 48). Mitochondria are crucial for the energy metabolism of sperm, primarily generating ATP through oxidative phosphorylation (OXPHOS) to fuel sperm motility (49). Damage to mitochondrial DNA can result in OXPHOS dysfunction (50), which in turn impairs ATP production and leads to reduced sperm motility (36, 37). Additionally, mitochondrial DNA damage

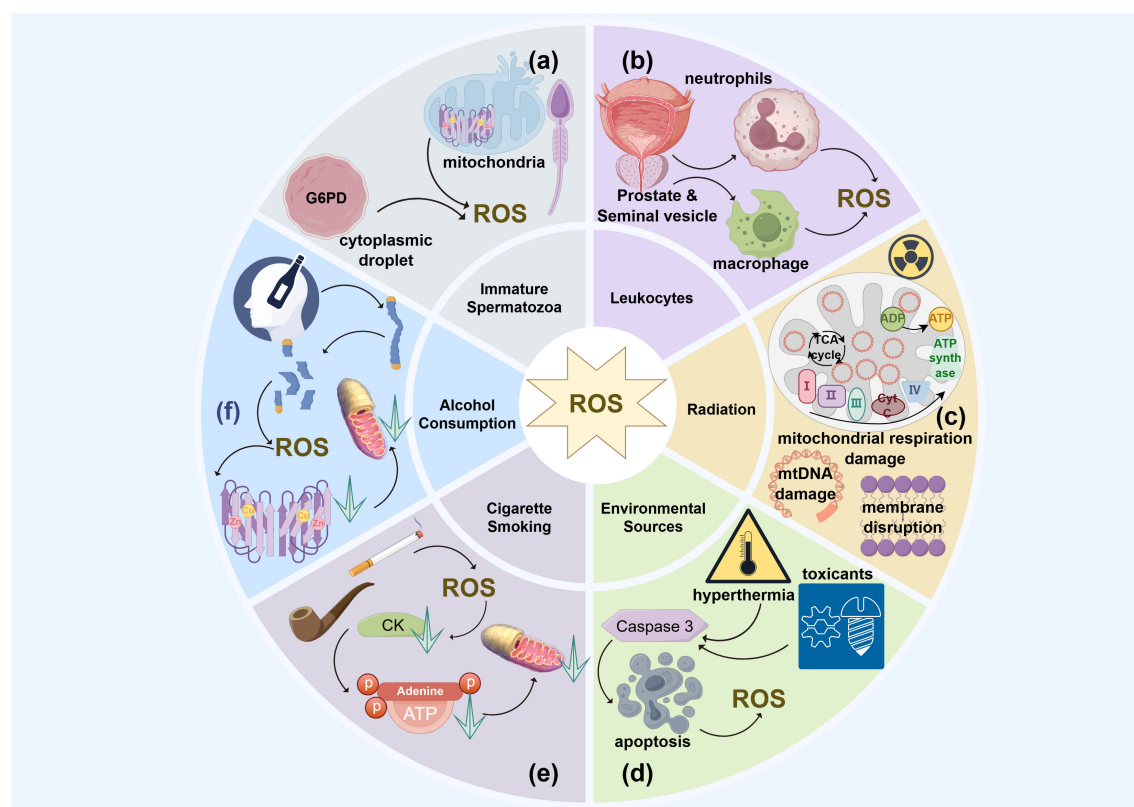


FIGURE 2

The endogenous and exogenous sources of ROS in idiopathic asthenozoospermia. (a) Immature spermatozoa; (b) Leukemia; (c) Radiation; (d) Environment resources; (e) Smoking; (f) Alcohol consumption.

can lead to excessive production of reactive oxygen species (ROS), with elevated ROS levels triggering oxidative stress that further impairs sperm function (51). Such damage may also interfere with the expression and function of mitochondria-related proteins. For instance, mitochondrial transcription factor A (TFAM) is critical for regulating mitochondrial DNA replication and transcription. Abnormal TFAM expression may be linked to mitochondrial DNA damage, consequently affecting sperm vitality (52). Moreover, mitochondrial DNA damage can compromise the structural integrity of mitochondria, resulting in reduced sperm motility (53). Finally, mitochondrial DNA damage can influence sperm survival and function by affecting apoptotic pathways. Research indicates that mitochondrial dysfunction may activate apoptotic signaling pathways, leading to sperm cell death (54). Therefore, mitochondrial DNA damage impacts sperm vitality through various mechanisms, including disruptions in energy metabolism, oxidative stress, alterations in mitochondrial membrane potential, abnormal protein expression, and apoptosis. These interconnected pathways collectively result in reduced sperm vitality, ultimately affecting male fertility.

Furthermore, ROS can compromise the integrity of mitochondrial membranes, potentially activating apoptotic signaling cascades and promoting the release of cytochrome C (55, 56). Apoptosis in spermatozoa is typically initiated by oxidative stress and lipid peroxidation, leading to the production of

mitochondrial ROS. This cascade results in a rapid loss of sperm motility, followed by caspase activation and the exposure of phosphatidylserine on the sperm surface (57). The Sperm Chromatin Structure Assay (SCSA) and active Caspase-3 levels correlate with the rate of motility decline post-ejaculation. Elevated levels of these markers suggest a faster decline in motility, indicating that apoptosis significantly impacts sperm vitality (58, 59). The phosphoinositide 3-kinase (PI3K) signaling pathway plays a role in regulating sperm apoptosis. Inhibition of PI3K activity triggers an apoptotic cascade characterized by loss of motility and oxidative DNA damage. Thus, impaired mitochondrial function due to mtDNA damage and mitochondrial apoptosis may be responsible for reduced sperm motility and idiopathic asthenozoospermia (Figure 1) (45).

3.2 Impaired sperm plasma membrane

Sperm plasma membrane may be the major target site of ROS through cascade signaling reaction (60). ROS affects the fluidity and integrity of sperm plasma membrane (12). The membrane fluidity of human spermatozoa depends on the polyunsaturated fatty acids (PUFA) in the sperm plasma membrane (61). Excessive ROS converts PUFA into 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), byproducts of LPO, to destroy the

membrane fluidity of spermatozoa. Meanwhile, the generation of 4-HNE and MDA also impaired mitochondrial electron transfer chain complexes, which resulted in reducing ATP production and corresponding sperm motility, and further increased ROS from mitochondria as a result of oxidative stress (62). Therefore, ROS causes damage to membrane fluidity of human spermatozoa through the generation of MDA and 4-HNE, which in turn lead to idiopathic asthenozoospermia (Figure 1) (63). What's more, loss of glutathione, a kind of antioxidants in the midpiece of spermatozoa, may also contributes to idiopathic asthenozoospermia (64).

In addition, lipid peroxidation (LPO) induced by excessive ROS could also adversely affect the fluidity of the sperm plasma membrane through oxidative stress (65). This process can result in the complete inactivation of membrane enzymes, subsequently leading to sperm DNA damage (57). Enzymes on the sperm membrane, such as phospholipase C (PLC) and phospholipase D (PLD), play crucial roles in regulating intracellular signal transduction and membrane lipid metabolism (66). Dysfunction of these enzymes can lead to disordered membrane lipid metabolism, increased ROS production, and consequently, oxidative stress and sperm DNA damage (67). For instance, overactivation of PLC can lead to an increase in intracellular calcium ion concentration, which in turn activates a series of downstream signaling pathways and increases ROS production (66). These ROS can attack the unsaturated fatty acids on the sperm membrane, triggering lipid peroxidation reactions that disrupt the membrane's integrity, ultimately leading to sperm DNA damage (67). Additionally, when the functions of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) are inhibited or their activities are reduced, ROS levels rise, leading to lipid peroxidation of the sperm membrane and consequently affecting the integrity of sperm DNA, resulting in decreased sperm motility (68).

3.3 Sperm DNA fragmentation

In spermatozoa, the integrity of DNA is crucial for protecting genetic material from environmental damage. Uncompacted DNA, due to its open structure, is more susceptible to attack by ROS, prompting mitochondria to produce apoptosis-inducing factor (AIF) and sperm DNA fragmentation (SDF) (69). DNA damage is assessed by the DNA fragmentation index (DFI) rate using the comet assay, the sperm chromatin dispersion assay, terminal deoxynucleotidyl transferase (TUNEL) assay, and sperm chromatin structure assay (70, 71). Studies have shown that the sperm DFI is significantly negatively correlated with progressive sperm motility (72, 73). Specifically, for every 10% increase in DFI, the probability of male conception may decrease by up to 30% (1, 74, 75). One study found that a sperm DFI greater than 30% is a threshold for a significant decline in conception rates; when DFI exceeds 30%, the success rates of natural conception and intrauterine insemination (IUI) are nearly zero (1, 74, 75). SDF is

a type of DNA damage that occurs under conditions involving sperm caspase and endonuclease activity, which subsequently affects the transition from histone to protamine (76). The reduction in sperm motility is also linked to the inhibition of this histone-to-protamine transition (77, 78). In patients with asthenozoospermia, the expression levels of protamine are typically lower, which may affect the motility and fertilization capacity of spermatozoa (79). Thus, SDF is one of the manifestations of idiopathic asthenozoospermia.

The relationship between SDF and reduced sperm motility is complex, involving various molecular mechanisms. Studies suggest that oxidative stress is a primary factor contributing to SDF. It leads to the excessive production of ROS within sperm cells, which attack DNA, causing strand breaks and thereby increasing DNA fragmentation (80). Moreover, oxidative stress can impair mitochondrial function, disrupting energy metabolism and resulting in diminished sperm motility (52). The chromatin packaging state of sperm is another critical factor. Research has shown that sperm with poorly packaged chromatin are more prone to DNA fragmentation and cell death during freeze-thaw processes (81). This inadequate chromatin packaging may be linked to insufficient protamine levels, which are essential for the high degree of chromatin compaction in spermatozoa (82). Additionally, sperm DNA fragmentation is correlated with the age of the sperm. As age advances, the integrity of sperm DNA declines, and DNA fragmentation increases. This is possibly due to age-related oxidative stress and a decline in antioxidant defense mechanisms (83).

3.4 Impaired flagella function

Human spermatozoa can utilize various carbohydrates to generate ATP necessary for sperm motility. However, even in the absence of impaired mitochondrial function, inhibition of glycolysis can also affect sperm motility (84). The flagella, which constitute most of the sperm tail structure, play a crucial role in facilitating sperm motility. For instance, cAMP can promote the phosphorylation of protein kinase A (PKA) in sperm flagella (85), which is followed by the activation of tyrosine kinases and the phosphorylation of tyrosine residues in sperm proteins, including AKAP3, AKAP4, FSIP2, CABYR, and VCP (86, 87). The cyclic AMP (cAMP)-mediated PKA signaling pathway in sperm has been shown to be downregulated due to oxidative stress in idiopathic asthenozoospermic males (84). Levels of cAMP are positively correlated with sperm motility (88). cAMP is activated by intracellular soluble adenylyl cyclase (sAC), which is encoded by the ADCY10 gene under the stimulation of Ca^{2+} , and is essential for sperm motility (89). Mutations in the ADCY10 gene can lead to a decline in sAC, resulting in idiopathic asthenozoospermia [106].

Meanwhile, AKAP3, a structural protein acting as the regulatory subunit of PKA, forms the fibrous sheath, maintaining the structural integrity of the sperm flagella in collaboration with AKAP4 (90). A deficiency in AKAP3 may impair sperm motility due to the abnormal accumulation of DNA and RNA metabolites (91).

Mutations in AKAP3 and AKAP4 can lead to structural abnormalities in the sperm tail's flagella (91). FSIP2 anchors cAMP-mediated PKA into AKAP4 to sustain sperm motility. Mutations in FSIP2, characterized by the absence of CPC, IDA, and ODA, can cause idiopathic asthenozoospermia due to the lack of AKAP4 protein (92). Thus, idiopathic asthenozoospermia can be monitored through AKAP3, AKAP4, and FSIP2 within the cAMP/PKA signaling pathway (93).

Additionally, the glycolysis process in the central part of the flagella provides sufficient ATP for its function to support sperm movement (94). Researchers have found that GPI, MDH1, PGAM1 and PGAM2A, the glycolysis-mediated proteins, were downregulated in the spermatozoa of patients with iAZS (95). Meanwhile, a significant number of glycolytic enzymes, including lactate dehydrogenase, phosphofructokinase, hexokinase, glyceraldehyde-3-phosphate dehydrogenase (GAPD), and phosphoglucose isomerase, have been identified in the sheath of the sperm flagella, maintaining its function (38). Additionally, in seminal plasma, researchers have demonstrated that citric acid, malic acid, succinic acid, which are associated with energy metabolism, and pyruvate were collectively reduced in the iAZS group, while lactate levels were elevated (96). These findings indicate a shift towards anaerobic glycolysis, resulting in decreased production of ATP compared to aerobic catabolism via the tricarboxylic acid cycle (96). This metabolic alteration likely contributes to reduced sperm motility (Figure 1).

4 The sources of ROS in human ejaculate

ROS is produced through both endogenous and exogenous pathways and play critical roles in sperm function. Human spermatozoa are significant sites of cellular ROS production (97, 98). Meanwhile in the context of iAZS, endogenous ROS are often produced in excess.

4.1 Endogenous sources and their effects on sperm motility

Human ejaculate contains a diverse array of round cell types, including human spermatozoa at various developmental stages, leukocytes, and epithelial cells (97, 98). The ROS contributed by these cells constitute the majority of the endogenous ROS pool, which is predominantly found in seminal plasma. Among them, immature spermatozoa and leukocytes, such as neutrophils and macrophages, are considered major endogenous sources of ROS (99, 100). The mechanisms of endogenous ROS generation in immature spermatozoa and leukocytes lied in two primary pathways: the reduced nicotinamide adenine dinucleotide (NADH)-dependent oxidoreductase system in the mitochondria and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system located in the spermatozoa plasma membrane (101).

The mitochondrial oxidoreductase system is responsible for the majority of ROS production within human spermatozoa, primarily due to the abundance of mitochondria, which supply continuous energy for sperm motility (101). Mitochondrial ROS generation is fundamentally linked to the process of respiration. NADH oxidoreductase plays a critical role by catalyzing the oxidation of O_2 to O_2^- , a precursor of sperm ROS, while transferring electrons from NADH to coenzyme Q10 (CoQ10) within the mitochondrial respiratory chain (102). If mitochondrial O_2 concentrations are elevated, coupled with increased respiratory rates, more superoxide is released (103).

In the plasma membrane of human spermatozoa, NADPH oxidase also contributes to the transformation of O_2 to superoxide (104). NOX5, a type of NADPH oxidase located in the acrosome and midpiece region of human spermatozoa, is activated through the binding of Ca^{2+} to its N-terminal cytoplasmic domain (105). These conformational changes facilitate the generation of superoxide, making the ROS generated by NOX5 as a major component of reactive oxygen species in human spermatozoa (105).

4.1.1 Immature spermatozoa

The synthesis of ROS in semen is influenced by the maturation level of spermatozoa (106). During their development and maturation, damaged or immature spermatozoa may retain residual cytoplasmic droplets, which are remnants of spermatogenesis. These droplets contain glucose-6-phosphate dehydrogenase (G6PD), a cytosolic enzyme that produces an excess of NADPH. This NADPH acts as a substrate for NADPH oxidase, facilitating the conversion of O_2 to O_2^- (107).

A significant concentration of mitochondria is found in the midpiece of spermatozoa, serving as energy reservoirs that support sperm motility (108). The diaphorase enzyme, an oxidoreductase in the mitochondrial respiratory chain, maintains a balance between the oxidized and reduced forms of NADH to sustain sperm motility. However, a reduction in diaphorase enzyme activity can lead to superoxide generation, resulting in mitochondrial dysfunction through ROS-induced oxidative stress, potentially even damaging the mitochondrial integrity of human spermatozoa (109). Damage to the mitochondrial membrane by excessive ROS can further exacerbate ROS generation (110) (Figure 2a).

4.1.2 Leukocyte

In patients with idiopathic asthenozoospermia, less than one million leukocytes per milliliter are typically found in naturally ejaculated semen (1). Most of the leukocytes from the prostate and seminal vesicles are activated leukocytes (106). Activated leukocytes, particularly peroxidase-positive types such as polymorphonuclear neutrophils (PMNs) and macrophages, are significant producers of ROS in human semen (111). These leukocytes enhance the production of NADPH, thereby increasing the activity of NADPH oxidases and resulting in elevated levels of superoxide O_2^- . Additionally, myeloperoxidase-positive neutrophils contribute to the oxidative conversion of O_2 (112).

In addition, leukocyte-mediated signaling can also lead to an imbalance between oxidative and antioxidative processes. Elevated proinflammatory cytokines, such as interleukin (IL)-8, and reduced levels of superoxide dismutase (SOD) promote ROS generation, triggering oxidative stress. This stress, exacerbated by excessive leukocytes, ultimately damages spermatozoa (Figure 2b) (74).

4.2 Exogenous sources of ROS and their effects on sperm motility

The influence of environmental factors on sperm quality and motility represents a complex and multifaceted challenge. Over recent years, an expanding corpus of research has been dedicated to elucidating the mechanisms by which these factors impact male fertility, with particular emphasis on sperm quality and motility. This review specifically examines the roles of environmental factors, encompassing high temperatures, toxicants, and occupational exposures, as well as lifestyle factors, including obesity, smoking, alcohol consumption, and daily electronic radiation, in the generation of ROS and their impact on sperm motility.

4.2.1 Environmental sources

Prolonged exposure to high temperatures and heat radiation can induce scrotal hyperthermia, promoting the generation of ROS. Studies have shown that high summer temperatures are associated with decreased sperm concentration and count, while variations in sunlight duration and humidity can also affect sperm quality (113). Research conducted in Argentina found that changes in sunlight duration and humidity are linked to reductions in sperm concentration, count, motility, and membrane integrity (113). The underlying mechanism involves the upregulation of Caspase 3, which induces apoptosis in Leydig and Sertoli cells of the human testis due to excessive ROS generated by heat stress (114, 115) (Figure 2c).

Chemical toxicants such as phthalates, originating from microplastic pollution, can lead to an overproduction of ROS in human spermatozoa and testicular germ cells (116, 117). This condition is characterized by a reduction in testicular antioxidants and hormone levels, causing mitochondrial dysfunction and decreased sperm motility as a result of oxidative stress (116, 117). Similarly, heavy metal ions like cadmium, copper, iron, and lead can reduce sperm motility and affect other sperm parameters. These effects are attributed to mitochondrial DNA damage caused by excessive ROS (118–120). A study involving coke oven workers identified a dose-response relationship between exposure to metal mixtures and diminished sperm quality (121). Furthermore, air pollution can also impact sperm motility by compromising the integrity of the spermatic plasma membrane through excessive ROS production. Research conducted in southern China has revealed a significant association between exposure to air pollutants like CO, NO₂, O₃, PM₁₀, and PM_{2.5} and reductions in sperm count and motility, particularly during critical periods of sperm development (122) (Figure 2d).

Occupational exposure and pesticides pose a global concern regarding male reproductive health, particularly in industrialized

nations (12, 123). Research has indicated that exposure to environmental toxicants such as cadmium, mercury and bisphenol A (BPA) can lead to male infertility, a condition associated with oxidative stress (123). These toxicants instigate oxidative stress, thereby disrupting the normal function of reproductive cells and consequently affecting the quality and motility of sperm (124). For instance, cadmium and mercury can interfere with the intracellular antioxidant defense systems, leading to an overproduction of ROS, which in turn damage sperm DNA and membrane lipids (125). BPA and pesticides may mimic or disrupt endocrine functions, thereby affecting the balance of reproductive hormones and subsequently influencing spermatogenesis (126, 127). Therefore, reducing occupational exposure and the use of pesticides is crucial for the protection of male reproductive health.

4.2.2 Lifestyle factors

Lifestyle factors and occupational exposures are considered significant influences on sperm quality. Studies indicate that obesity and irregular sleep patterns are associated with declines in sperm quality (128). In a study involving 1,060 participants, these lifestyle factors were significantly correlated with lower sperm quality (128). Research indicates that obesity leads to an increased accumulation of body fat, thereby triggering oxidative stress. This condition has a negative impact on sperm quality, particularly contributing to the occurrence of AZS (129). The oxidative stress induced by obesity not only affects the quality of sperm but may also exacerbate reproductive dysfunction by influencing the function of the reproductive axis (130). For instance, the disruption of tightly regulated metabolic pathways can lead to adverse reproductive outcomes, such as an inefficient energy supply to germ cells, defects in sperm motility, or arrest of spermatogenesis (129). Moreover, testicular metabolic alterations induced by obesity may also result in mitochondrial dysfunction, which is closely associated with the overproduction of ROS and oxidative stress readily targeting spermatozoa DNA and lipids, thereby contributing to a decrease in sperm quality (129).

Cigarette smoking is a major etiological factor in idiopathic asthenozoospermia. A meta-analysis of 20 studies conducted by Sharma et al. highlighted that cigarette smoking has an overall negative effect on sperm motility and other semen parameters (131). The accumulation of excessive ROS due to hazardous chemicals, carcinogens, and mutagenic substances in tobacco can damage mitochondrial DNA, inducing oxidative stress. Substances such as nicotine in cigarettes ultimately impair sperm motility by causing oxidative damage to the integrity of plasma membranes, altering protein and enzyme conformations and activation, and compromising the mitochondrial DNA sequence integrity. Creatine kinase (CK), a protein serving as a cellular energy reserve for fast ATP buffering and rebuilding in human spermatozoa, exhibits decreased activity in smokers. Elevated levels of reactive oxygen species (ROS) lead to additional oxidative damage to mitochondrial DNA, reducing ATP production and available energy, which, coupled with reduced CK activity, results in a rapid decline in sperm motility. Furthermore, the decline in sperm motility due to smoking is associated with protein

phosphorylation, inhibition of histone-to-protamine transition, and disruptions in the expression of microRNA (miRNAs). Additionally, second-hand smoke also damages sperm motility through mitochondrial DNA damage and methylation, caused by excessive ROS (Figure 2e) (99, 100).

Sperm motility, concentration and morphology are deleteriously affected by excessive alcohol consumption as a result of spermatogenic chromatin abnormalities through apoptosis, oxidative stress for elevated ROS production and mitochondrial DNA damage (132). Ethanol from alcohol consumption leads to mitochondrial dysfunction and decreased ATP generation in hepatic metabolic processes (110). Cytochrome P450 enzymes (CYP2E), as a kind of catalyst for NADPH oxidase in oxidative stress, promote the concentrations of Cu^{2+} and Fe^{3+} under alcohol intake, which, through various pathways, enhance the generation of ROS (133). The generation of nitric oxide (NO) from inducible nitric oxide synthase (iNOS), which is secreted by macrophages, and its metabolite peroxynitrite will induce mitochondrial dysfunction under the stimulus of excessive ROS (Figure 2f) (134).

The biological impact of radiation on sperm motility is influenced by the type of radiation, as well as the dose and duration of exposure (135). In recent years, electronic devices such as mobile phones, computers, and microwave ovens have significantly increased exposure to ionizing radiation (136). It has been demonstrated that mobile phone radiation negatively affects the count, morphology, and motility of spermatogenic cells and spermatozoa (137). This radiation can damage the integrity of the plasma membrane and activate NADPH oxidase, leading to oxidative stress driven by elevated ROS and lipid peroxidation (LPO) (138). Electromagnetic radiation emitted by computers and mobile phones exerts adverse effects on sperm motility, capacitation, and acrosome reaction through oxidative stress induced by radiofrequency. This stress results from damage to mitochondrial DNA and disruptions in the electron transport chain within the mitochondrial respiratory complex (20). Additionally, sperm motility affected by oxidative stress is exacerbated by radiofrequency radiation due to decreased glutathione levels and compromised plasma membrane integrity (136). Furthermore, exposure to microwave radiation for two hours daily over 35 days has been shown to induce oxidative stress in human spermatozoa (Figure 2c) (139).

5 Therapeutic strategy of idiopathic asthenozoospermia

Currently, there is no radical treatment for idiopathic asthenozoospermia that can fundamentally preserve sperm motility, primarily due to genetic alterations caused by ROS-mediated oxidative stress (140, 141). However, appropriate antioxidants and healthy lifestyle choices can help protect sperm motility (142). As previously mentioned, unhealthy lifestyle habits and endogenous sources such as immature spermatozoa and leukocytes contribute to excessive ROS in idiopathic asthenozoospermia (143–145).

Antioxidants mainly function by suppressing ROS levels, inactivating ROS generated by metabolic processes and enzymatic reactions, thereby preventing lipid peroxidative damage to the plasma membrane of human spermatozoa (36, 37). Most antioxidants primarily have positive effects on reducing ROS levels (146), while a few can also repair oxidative stress damage in human spermatozoa caused by excessive ROS. Agarwal et al. found that approximately 85.6% of urologists and andrologists prescribed oral antioxidants to patients with abnormal semen parameters (147), demonstrating therapeutic effects on idiopathic asthenozoospermia (148, 149). Besides improving sperm motility, antioxidants may also upregulate the expression of fertility-associated sperm proteins in patients with idiopathic asthenozoospermia (150). Antioxidants protecting sperm motility include vitamins E and C, glutathione, hypotaurine, albumin, taurine, as well as superoxide dismutase (SOD) and catalase, while those elevating sperm motility are CoQ10 and N-acetyl cysteine (Figure 3) (12).

5.1 Vitamin C

Vitamin C, as an antioxidant, plays a critical role in alleviating oxidative stress, a recognized factor contributing to male infertility. It safeguards sperm from oxidative damage, thereby enhancing sperm quality and motility. Combination therapies that include Vitamin C have demonstrated promising results in improving sperm motility. For example, one study reported that while individual parameters such as sperm concentration and motility did not show significant changes, a regimen incorporating multiple antioxidants, including Vitamin C, significantly increased the total number of motile sperm (151). Another study emphasized the role of Vitamin C as an adjunct therapy following varicocele surgery, where it significantly improved sperm motility and morphology, highlighting its potential to enhance sperm quality post-surgery (152). Vitamin C effectively mitigates the adverse effects of environmental stressors, such as cigarette smoke and tetrahydrocannabinol exposure. It improved the motility and morphology of sperm exposed to cigarette smoke, underscoring its protective antioxidant properties (153). *In vitro* studies further demonstrated that Vitamin C could alleviate reductions in sperm motility and kinematics caused by tetrahydrocannabinol, further supporting its role in protecting sperm from various stressors (Figure 3) (153).

5.2 Vitamin E

Vitamin E serves as an oxygen radical scavenger, protecting sperm motility from reactive oxygen species (ROS)-mediated oxidative stress. It prevents the propagation of ROS, thus ensuring the integrity of the membrane and plasma lipoproteins of human spermatozoa (154). The level of malondialdehyde (MDA), a biomarker of lipid peroxidation (LPO) in oxidative stress, can be reduced by Vitamin E, thereby improving sperm motility (155). Additionally, Vitamin E can prevent DNA damage and fragmentation in human spermatozoa and their mitochondria

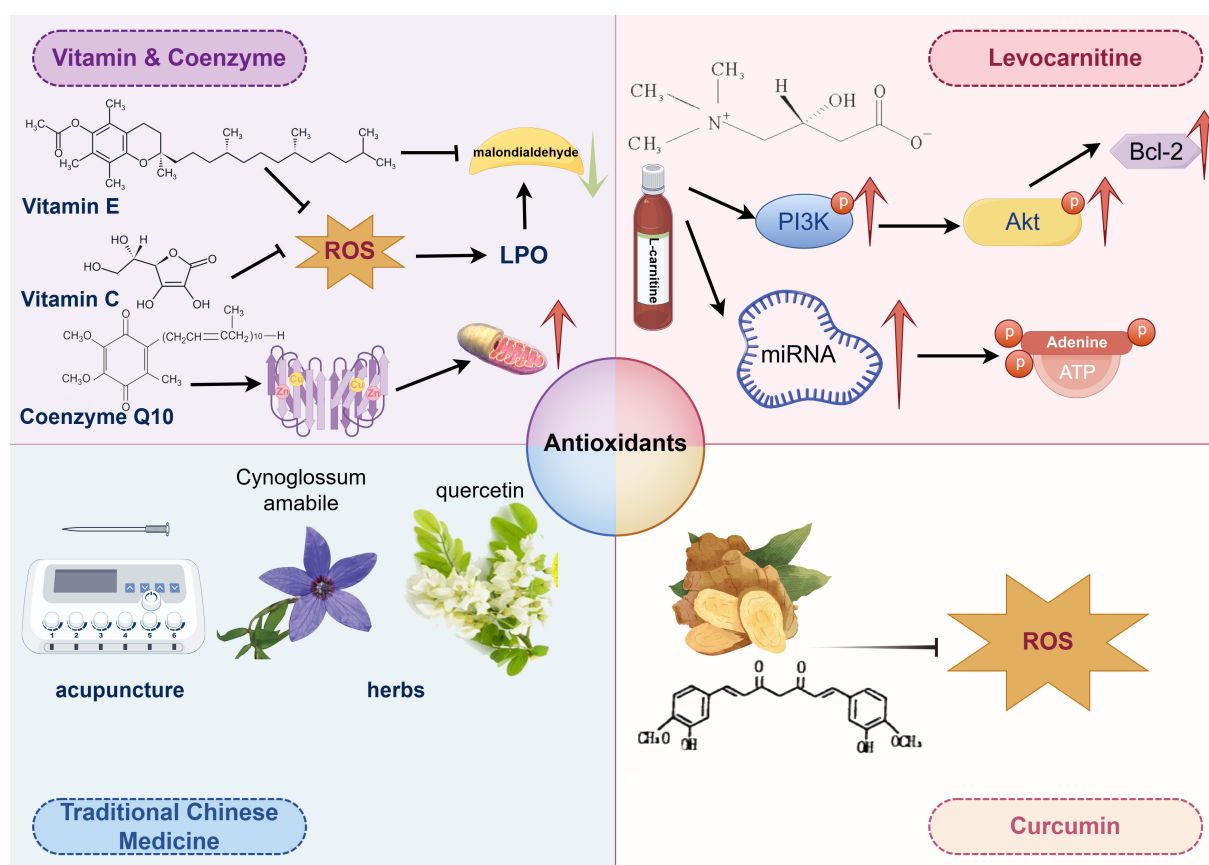


FIGURE 3

The therapeutic strategy of idiopathic asthenozoospermia.

caused by ROS (156, 157). Therefore, Vitamin E is potentially an effective treatment strategy for idiopathic asthenozoospermia, warranting a level B recommendation (Figure 3).

5.3 Coenzyme Q10

Coenzyme Q10 (CoQ10) primarily participates in the electron transport of oxidative phosphorylation during the respiratory process (158). It receives electrons from complex I and complex II, transferring them to complex III, to generate sufficient ATP necessary for maintaining sperm motility. Additionally, CoQ10 plays a role in transferring protons from fatty acids to the matrix (159). CoQ10 may positively influence nutrient uptake through the outer mitochondrial membrane, supporting the mitochondrial function of human spermatozoa (Figure 3) (160, 161).

5.4 Levocarnitine

Levocarnitine is a naturally occurring compound that has been demonstrated to enhance sperm motility, rendering it a promising candidate for the treatment of asthenozoospermia. The enhancement in sperm motility is attributed to several mechanisms, notably its role in

energy metabolism and its influence on various molecular pathways. Specifically, levocarnitine upregulates the expression of PI3K, p-Akt, and BCL-2 proteins, thereby decreasing sperm cell apoptosis and improving both sperm count and motility (162). Additionally, levocarnitine modulates the expression of specific miRNAs, such as Hsa-mir-27b-3p and hsa-MIR-206, which are integral to energy metabolism pathways like ATP synthase activity and cAMP signaling. These pathways are crucial for sperm motility, providing a molecular foundation for the effectiveness of levocarnitine in the treatment of asthenozoospermia (163). In a randomized controlled trial, levocarnitine significantly enhanced sperm motility, morphology, and concentration when compared to coenzyme Q10 and vitamin E (164). It also increased testosterone and luteinizing hormone levels, suggesting a more extensive hormonal impact. A meta-analysis corroborated that levocarnitine and its derivatives substantially improve sperm motility and morphology relative to placebo, albeit without significant effects on serum hormone levels (165). Although levocarnitine demonstrates significant potential in treating asthenozoospermia, its precise molecular mechanisms remain partially elucidated, necessitating additional research to fully explore its capabilities and long-term effects. Moreover, while levocarnitine is efficacious, its combination with other therapeutic modalities may enhance its benefits, indicating that a multifaceted approach could be more beneficial for patients (Figure 3).

5.5 Curcumin

Curcumin, a natural compound derived from *Curcuma longa* (turmeric), exhibits numerous biological effects, including anti-inflammatory, antioxidant, anti-proliferative, and anti-metastatic activities. It is also recognized as a scavenger of reactive oxygen species (ROS) in both *in vitro* and *in vivo* settings (166, 167). Adequate levels of curcumin can enhance sperm motility by binding to promoters of antioxidant genes, thereby promoting the release of antioxidative enzymes and upregulating the expression of these genes to suppress ROS generation (158). It is also believed to aid in the cryopreservation of spermatozoa. However, excessive curcumin has been reported to mediate oxidative stress in the testes of rats. Notably, it is renowned as a potent non-steroidal contraceptive due to its ability to block sperm motility within the female reproductive tract (168). Therefore, the dosage of curcumin is crucial for regulating sperm motility (Figure 3).

5.6 Traditional Chinese medicine

The clinical application of Traditional Chinese Medicine in enhancing sperm motility involves a multifaceted approach combining herbal medicine, acupuncture, and integrative therapies. These methods have shown promising results in improving sperm motility and overall semen quality, providing a complementary treatment option for male infertility. One study indicated that acupuncture at the Fuxi point combined with tamoxifen citrate tablets significantly improved sperm motility parameters in patients with asthenozoospermia. This combination therapy enhanced sperm motility, average path velocity, and the percentage of motile sperm, outperforming tamoxifen alone (169). *Cynoglossum amabile*, a traditional Chinese herb, contains bioactive compounds with various pharmacological activities, including anti-inflammatory and cardiovascular effects. Although there is insufficient evidence for its direct application to sperm motility, its traditional use in treating reproductive issues suggests potential benefits (Figure 3) (170).

The multi-target approach of Traditional Chinese Medicine, involving compounds like kaempferol and quercetin, has been shown to regulate hormones, reduce oxidative stress, and improve sperm quality. These components are integral to the effectiveness of Traditional Chinese Medicine in treating male infertility (171). An integrated approach combining data mining, network pharmacology, and experimental validation has identified key components and mechanisms of Traditional Chinese Medicine prescriptions that enhance sperm motility. This approach underscores the multi-component, multi-target strategy of Traditional Chinese Medicine in treating male infertility (171).

While Traditional Chinese Medicine offers promising avenues for improving sperm motility, potential risks must be considered, such as hepatotoxicity associated with certain herbs like *Cynoglossum amabile*. Further research and clinical trials are needed to validate these therapies and ensure their safety and efficacy in broader applications (170).

6 The limitations and future prospects

Despite significant research into the relationship between ROS and sperm motility, several limitations have also persisted. For instance, accurately measuring ROS levels in semen and effectively assessing the efficacy of antioxidant treatments require further investigation (16). The short half-life of ROS poses challenges for their direct detection in human specimens (8). In addition, despite the availability of various antioxidant therapies for treating idiopathic asthenozoospermia, there is still no more effective clinical strategy to develop sperm motility. Furthermore, individual variability and the complex mechanisms of ROS and oxidative stress add to the challenges of research in this area (16). Consequently, future studies should delve deeper into the specific mechanisms by which oxidative stress affects sperm motility. The development of more effective diagnostic and therapeutic strategies is crucial to enhancing treatment outcomes for male infertility.

In summary, ROS plays a dual role in maintaining sperm motility. A moderate amount of ROS is essential for normal sperm function as they participate in energy acquisition, motility, and the capacitation process. However, excessive ROS can lead to oxidative stress, damaging the lipid bilayer structure of sperm membranes, impairing mitochondrial function, and affecting DNA integrity, which significantly reduces sperm motility and fertilization capacity. Therefore, maintaining an appropriate balance of ROS is crucial for ensuring male reproductive health. Further research should focus on exploring the potential benefits of antioxidant supplementation and its application in improving sperm quality and enhancing the effectiveness of male infertility treatments.

Author contributions

ZW: Conceptualization, Data curation, Formal Analysis, Writing – original draft. DL: Data curation, Formal Analysis, Investigation, Writing – original draft. GZ: Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft. ZX: Data curation, Methodology, Project administration, Resources, Writing – original draft. XW: Data curation, Methodology, Writing – original draft. ST: Formal Analysis, Writing – original draft. ZL: Formal Analysis, Investigation, Writing – original draft. XL: Supervision, Validation, Visualization, Writing – review & editing. CS: Funding acquisition, Software, Supervision, Validation, Visualization, Writing – review & editing. SY: Software, Supervision, Validation, Visualization, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This research was funded by the Hospital Research Fund of SAHSYSU (ZSQYLCKYJJ202317), Research Start-up Fund of Post-doctoral of Shenzhen Municipality and Lateral Research Project of Sun Yat-sen University (ZSQY-XQ-2309-0884).

Acknowledgments

The figures were created using Figdraw 2.0 on Home for Researchers. We thank the investigators whose studies have not yet been cited.

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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