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Analyzing male reproductive risk, understanding molecular targets, and developing treatments

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Xiang Xiao, Cibele S. Borges, Huitao Li and
C. Yan Cheng

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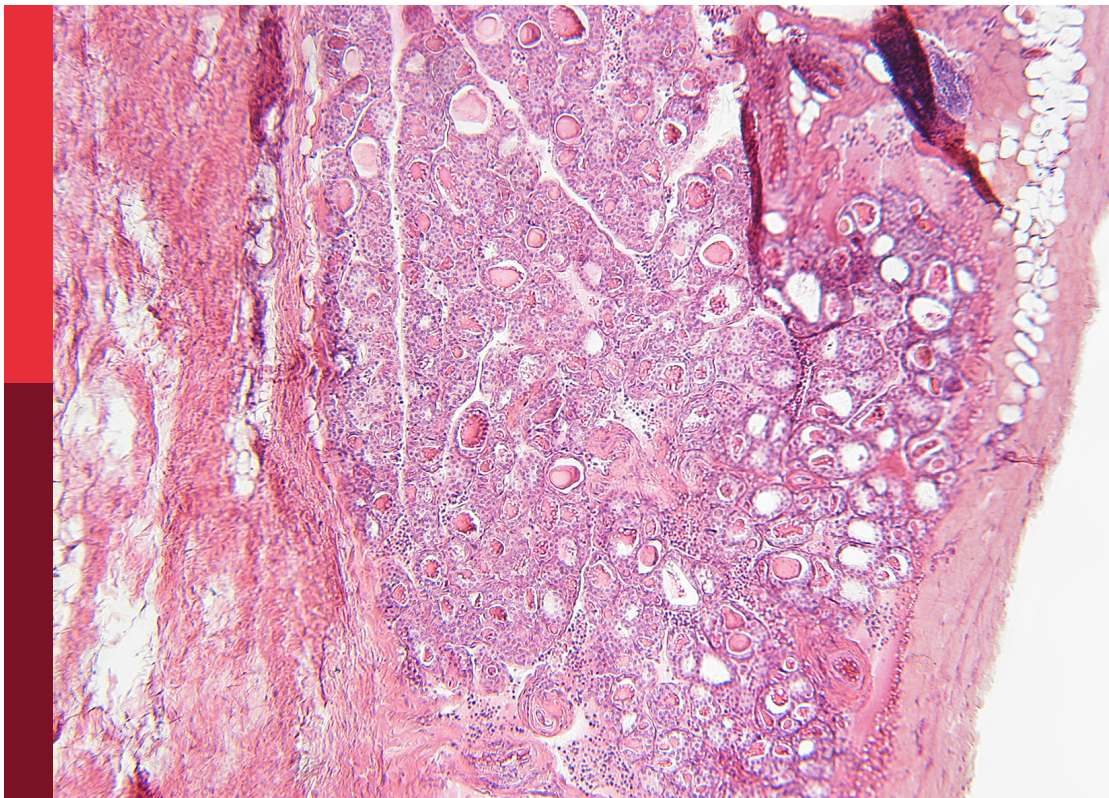

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Analyzing male reproductive risk, understanding molecular targets, and developing treatments

Topic editors

Xiang Xiao — Hangzhou Medical College, China

Cibele S. Borges — Federal University Rural Semi-Arid, Brazil

Huitao Li — Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, China

C. Yan Cheng — St. Francis College, United States

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EDITED AND REVIEWED BY
Richard Ivell,
University of Nottingham, United Kingdom

*CORRESPONDENCE
Xiang Xiao
xxiao@zjams.cn

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Editorial: Analyzing male reproductive risk, understanding molecular targets, and developing treatments

Xiang Xiao[1*], Huitao Li[2,3], Cibele S. Borges[4] and C. Yan Cheng[5]

[1]Center for Reproductive Health, School of Pharmaceutical Sciences, Hangzhou Medical College (Zhejiang Academy of Medical Sciences), Hangzhou, China, [2]Zhejiang Engineering Research Center for Innovation and Application of Intelligent Radiotherapy Technology, and Department of Anesthesiology and Perioperative Medicine, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, China, [3]Key Laboratory of Environment and Male Reproductive Medicine of Wenzhou, Wenzhou, China, [4]Laboratory of Tissue Biology and Developmental Toxicology, Department of Bioscience - CCBS, UFERSA - Federal Rural University of Semi-Arid Region, Mossoró, Brazil, [5]Department of Urology and Andrology, Sir Run-Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China

KEYWORDS

male fertility, sperm, testis, semen quality, endocrine disruption

Editorial on the Research Topic
Analyzing male reproductive risk, understanding molecular targets, and developing treatments

1 Introduction

Male fertility is profoundly impacted by environmental factors from lifestyle, diseases, and toxicants. However, the specific molecular mechanisms of these external influences on male reproductive health remain unclear. This Research Topic aimed to gain insights into how elements like toxicants, diseases, medications, and nutrition affect male fertility at the cellular and molecular levels. Better characterizing pathways linking diverse external stimuli to outcomes could assist in improving diagnostic and therapeutic approaches for infertility. Identifying shared regulatory mechanisms across conditions may highlight targets for non-invasive strategies or restoring function. Ultimately, a deeper understanding of resilience and vulnerability could accelerate enhancing diagnosis and treatment impacted by environment and health. This editorial provides an overview of key contributions advancing the understanding of male fertility regulation against stressors.

2 Spermatogenesis processes and regulation

Several studies featured in this Research Topic provided important new understandings of the dynamic cellular processes governing normal spermatogenesis and their disruption. Xiao et al. revealed a novel role for the circulating signaling molecule sICAM-1 in regulating the integrity of the blood-testis barrier and adhesion between Sertoli cells and germ cells through inhibition of SRC family kinase signaling pathways in Sertoli cells. By

downregulating SRC activity, sICAM-1 was shown to facilitate the essential transport of germ cells through the seminiferous epithelium required for sperm production.

Li et al. reported the intriguing finding of positive trends over time in specific sperm motility parameters like velocity, even as more conventional measures of semen quality like count declined in their analysis of over 49,189 semen samples. Sperm motility is a highly heritable trait that could reflect adaptive responses to environmental pressures. This study points to the possibility of compensatory mechanisms offsetting certain impairments and warrants further exploration of factors modulating this critical fertility trait. Together, these articles provided novel mechanistic insights into the dynamic cellular adhesion events and regulatory pathways that govern normal human spermatogenesis.

3 Disease states and lifestyle impacts

Several studies investigated the effects of specific disease conditions and short-term lifestyle influences on male reproductive health at the molecular level. Osadchuk et al. provided a comprehensive analysis linking cigarette smoking to multi-level impairments in semen quality indicators and general male health through disrupted zinc homeostasis, oxidative damage, metabolic dysregulation, and inflammation within a large Russian population. This work demonstrated ethnicity-dependent sensitivities to smoking toxicity.

Liu et al. associated fluctuations in Leydig cell lipid metabolism and related hormone levels with the ability to retrieve focal sperm samples from patients of different ages with Klinefelter syndrome, indicating potential therapeutic targets related to age. Sun et al. identified progressive transcriptional changes driving impaired spermatogenesis in undescended testes from cryptorchidism patients through RNA sequencing and bioinformatics analyses.

Additionally, Falvo et al. demonstrated how even a short five-week high-fat diet period impaired rat testicular functions through disruptions to mitochondria, antioxidant defenses, barrier integrity, and signaling cascade. These studies provided novel disease- and lifestyle-specific insights into pathological influencers of male reproductive health.

4 Connections to broader health

Several contributions examined relationships between male fertility and broader indicators of physiological wellness. Huang et al. analyzed data from over 3,625 American males, finding that visceral adiposity assessed through a novel metric strongly predicted the prevalence of erectile dysfunction. This highlights the clinical utility of analyzing body fat distribution patterns for sexual health risk evaluation.

Additionally, Simón et al. reviewed evidence that various bioactive compounds commonly found in plants may help mitigate potential infertility risks associated with cancer therapies by protecting against oxidative stress, inflammation, and other damaging effects in testicular cells through diverse protective mechanisms revealed in animal and cellular research. Evaluating natural agents as alternative or adjuvant options represents an impactful area for future therapeutic development. Together, these studies delineated links between specific health parameters and male reproductive function.

5 Toxicant exposure propagation

Lee et al. provided a clear example of how environmental toxicity can propagate impairment from one organ system to negatively impact male fertility through the disruption of metabolic crosstalk. Their study linked exposure to the ubiquitous contaminant perfluorooctane sulfonate (PFOS) to perturbed hepatic lipid metabolism and related gene expression in mice. This was shown to subsequently influence testicular structure and function through increases in fatty acid metabolites and perturbation of testicular lipid pathways.

The findings showed that PFOS exposure impairs male reproductive health by disrupting the normal balance of fatty acid metabolism between the liver and testes. This work exemplified how elucidating causative toxicological pathways can reveal promising molecular targets for monitoring or mitigating contamination-related health effects, here pointing to lipid regulatory networks. It highlighted the utility of investigating connectivity between organ toxicodynamics to fully characterize toxicity mechanisms.

6 Significant advances and conclusions

Collectively, the diverse studies on this topic significantly advanced the understanding of male fertility as resilient yet vulnerable to various stressors. Common mechanisms and molecular signatures were identified across studies, providing opportunities for future research. Novel insights were provided into dynamic processes like cellular junctions, motility, and transcriptional profiles, as well as tissue crosstalk and lifestyle influences on fertility regulation.

Outstanding questions warranting further exploration were also highlighted, including compensatory responses, ethnicity influences, and optimizing targeted therapies. Achieving deeper insights into interconnected regulatory pathways influencing fertility across conditions will guide development of improved diagnostics, personalized infertility treatment strategies, and prevention methods.

Continued multidisciplinary efforts to map the systemic complexities of fertility regulation and identify intervention points at multiple scales will further the overarching goal of enhancing global reproductive health. This Research Topic meaningfully advances that aim through advancing knowledge of spermatogenesis, disease pathogenesis, toxic impacts, and links between overall health and fertility outcomes. Overall, the work

here collectively pushes boundaries in understanding male reproductive resilience and vulnerabilities.

Author contributions

XX: Conceptualization, Writing – original draft, Writing – review & editing. HL: Writing – review & editing. CB: Writing – review & editing. CC: Writing – review & editing.

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Conflict of interest

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

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EDITED BY
Cibele Borges,
Federal University Rural Semi-Arid, Brazil

REVIEWED BY
Lucia Rocco,
University of Campania Luigi Vanvitelli, Italy
Leonardo Nascimento,
University of Ribeirão Preto, Brazil

*CORRESPONDENCE
Yuchuan Zhou
zhouych@sibcb.ac.cn
Chunhua Tang
tangchunhua@sibcb.ac.cn

†These authors have contributed equally to this work

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Trends in sperm quality by computer-assisted sperm analysis of 49,189 men during 2015–2021 in a fertility center from China

Yanquan Li[1,2†], Tingting Lu[1,2†], Zhengmu Wu[1], Zhengquan Wang[1], Ting Yu[1,2], Hanshu Wang[1,2], Chunhua Tang[1,2*] and Yuchuan Zhou[1,2*]

[1]International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China, [2]Shanghai Key Laboratory of Embryo Original Diseases, Shanghai, China

Background: Sperm quality, including semen volume, sperm count, concentration, and total and progressive motility (collectively, "semen parameters"), has declined in the recent decades. Computer-assisted sperm analysis (CASA) provides sperm kinematic parameters, and the temporal trends of which remain unclear. Our objective is to examine the temporal trend of both semen parameters and kinematic parameters in Shanghai, China, in the recent years.

Methods: This retrospective study analyzed semen parameters and kinematic parameters of 49,819 men attending our reproductive center by using CASA during 2015–2021. The total sample was divided into two groups: samples that surpassed the WHO guideline (2010) low reference limits ("above reference limit" group, ARL; n = 24,575) and samples that did not ("below reference limit" group, BRL; n = 24,614). One-way analysis of variance, Kruskal–Wallis test, independent samples *t*-test, and covariance analysis were used to assess the differences among groups. Year, age, and abstinence time were included in the multiple linear regression model of the ARL group to adjust the confounders and depict the trends in sperm quality.

Results: Among all the total sample and the ARL and BRL groups, the age of subjects increased in recent years. Semen volume and sperm count showed declined tendency with years in the total sample, the ARL and BRL groups, and the subgroup of age or abstinence time, whereas sperm velocities showed increased tendency with years on the contrary. The multiple linear regression model of the ARL group, adjusting for age and abstinence time, confirmed these trends. Semen volume (β1= −0.162; CI: −0.172, −0.152), sperm count (β1= −9.97; CI: −10.813, −9.128), sperm concentration (β1 = −0.535; CI: −0.772, −0.299), motility (β1 = −1.751; CI: −1.830, −1.672), and progressive motility (β1 = −1.12; CI: −0.201, −0.145) decreased with year, whereas curvilinear line velocity (VCL) (β1 = 3.058; CI: 2.912, 3.203), straight line velocity (VSL) (β1 = 2.075; CI: 1.990, 2.161), and average path velocity (VAP) (β1 = 2.305; CI: 2.224, 2.386) increased over time (all $p < 0.001$). In addition, VCL, VSL, and VAP significantly declined with age and abstinence time.

Conclusion: The semen parameters declined, whereas the kinematic parameters increased over the recent years. We propose that, although sperm count and motility declined over time, sperm motion velocity increased, suggesting a possible compensatory mechanism of male fertility.

KEYWORDS

sperm quality, temporal trends, computer-assisted sperm analysis, kinematic parameters, multiple linear regression model

Introduction

Semen quality has been drawing increasing concerns in the recent decades as the fertility rate has declined and infertility problems have become cumulatively serious (1, 2). In the late 20th century, sperm concentration and semen volume received the most attention after a systematic review reporting that the two parameters had declined by about 50% over the 50 years from 1930 to 1991 (3). Since then, reports from various regions demonstrated the sperm quality–deteriorated trend including decreased sperm count, concentration, normal morphology rate, and total and progressive motility rates (4–13). In China, scholars held a similar view, as reported in studies from Hunan (14), Shandong (15), Henan (16), and other regions (17, 18). Recently, a systematic review and meta-regression analysis revealed that sperm concentration and total sperm count declined worldwide between 1973 and 2018, and the decline in the 21st century was more rapid than that in the last century (19). However, the decline remains controversial as some studies reported no significant change or even an increase in these parameters (20–23). Evidently, male fertility decline is destined to be a crucial long-term issue.

Computer-assisted sperm analysis (CASA) is a notable technological advancement that has gradually replaced manual sperm assessment in reproductive centers worldwide. The advantages of CASA lie in its rapid and automatic semen analysis, providing sample statistics and sperm kinematic parameters including curvilinear line velocity (VCL, μm/s), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), straightness (STR, %), linearity (LIN, %), the amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), and wobble (WOB, %) (24). These parameters were difficult to acquire by manual analysis. Only a few studies have reported on the clinical implications or trends alterations associated with these parameters (25–28) especially temporal trend of kinematic parameters. The conclusions drawn from CASA parameters or the significance of these parameters need to be evaluated by more investigations and researches.

Sperm count and motility rate as well as kinematic status are pivotal for fertilization. Activated and hyperactivated sperm display specific movement patterns, characterized by high VSL or high VCL and ALH, aimed to propel sperm migration from the cervix to the oviduct and to enhance cumulus cell layers and zona pellucida penetration during fertilization (29–33). VCL and STR are important references for choosing the therapeutic regimen in assisted reproductive technology. *In vitro* fertilization (IVF) should be considered instead of intracytoplasmic sperm injection if VCL > 65 μm/s and STR > 40 μm/s (34, 35). VSL, VCL, and VAP are valuable in predicting the fertilization potential of spermatozoa in IVF (36–38), whereas STR and BCF can help predict sperm DNA damage (39). The clinical significance of sperm kinematic parameters for fertilization remains open.

This study explored changes in sperm count, motility, and kinematic parameters in the recent years, analyzing these parameters by year, age, and abstinence time. A comprehensive sperm quality evaluation could provide new ideas on elucidating unexplained male infertility and suggest reference values for the kinematic parameters.

Materials and methods

Study population and semen samples

We retrieved data on 83,708 samples assessed at the reproductive center of the International Peace Maternity and Child Health Hospital, Shanghai, China, between January 2015 and July 2021. Subsequently, the samples were screened by the following participants and sample characteristics (1): age, 18–60 (2); abstinence time, 2–7 days; and (3) properly formatted and complete information. Only the first sample report was used when more than one sample from the same individual was available. Finally, 49,189 samples were retained. Although the justifications for sperm detection were not specifically analyzed, most analyses were performed in conjunction with pre-pregnancy clinic and infertility clinic including infertility workup of the patient and his partner, as well as oligo-atheno-spermia and sperm freezing for Assisted Reproductive Technology (ART) cycles. Nearly half of the latter category would have subfertility in the male partner. Because of this, the study population would have been biased toward subfertility and not a random cross section of men of reproductive age.

Ethical approval

This study was under the approval of the Ethics Committee on human subjects of International Peace Maternity and Child Health Hospital (GKLW2018-03).

Semen analysis

The semen samples were collected by masturbation and liquefied at 37°C for at least 30 min. Two well-trained technicians performed all diagnostic semen analyses, and the results were verified by an andrology-trained laboratory director. Sperm volume was evaluated by reading the values directly from the graduated container. Other semen and kinematics parameters were assessed by CASA (Hamilton-Thorne, Beverly, MA, USA). Each sample (5 µl) was loaded into a counting chamber (Leja Products B.V, Nieuw-Vennep, The Netherlands) and analyzed at once. Ten microscopic fields of each chamber were analyzed, evaluating at least 200 spermatozoa. The following parameters were recorded: total count (million), sperm concentration (million/ml), motility (%), progressive motility (%), VAP (µm/s), VSL (µm/s), VCL (µm/s), STR (%), LIN (%), ALH (µm), BCF (Hz), and WOB (%). Sperm were classified as motile when their path velocity exceeded 5 µm/s. Those sperm with path velocities >25 µm/s and linearity > 80% were classified as "progressively motile sperm."

The samples were classified as two groups: samples that surpassed the low reference limits ("above reference limit" group, ARL; n = 24,575) and samples that did not ("below reference limit" group, BRL; n = 24,614) according to the World Health Organization laboratory manual for the examination and processing of human semen (fifth edition) (sperm volume, 1.5 ml; sperm count, 39 million; sperm concentration, 15 million/ml; total motility, 40%; and progressive motility, 32%). The samples were also grouped by age (18–24, 25–29, 30–34, 35–39, 40–44, and 45–60 years) and abstinence time (2, 3, 4, 5, 6, and 7 days) to observe trends in these parameters.

Data analysis

IBM SPSS Statistics for Windows (version 22.0; IBM Corp., Armonk, NY, USA), GraphPad Prism Software (version 8.3; GraphPad Inc., San Diego, CA, USA), and R studio (v.4.1.1; Platform, 64 bit) were used for the statistical analysis and plotting. Semen parameters showed a positively skewed distribution, whereas kinematic parameters were nearly normally distributed. Nonnormally distributed data, semen volume, sperm count, motility, and progressive motility were presented as medians and interquartile range in the tables, and Kruskal–Wallis test was used in the variation analysis, whereas kinematic parameters were shown as means and standard deviation and one-way analysis of variance (ANOVA), independent samples *t*-test, and covariance analysis were used to assess the differences among groups. Means with confidence intervals (95% CIs) were used in line graphs to depict original trend of parameters. A multiple linear regression model was used to explore the changes in the ARL group parameters controlling for two of three factors: year, age, and abstinence time. The R packages "ggpredict" and "ggplot2" were used to visualize the generalized linear model outcomes. The statistical significance is represented as $p < 0.05$, $p < 0.01$, and $p < 0.001$ or non-significance with $p \geq 0.05$.

Results

Distribution of samples

The dataset includes 49,189 semen samples, divided into ARL (n = 24,575, 49.96%) and BRL (n = 24,614, 50.04%) groups, and the samples of each year were shown in Figure 1A. As shown in Figures 1B, C and Table 1, the age of the outpatients differed significantly among years in all groups (all $p < 0.001$) and showed an increase toward. Abstinence time had significant difference among years with fluctuation toward. The mean age of participants was 32.59 ± 4.50 years, and the mean abstinence time was 4.50 ± 1.70 days in the total sample.

Changes in semen parameters

Tables 2, 3 illustrated semen parameters' medians and interquartile range in the total sample and ARL group over the years. The means and 95% CIs of the parameters in the total sample and ARL and BRL groups were shown in Figures 2A–E. These results displayed that semen volume (Figure 2A) and sperm count (Figure 2B) declined over the years, whereas sperm concentration (Figure 2C), motility (Figure 2D), and progressive motility (Figure 2E) had no significant trends.

Changes in sperm kinematic parameters

Representative kinematic parameters in the total sample and the ARL group sample over the years were shown in Tables 4, 5,

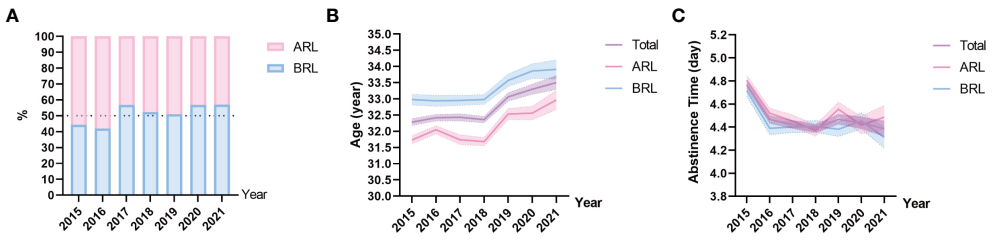

FIGURE 1
Sample distribution on type, age, and abstinence time. **(A)** Proportions of the above reference limit group (ARL) and the below reference limit group (BRL). **(B, C)** Line charts of age **(B)**, and abstinence time **(C)** based on means with 95% confidence intervals (CIs) over the years. The purple line represents the total sample, the pink line represents the ARL group, and the blue line represents the BRL group.

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TABLE 1 Age and abstinence time distribution in the total sample and the ARL and BRL groups over the years.

Year	n	Total sample		n	ARL group		n	BRL group	
		Age (year)	Abstinence time (day)		Age (year)	Abstinence time (day)		Age (year)	Abstinence Time (day)
2015	9,410	32.28 ± 5.09	4.77 ± 1.64	5,240	31.73 ± 4.65	4.81 ± 1.59	4,170	32.98 ± 5.52	4.72 ± 1.69
2016	9,433	32.42 ± 5.11	4.47 ± 1.71	5,467	32.05 ± 4.70	4.52 ± 1.68	3,966	32.94 ± 5.58	4.39 ± 1.75
2017	8,580	32.43 ± 5.20	4.42 ± 1.68	3,710	31.74 ± 4.68	4.45 ± 1.63	4,870	32.95 ± 5.51	4.40 ± 1.72
2018	9,142	32.36 ± 5.02	4.39 ± 1.74	4,361	31.68 ± 4.47	4.37 ± 1.68	4,781	32.98 ± 5.40	4.41 ± 1.79
2019	6,058	33.06 ± 5.15	4.47 ± 1.73	2,965	32.53 ± 4.66	4.56 ± 1.69	3,093	33.57 ± 5.54	4.38 ± 1.76
2020	4,217	33.30 ± 5.20	4.44 ± 1.71	1,822	32.56 ± 4.63	4.42 ± 1.66	2,395	33.86 ± 5.54	4.45 ± 1.74
2021	2,349	33.50 ± 5.25	4.39 ± 1.69	1,010	32.97 ± 4.79	4.49 ± 1.67	1,339	33.91 ± 5.53	4.31 ± 1.71
Total	49,189	32.59 ± 4.50	4.50 ± 1.70	24,575	32.00 ± 4.65	4.54 ± 1.66	24,614	33.18 ± 5.52	4.45 ± 1.74
p^a		< 0.001	< 0.001		< 0.001	< 0.001		< 0.001	< 0.001

Values are presented as mean ± standard deviation. ARL, above reference limit group; BRL, below reference limit group.
p^a, One-way analysis of variance (ANOVA) of differences among the 7 years (2015–2021).

respectively. The means and 95% CIs of the parameters in the total sample and the ARL and BRL groups were shown as line graphs in Figures 3A–D. The data showed that VCL (Figure 3A), VSL (Figure 3B), VAP (Figure 3C), and BCF (Figure 3D) had increased toward with time.

The kinematic parameters of the ARL group—VCL, VSL, VAP, ALH, LIN, and STR—were higher than those of the BRL group (all $p < 0.001$; Table 6), even after adjusting for age and abstinence time. These parameters could discriminate the sperm motility coincident with semen parameters. Whereas, BCF and WOB of the ARL group were lower than BRL group after the adjustment ($p < 0.001$).

Multiple linear regression model of ARL group

To exclude the potential effects of discrepant age, abstinence time, and possible diseases in participants of the BRL group on temporal trends in sperm quality, we analyzed the ARL group separately by dividing it into subgroups by age or abstinence time (Figure 4). The results showed that the individual age (Figures 4A, C, E, G) or abstinence time (Figures 4B, D, F, H) groups had similar trends over time, although age and abstinence time distributions differed among years. To exhibit the effect of age and abstinence time on sperm quality, line charts of semen and kinematic parameters in the different age and abstinence time groups in the total sample and the ARL and BRL groups were shown in Figures S1, S2.

We established multiple linear regression models to further explore the relationship between semen parameters with year, age, and abstinence time in the ARL group (Table 7). After adjusting for age and abstinence time, we found significant declines with year in the semen parameters, including semen volume (β1 = −0.162; CI: −0.172, −0.152; Figure 5A), sperm count (β1 = −9.97; CI: −10.813, −9.128; Figure 5B), sperm concentration (β1 = −0.535; CI: −0.772, −0.299; Figure 5C), total motility (β1 =

TABLE 2 Semen parameters in the total sample during 2015–2021.

Year	n	Semen volume (ml)	Total sperm count (× 10^6)	Sperm concentration (× 10^6/ml)	Total motility(%)	Progressive motility (%)
2015	9,410	3.0 (2.0–4.5)	116.96 (57.71–214.06)	38.65 (20.10–64.79)	53.40 (35.93–67.50)	41.96 (26.53–55.43)
2016	9,433	2.8 (2.0–4.0)	116.18 (58.11–206.02)	42.47 (22.66–71.37)	53.20 (37.10–66.10)	44.15 (29.19–56.58)
2017	8,580	2.5 (2.0–4.0)	110.10 (56.67–188.22)	42.21 (24.19–68.89)	41.90 (27.40–54.00)	35.06 (22.16–46.59)
2018	9,142	2.5 (2.0–4.0)	100.68 (51.59–174.02)	38.47 (21.72–63.03)	43.50 (30.10–54.80)	37.50 (25.00–48.13)
2019	6,058	2.5 (2.0–3.1)	87.90 (45.34–159.23)	36.55 (20.70–61.81)	46.10 (32.50–57.60)	39.22 (26.63–50.24)
2020	4,217	2.5 (2.0–3.0)	95.58 (48.67–161.49)	39.06 (21.82–63.32)	42.70 (28.40–54.60)	34.25 (21.97–46.12)
2021	2,349	2.0 (2.0–3.0)	83.75 (44.38–142.27)	37.78 (21.60–63.33)	44.00 (29.35–56.00)	35.68 (22.21–46.64)
p^a		*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*

Values are presented as median (interquartile range).
p^a, The Kruskal–Wallis test of the medians among the years.

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TABLE 3 Semen parameters in the ARL group during 2015–2021.

Year	n	Semen volume (ml)	Total sperm count ($\times 10^6$)	Sperm concentration ($\times 10^6$/ml)	Total motility (%)	Progressive motility (%)
2015	5,240	3.2 (2.5–5.0)	164.68 (101.89–258.52)	48.07 (31.33–73.80)	63.68 (53.89–73.00)	52.19 (42.93–61.16)
2016	5,467	3.0 (2.2–4.1)	157.86 (97.33–250.85)	50.86 (32.62–79.28)	61.70 (52.70–71.00)	52.45 (43.59–61.65)
2017	3,710	3.0 (2.0–4.0)	152.31 (98.15–230.90)	51.94 (34.62–76.54)	53.60 (47.00–61.40)	46.30 (40.03–53.62)
2018	4,361	3.0 (2.0–4.0)	134.51 (86.80–206.47)	47.11 (32.02–69.98)	53.40 (47.00–61.10)	46.57 (40.65–54.42)
2019	2,965	3.0 (2.0–3.5)	125.61 (81.08–193.60)	45.67 (29.79–70.14)	55.50 (48.10–63.20)	48.06 (41.08–55.66)
2020	1,822	3.0 (2.0–3.5)	132.43 (87.60–197.72)	48.97 (32.64–71.62)	54.65 (47.60–62.20)	46.23 (39.96–53.24)
2021	1,010	2.0 (2.0–3.0)	116.89 (78.12–170.56)	48.09 (32.14–72.53)	55.15 (48.30–63.30)	45.96 (40.07–53.95)
p^a		*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*

Values are presented as median (interquartile range).
p^a, The Kruskal–Wallis test of the medians among the years.

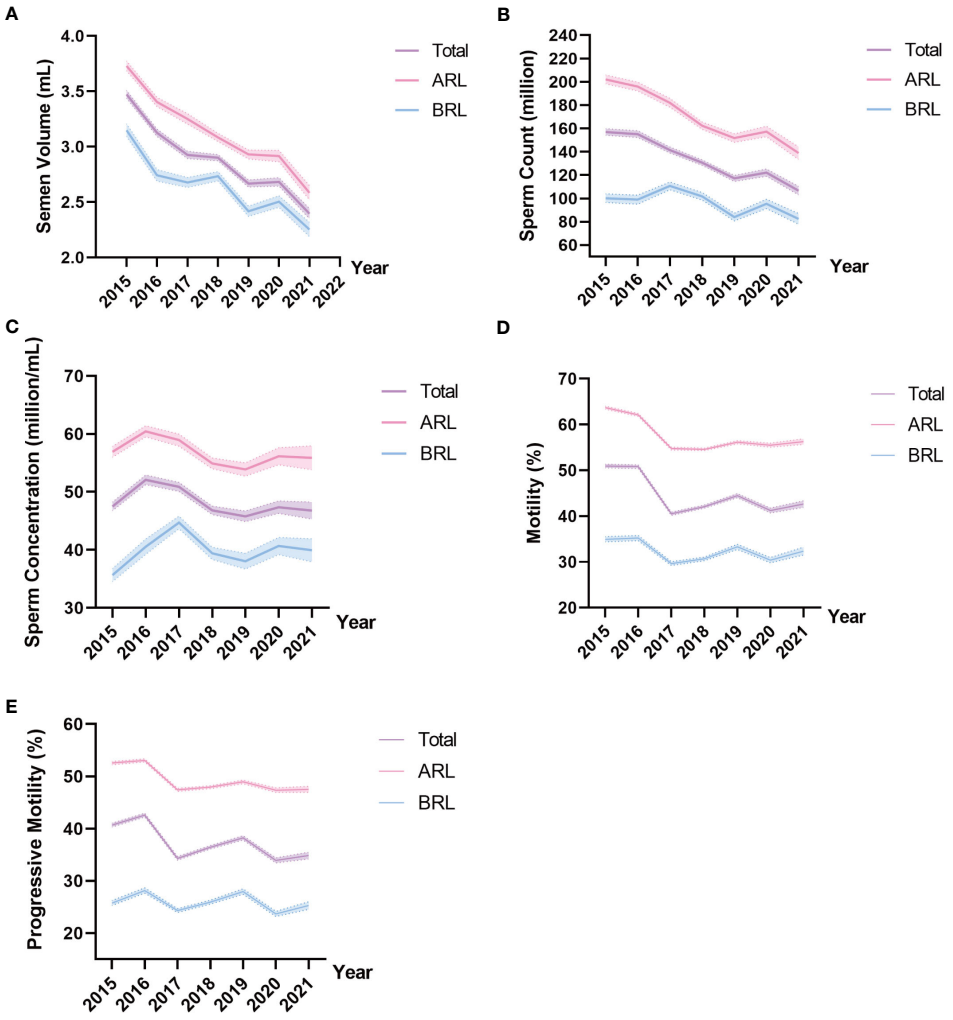

FIGURE 2
Line charts of semen parameters in the three type groups. Means and 95% CIs of semen volume **(A)**, sperm count **(B)**, sperm concentration **(C)**, motility **(D)**, and progressive motility **(E)** in the groups over the years. The purple line represents the total sample, the pink line represents the ARL group, and the blue line represents the BRL group.

TABLE 4 Sperm kinematic parameters of the total sample during 2015–2021.

Year	n	VCL	VSL	VAP	BCF
2015	9,410	86.05 ± 25.22	43.08 ± 12.76	53.45 ± 13.98	23.88 ± 5.60
2016	9,433	91.31 ± 23.77	44.19 ± 11.94	55.84 ± 12.81	23.45 ± 6.20
2017	8,580	90.09 ± 21.94	42.05 ± 11.62	54.52 ± 12.10	23.07 ± 6.09
2018	9,142	94.94 ± 21.09	40.94 ± 9.37	55.54 ± 10.22	22.45 ± 5.80
2019	6,058	99.88 ± 23.29	47.99 ± 13.98	60.99 ± 13.58	24.53 ± 5.39
2020	4,217	100.55 ± 27.08	56.30 ± 16.91	65.98 ± 17.40	27.40 ± 7.04
2021	2,349	105.93 ± 27.48	60.34 ± 15.05	69.85 ± 16.47	27.99 ± 5.30
p^a		*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*

Values are presented as mean ± standard deviation.
p^a, The one-way ANOVA of the kinematics parameters among the years.

−1.751; CI: −1.830, −1.672; Figure 5D), and progressive motility (β1 = −1.12; CI: −0.201, −0.145; Figure 5E). Conversely, we noted significant increases over time in VCL (β1 = 3.058; CI: 2.912, 3.203; Figure 5F), VSL (β1 = 2.075; CI: 1.990, 2.161; Figure 5G), VAP (β1 = 2.305; CI: 2.224, 2.386; Figure 5H), and BCF (β1 = 0.467; CI: 0.441, 0.492; Figure 5I; all $p < 0.001$). The estimates should be interpreted as semen volume, sperm count, sperm concentration, motility, and progressive motility decreased: 0.162 ml/year, 9.97 × 10^6/year, 0.535 × 10^6/ml/year, 1.751%/year, and 1.12%/year, respectively; whereas, VCL, VSL, VAP, and BCF increased: 3.058 μm/s, 2.075 μm/s, 2.305 μm/s, and 0.467 Hz per year, respectively.

In addition, most semen parameters declined with age (Table 7), including sperm count, semen volume, total motility, and progressive motility, as well as kinematic parameters VCL (Figure 6A), VSL (Figure 6C), and VAP (Figure 6E; all $p < 0.001$). Sperm concentration and BCF (Figure 6G, both $p < 0.001$) increased with age. Moreover, abstinence time was associated with increased semen volume, sperm count, sperm concentration, and BCF (Figure 6H; all $p < 0.001$). Conversely, total motility, progressive motility, VCL (Figure 6B), VSL (Figure 6D), and VAP (Figure 6F; all $p < 0.001$) declined with abstinence time.

Discussion

Male sperm quality decline over the years is a worldwide trend. This study found declining trends over the years in semen volume, sperm count, and motility, consistent with most previous studies (4–18). Unexpectedly, a further novel finding is the increasing trend with year in the kinematics parameters VCL, VSL, and VAP, even after correcting for age and abstinence time. In addition, sperm velocity decreased with age and abstinence time as previous studies have reported (25–28). This result suggests that continuous attention and improvement of male fertility remain important scientific issues in the field of reproduction.

The cause and clinical significance of the kinematic parameters' increase remain unclear. Sperm velocity is inextricably related to the sperm motility state, which is used to discriminate sperm motile state in CASA and increases during capacitation and hyperactivation (40–42). It was also reported that a higher percentage of hyperactivated spermatozoa was related to higher fertilization rates in conventional IVF (40). Our results hinted that sperm movement velocity increased despite a decrease in the amount and rate of motile sperm, which probably compensates to some extent for male fertility. However, we

TABLE 5 Sperm kinematic parameters in the ARL group during 2015–2021.

Year	n	VCL	VSL	VAP	BCF
2015	5,240	94.43 ± 21.55	47.82 ± 10.59	58.84 ± 11.20	24.04 ± 3.42
2016	5,467	97.79 ± 20.60	47.58 ± 10.36	59.92 ± 10.57	23.37 ± 3.33
2017	3,710	98.31 ± 19.10	46.20 ± 10.89	59.63 ± 10.36	23.00 ± 3.29
2018	4,361	101.23 ± 18.40	43.92 ± 8.56	59.34 ± 8.44	22.45 ± 2.79
2019	2,965	106.12 ± 20.37	51.17 ± 13.35	65.05 ± 12.03	24.50 ± 3.80
2020	1,822	110.18 ± 21.33	62.16 ± 14.89	72.83 ± 14.01	27.25 ± 3.64
2021	1,010	115.67 ± 21.28	67.01 ± 10.52	77.29 ± 11.26	28.26 ± 2.97
p^a		*< 0.001*	*< 0.001*	*< 0.001*	*<0.001*

Values are presented as mean ± standard deviation.
p^a, The one-way ANOVA of the kinematics parameters among the years in the ARL group.

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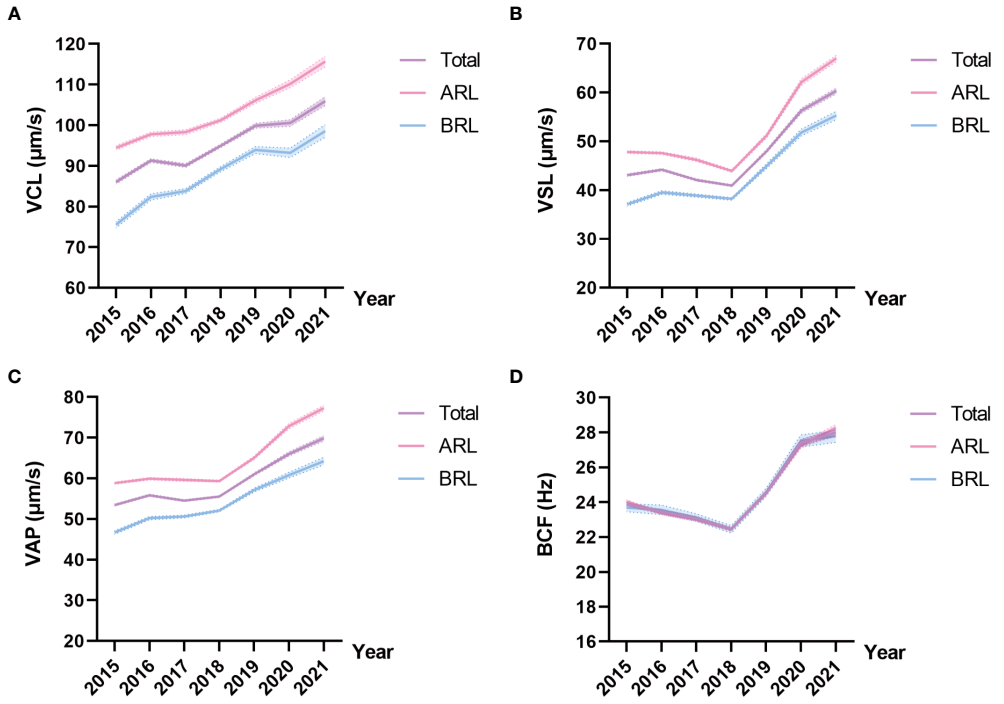

FIGURE 3
Line charts of kinematic parameters in the three type groups. Means and 95% CIs of VCL **(A)**, VSL **(B)**, VAP **(C)**, and BCF **(D)** in the groups over the years. The purple line represents the total sample, the pink line represents the ARL group, and the blue line represents the BRL group.

cannot exclude the possibility that the activation of sperm motility might be related to premature hyperactivation, which might cause spontaneous acrosome reaction and unexplained fertilization failure (43). This sequence of events has a detrimental effect on male fertility, especially in men with astheno- and oligozoospermia, possibly another indicator of declining male fertility. However, the data of kinematic parameters are not been reported online enough, which made the verification of our results difficult. We hope that more colleagues will pay attention to these data to discuss the accuracy and significance of this result. The causes and clinical implications of these results also should be explored in future prospective and experimental studies.

The age of subjects included in this study increased between 2015 and 2021. The main reason for this increase may be the introduction of the Chinese fertility policy that eased the one-child policy, causing many older men to visit clinical fertility centers. Moreover, there is a tendency to postpone the fatherhood age with the increase in educational requirements for employment. Therefore, we must consider the distribution of population age when studying the trends in semen parameters over time.

Furthermore, age was a significant factor in sperm quality. Many studies have indicated that age was related to sperm concentration, sperm count, total motility, and progressive motility (28, 44). The

TABLE 6 Variation analysis of kinematic parameters in the different groups.

Parameter	Total sample n = 49,819	ARL n = 24,575	BRL n = 24,614	p^a	p^b
VCL	93.3123 ± 24.3496	100.4208 ± 21.0102	86.2151 ± 25.3642	*<0.001*	*<0.001*
VSL	45.2764 ± 13.6507	49.0876 ± 12.4259	41.4713 ± 13.7570	*<0.001*	*<0.001*
VAP	57.2697 ± 14.0085	61.8333 ± 11.9238	52.7134 ± 14.4454	*<0.001*	*<0.001*
LIN	50.4537 ± 9.3838	50.6666 ± 9.0333	50.2412 ± 9.7166	*<0.001*	*<0.001*
ALH	5.0330 ± 1.3248	5.2976 ± 1.2017	4.7687 ± 1.3878	*<0.001*	*<0.001*
STR	77.5406 ± 8.5594	77.8576 ± 7.8030	77.2240 ± 9.2425	*<0.001*	*<0.001*
BCF	23.9664 ± 6.1385	23.9188 ± 3.6441	24.0139 ± 7.8766	*0.086*	*<0.001*
WOB	63.0401 ± 5.9304	62.9979 ± 5.5934	63.0823 ± 6.2486	*0.114*	*<0.001*

Values are presented as mean ± standard deviation.
p^a, The independent samples t-test of the kinematic parameters between the ARL and BRL groups.
p^b, Covariance analysis of the kinematic parameters between the ARL and BRL groups, adjusted for age and abstinence time.

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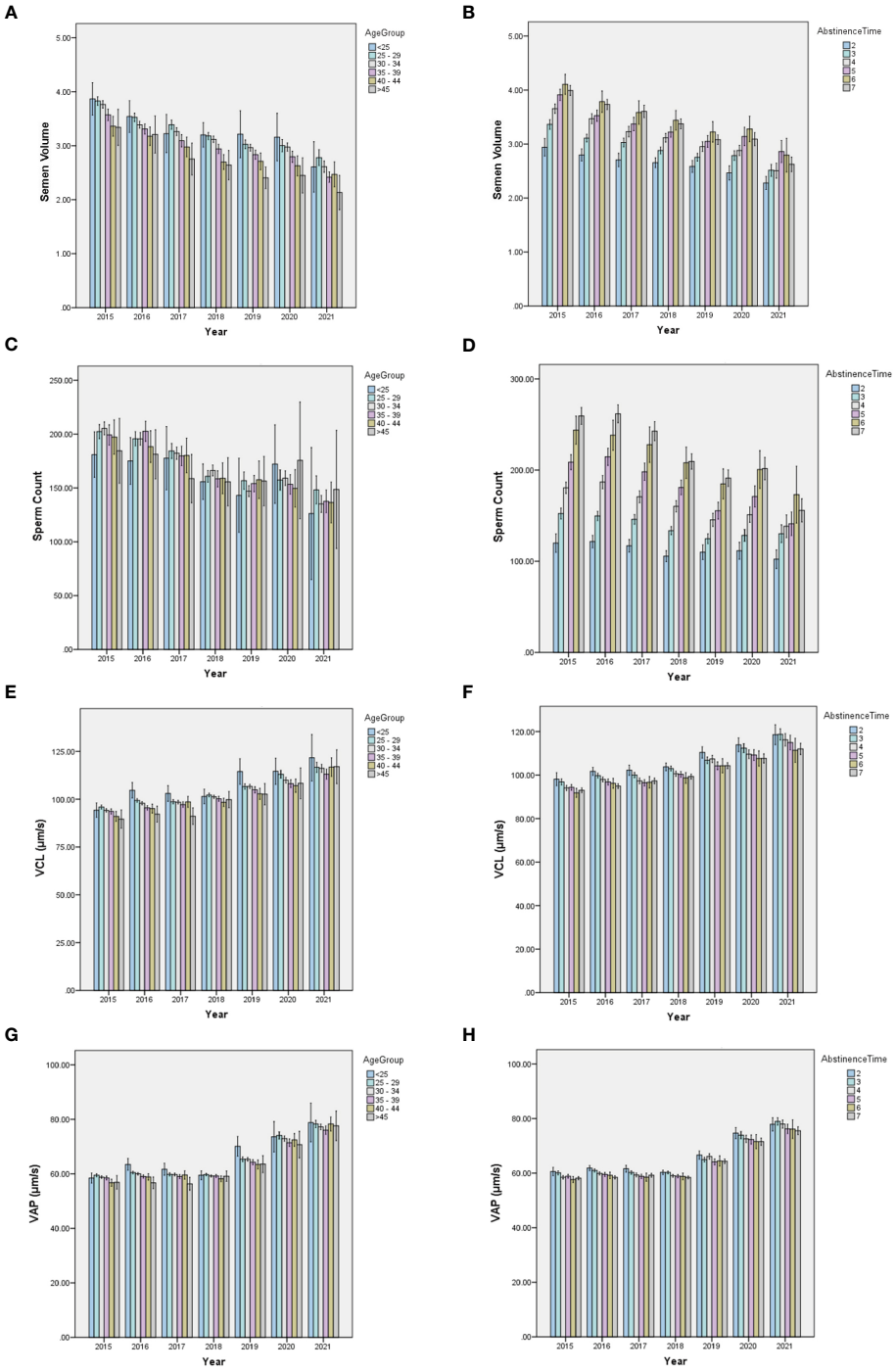

FIGURE 4
Bar charts of semen quality parameters in subdivided ARL groups by age or abstinence time. The yearly distribution of semen volume, sperm count, VCL, and VAP among the age **(A, C, E, G)** and abstinence time **(B, D, F, H)** groups.

kinematic parameters assessed by CASA were also associated with age. Studies showed that ALH, VCL, VSL, VAP, LIN, STR, and head width–to–length ratio significantly decreased with age, suggesting a decline in sperm velocity (25–28). Our results supported this point (Table 7 and Supplementary Figure 1). To exclude the impact of age increase in our study, we compared the semen quality and kinetic parameters of the same age group every year and found that the conclusion that semen quality decreased and sperm motility velocity increased year by year did not change. In addition, age was considered a confounder when analyzing the sperm parameters' temporal trends in regression models.

Abstinence time can also impact sperm quality (45–47). Sperm count, volume, and concentration increased gradually with abstinence time of up to 10 days (25, 46). Recent studies focusing on clinical assisted reproduction recommended a short ejaculatory abstinence interval to obtain better sperm quality, especially in infertile men (47–

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TABLE 7 Multiple regression linear model of semen parameters.

		Semen volume	Sperm count	Sperm concentration	Total motility	Progressive motility	
	Predictors	Estimates (95% CI)	Estimates (95% CI)	Estimates (95% CI)	Estimates (95% CI)	Estimates (95% CI)	*p*
Semen parameters	Year (β1)	−0.162 (−0.172, −0.152)	−9.97 (−10.813, −9.128)	−0.535 (−0.772, −0.299)	−1.751 (−1.830, −1.672)	−1.12 (−1.195, −1.044)	<0.001
	Age (β2)	−0.029 (−0.033, −0.026)	−0.554 (−0.869, −0.239)	0.374 (0.285, 0.462)	−0.139 (−0.168, −0.109)	−0.173 (−0.201, −0.145)	<0.001
	Abstinence Time (β3)	0.150 (0.130, −0.160)	23.033 (22.148, 23.917)	4.666 (4.418, 4.915)	−0.313 (−0.396, −0.229)	−0.527 (−0.60, −0.448)	<0.001
	R^2	0.080	0.119	0.057	0.077	0.046	
Kinematic parameters		VCL	VSL	VAP	BCF		
	Predictors	Estimates (95% CI)	Estimates (95% CI)	Estimates (95% CI)	Estimates (95% CI)	*p*	
	Year (β1)	3.058 (2.912, 3.203)	2.075 (1.990, 2.161)	2.305 (2.224, 2.386)	0.467 (0.441, 0.492)	<0.001	
	Age (β2)	−0.247 (−0.301, −0.192)	−0.045 (−0.077, −0.013)	−0.091 (−0.122, −0.061)	0.01 (0.001, 0.020)	<0.05	
	Abstinence Time (β3)	−0.967 (−1.120, −0.814)	−0.199 (−0.289, −0.109)	−0.389 (−0.473, −0.304)	0.092 (0.065, 0.118)	<0.001	
	R^2	0.074	0.086	0.119	0.051		

Multiple linear regression model of sperm analysis parameters. Year, age, and abstinence time were included as factors. β1, β2, and β3 represent the slope of liner regression by year, age, and abstinence time, respectively, weighted by the other two factors. R^2, R-squared represented the fitness of the model. VCL ($p < 0.001$), VSL ($p = 0.006$), VAP ($p < 0.001$), and BCF ($p = 0.035$) declined with age.

52). Our results showed that semen volume, sperm count, and concentration increased, whereas total and progressive motility, VCL, VSL, and VAP decreased with abstinence time (within the range of 2–7 days). The longer the sperm were stored in the epididymis, the poorer its motility was once ejaculated, especially in the BRL group (Figure S2). Sperm are continuously stored and naturally renewed in the epididymis. However, many studies suggested that the quality of sperm is closely related to the abstinence time or storage time in the epididymis, especially under pathological or harmful environmental conditions (45, 47). First, previous reports demonstrated that human sperm are susceptible to damage induced by reactive oxygen species (ROS) (53). Increased abstinence periods enhance the ROS-exposed time and bring about lipid peroxidation and malondialdehyde that is harmful to acrosome, mitochondrial activity, and DNA integrity (49, 54–58). For subfertile men, abnormal factors may result in excessive production of ROS in the epididymis, leading to decreased sperm motility and an infertile status (59–61). Impaired antioxidant capacity of abnormal spermatozoa also makes infertile sperm more prone to be damaged by ROS (62–65). Second, longer abstinence period affects secretions of epididymis, prostate, and seminal vesicle, which are related to the pH and biochemical of final semen and affect sperm motility (24, 66, 67). Moreover, longer abstinence time is related to a higher bacterial load and diversity in semen, more pro-inflammatory cytokines (68), as well as decreased rate of morphologically normal sperm (46, 69). Therefore, a shorter abstinence time is suggested for patients with oligo-atheno-zoospermia as it increases the probability of obtaining higher-quality sperm.

The rate of decline in sperm count and sperm concentration derived from this study was slightly higher than previous studies (5, 18, 19) (non-selected population fraction or reproductive center data). This may be related to the demographic and employment characteristics of the city as an economic center. Our research center is located in a mega city of China with high talent competition and socioeconomic burden, and one notable feature that sets it apart from other cities is the high level of work pressure, which, in turn, leads to reduced sleep time, high psychosocial stress, and irregular eating. Studies reported that short sleep durations, late bedtime, and poor sleep quality were associated with impaired sperm health (70, 71). Psychosocial stress was also negatively associated with sperm concentration, total count, and motility (72–74). Unbalanced diet is reported among Shanghai population (75) and is harmful to sperm quality, which includes overconsumption of cooking oil and salt as well as under-intake of dairy, vegetables and fruits, soybeans, fish and shrimps, and eggs (75–79). These lifestyles can easily lead to male nutritional disorders and endocrine dysfunction. For instance, overweight and obesity brought by unhealthy lifestyle are positively associated with high estradiol concentrations and lower sperm quality (80, 81). In addition, as China's main industrial center, Shanghai has serious air pollution that adversely impacts on sperm quality (82).

The strengths of this study are as follows. Most previous studies reported that semen parameters declined in recent decades, whereas this study focused on sperm kinematic parameters, showing increasing trends of sperm velocity over the years. Furthermore, we used a multiple linear regression model to analyze the data after

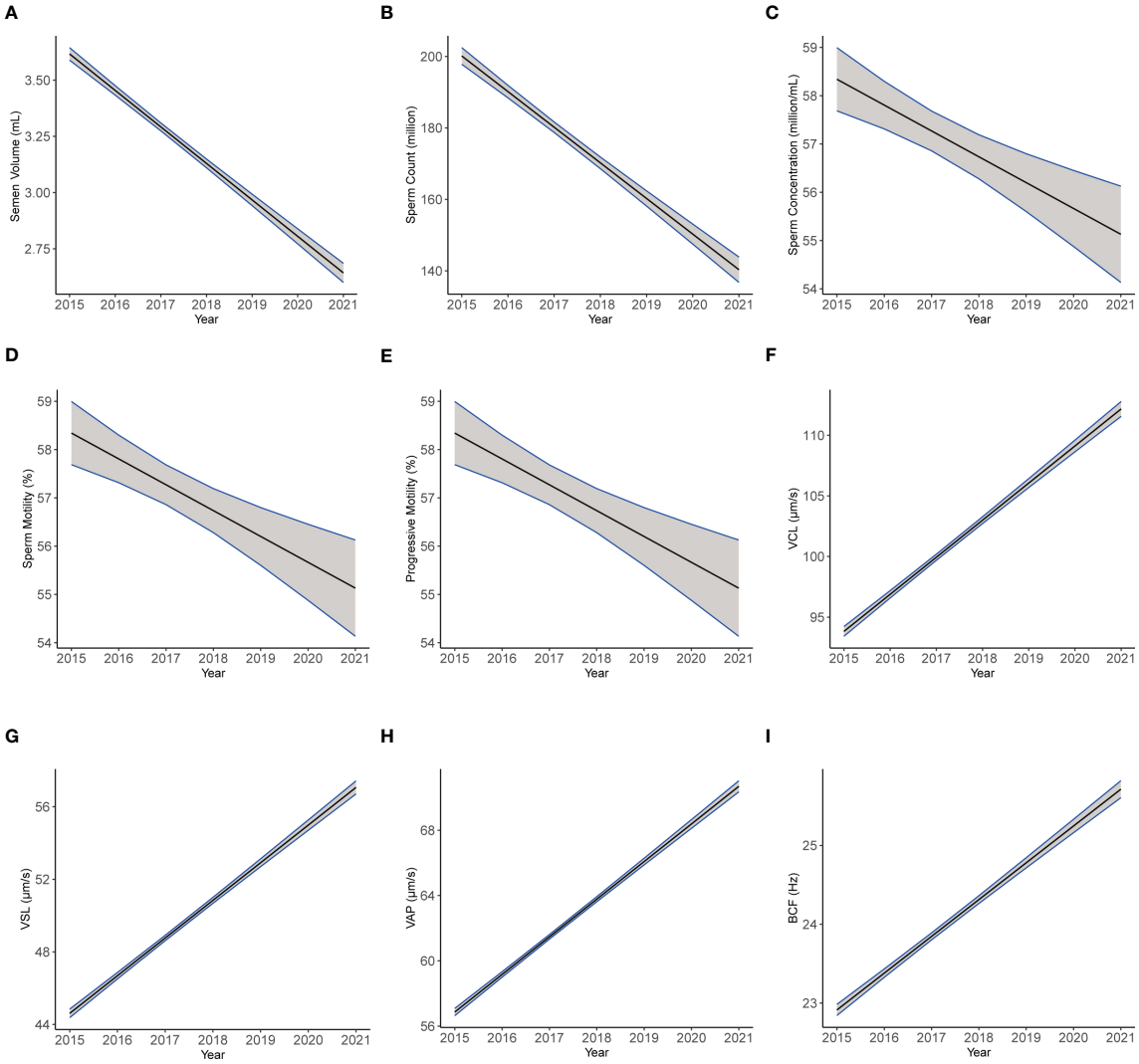

FIGURE 5
The plotting of multiple regression linear model of semen and kinematics parameters by year. Images of multiple regression linear models fitting of semen parameters: semen volume **(A)**, sperm count **(B)**, sperm concentration **(C)**, motility **(D)**, and progressive motility **(E)** and kinematics parameters: VCL **(F)**, VSL **(G)**, VAP **(H)**, and BCF **(I)** by year adjusted for age and abstinence time.

factoring in the effects of age and abstinence time considering that they may have some impacts on the results. Moreover, systematic error in data was low as the methods, personnel, and instruments were consistent throughout the study period.

However, the study had several limitations. First, the data came from a single reproductive center, and the subjects included healthy and infertile or potentially infertile men, which might not accurately reflect the trends in the general population. Second, we only corrected for age and abstinence time. Other potentially influencing factors were not recorded or analyzed. Third, the alterations in sperm quality do not necessarily indicate changes in fertility, and the fertilization outcomes were unknown. Despite the shortcomings of this research, the analysis of the results is reasonable and reliable. The majority of sperm quality studies are from single-centers with minor instrument and manual operation errors. Statisticians afterward could collect many studies' data for systematic review and meta-analysis and summarize widely conclusions. As for this study, the amount of sample is large in this study, and this can offset the impact caused by unselected subjects. We have further subdivided the total sample into different groups to diminish the potential interference. In addition, the conclusion that semen quality is deteriorating year by year is in good agreement with previous reports. Thus, the alterations of sperm kinematics parameters based on mutual reports are analogously convincing in this study. Indeed, semen quality is influenced by factors such as occupational factors, living habits, body mass, and environmental adverse factors. It is extremely difficult to eliminate all factors. Thus, it is crucial to overcome these shortcomings for future prospective experimental research design.

The decrease in semen parameters and increase in the kinematic parameters over the years might be the current trend in male fertility. Sperm kinematic parameters reflect information on sperm movement and functional state. The clinical significance of these alterations needs further studies to be verified and explored, which

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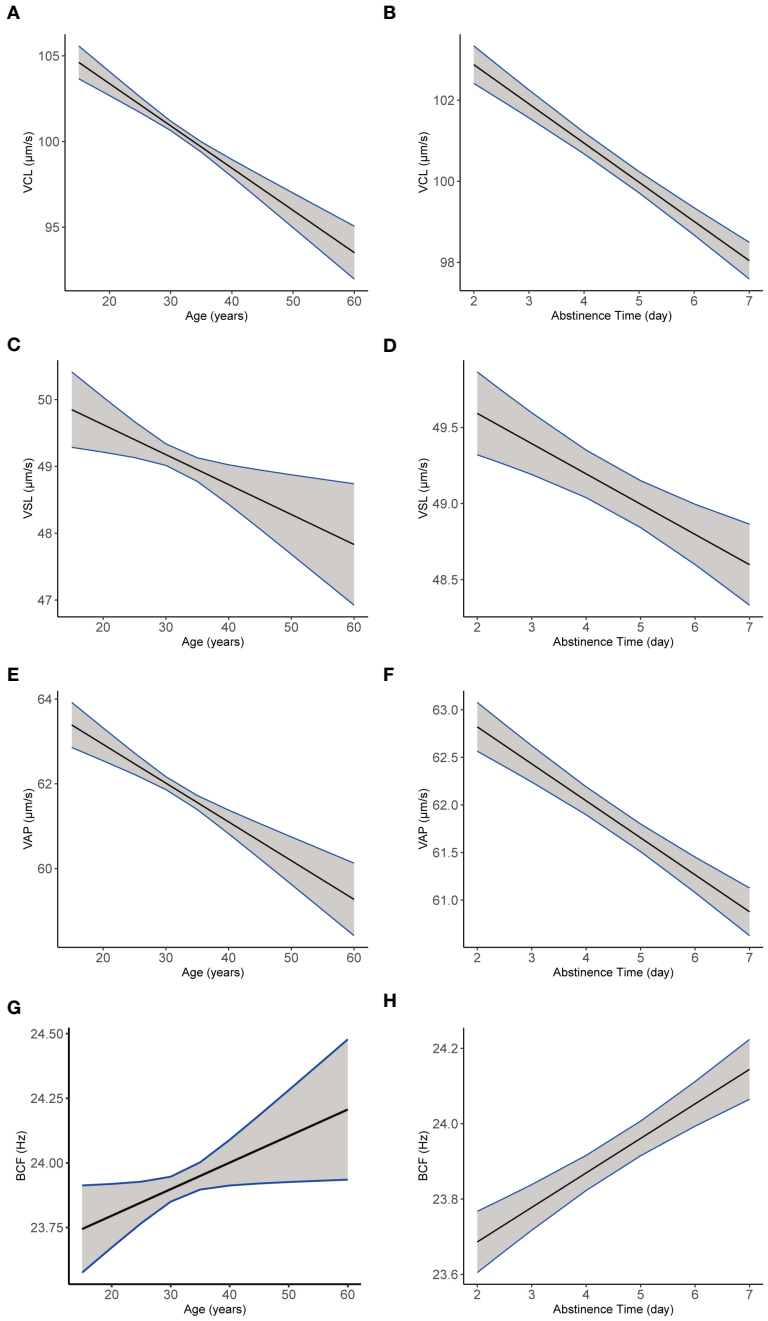

FIGURE 6
The plotting of multiple regression linear model of semen and kinematics parameters by age or abstinencetime. Images of multiple regression linear models fitting of sperm kinematic parameters VCL **(A)**, VSL **(C)**, VAP **(E)**, and BCF **(G)** by age, adjusted for the year and abstinence time. Images of multiple regression linear models fitting of sperm kinematic parameters VCL **(B)**, VSL **(D)**, VAP **(F)**, and BCF **(H)** by abstinence time (b, d, f, h), adjusted for age and the year.

could provide new reference values for guideline formulation and more diagnosis basis for unexplained infertility.

Conclusion

We presented temporal trends in sperm quality that comprised decreased semen parameters and increased kinematic parameters. Our results implied that enhancive velocity of sperm may be a potential compensatory mechanism of decreased sperm count and motility.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: The dataset belongs to our institution and data underlying this article will be shared on reasonable request to the

corresponding authors. Requests to access these datasets should be directed to zhouych@sibcb.ac.cn.

Ethics statement

This study was under approval by the Ethics Committee on human subjects of International Peace Maternity and Child Health Hospital (GKLW2018-03). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

YZ, CT, YL, and TL conceived and designed the study. YL and TL completed most of the data collection and analysis. ZMW, ZQW, HW, and TY participated in the sample collection, data analysis, and further explanation. YZ, CT, YL, and TL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1194455/full#supplementary-material

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EDITED BY
C. Yan Cheng,
St. Francis College, United States

REVIEWED BY
Jingbo Dai,
Northwestern Medicine, United States
Mariana Pereira Antoniassi,
Federal University of São Paulo, Brazil

*CORRESPONDENCE
Ludmila Osadchuk
losadch@bionet.nsc.ru;
losadch@bionet.nsc.ru

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Effects of cigarette smoking on semen quality, reproductive hormone levels, metabolic profile, zinc and sperm DNA fragmentation in men: results from a population-based study

Ludmila Osadchuk*, Maxim Kleshchev and Alexander Osadchuk

Department of Human Molecular Genetics, Federal Research Center 'Institute of Cytology and Genetics', the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia

Background: Cigarette smoking seems to have a negative impact on men's reproductive health, but our knowledge of its effects on the reproductive function of Russian men is still very limited. The purpose of this study was to evaluate the effect of cigarette smoking on semen quality, including sperm DNA fragmentation, hormonal, zinc and metabolic status in young men from the general multi-ethnic Russian population (n=1,222, median age 23 years) and to find out the ethno-specific effects of smoking by comparing male groups of different ethnicity.

Methods: Each participant filled out a standardized questionnaire, provided one blood and semen sample. Semen parameters, serum reproductive hormones, lipids, glucose, uric acid and seminal zinc were analyzed. Participants were classified as smokers (n=450) and non-smokers (n=772), and smokers were stratified into moderate (≤10 cigarettes/day) and heavy (>10 cigarettes/day) smokers.

Results: In the entire study population, heavy smokers were characterized by a decrease in semen volume, total sperm count, sperm concentration and motility, and an increase in sperm DNA fragmentation and teratozoospermia compared with non-smokers ($p<0.05$). There was also a reduction in the serum and seminal zinc level as well as an impairment in metabolic health in smokers compared with non-smokers ($p<0.05$). No significant differences between smokers and non-smokers were found for serum levels of LH, FSH, inhibin B, testosterone and estradiol. In the second part of our study, the most numerous ethnic groups of Slavs (n=654), Buryats (n=191), and Yakuts (n=125) were selected from the entire study population. Among three ethnic groups, the smoking intensity was higher in Slavs than in Buryats or Yakuts suggesting a greater tobacco addiction in Slavs than in Asians. A decrease in semen parameters and seminal zinc levels, and an increase in sperm DNA fragmentation and teratozoospermia was observed only in smoking Slavs ($p<0.05$); moderate decrease in testosterone and increase in

triglyceride levels were revealed in smoking Yakuts ($p<0.05$), but no significant changes were detected in smoking Buryats.

Conclusion: We concluded that cigarette smoking has an ethno-specific effect on male reproductive function, probably due to the different activity of the seminal antioxidant system, which is yet to be elucidated.

KEYWORDS

cigarette smoking, semen quality, sperm DNA fragmentation, reproductive hormones, metabolic profile, seminal zinc

Introduction

In recent decades, the demographic crisis observed in highly developed countries, including Russia, has been combined with a progressive decline in the reproductive potential of human populations, which raises the question of the underlying causes of this phenomenon. In different regions of the world, adverse secular trends in male reproductive health has been observed, which are expressed in poor semen quality, an increase in the proportion of male factor in infertile couples and the growth of congenital anomalies of the male reproductive system, resulting to infertility (1–4). In addition to a reduction of semen volume, sperm concentration and motility and an increase in sperm morphological abnormalities, several epidemiological studies have indicated a population-level decline in testosterone, suggesting that all these facts may be interrelated (5–7).

Since adverse changes in male fertility have occurred in a short period of time and in parallel in different parts of the world, the decline in semen quality is probably due to environmental rather than genetic factors (4). In recent years, it has been well documented that adverse changes in male fertility seem to be primarily associated with primarily anthropogenic environmental pollution, occupational exposure and individual lifestyle (8–11). Lifestyle, especially bad habits such as smoking, alcohol consumption, physical inactivity or excessive nutrition, have a negative impact not only on general health, but also reduce sperm quality and can lead to male sub-fertility or infertility (11–13). Some authors believe that the worldwide decline in men's reproductive potential might be due, at least in part, to lifestyle factors (4, 8, 9).

Tobacco smoking is considered among the most significant lifestyle factors affecting male reproductive function. Smoking is still widespread all over the world, including Russia. According to the World Health Organization, 22.3% of the global population, 36.7% of men and 7.8% of women, used tobacco in 2020 (14). Its prevalence in the United States is about 21-22% among men of reproductive age (9, 15). The Russian Public Opinion Research Center (VCIOM) reports that the proportion of smokers in Russia has remained unchanged over the past five years and today it is a third of total number of citizens (33%), while smoking men are much more than women (47% vs 21%) (16).

It is generally recognized that tobacco smoking has a negative impact on overall health and is one of the leading preventable causes of death. Tobacco smoking is associated with more than 27 diseases, including respiratory and cardiovascular diseases, lung, kidney, urinary bladder, pancreas cancer, type 2 diabetes and other medical conditions (17, 18). Despite the fact that smoking is a well-established cause of many hard diseases, the mechanism by which smoking causes such a wide range of diseases is not fully elucidated. Although many are aware of the dangers of tobacco use, smoking continues to be a public health concern. Only recently, due to the growth of male infertility and subfertility, increased attention has been paid to the effects of smoking on men's reproductive health (19). Cigarette smoking is a potential risk factor for impaired male fertility and the relationship between smoking and male infertility is actively being discussed. A decrease in semen volume, sperm concentration, viability, motility and normal morphology has been shown in heavy tobacco smokers (8, 9, 19–22). Moreover, in ART programs, chronic smoking of male partner was related to poor embryo quality and the reduced success of *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (8, 23–25). Although many studies have reported a decrease in one or more conventional semen parameters or altered sperm functions in tobacco smokers, some studies have not established or shown very weak negative changes in semen quality, especially in infertile and sub-fertile men (20, 21, 23, 26, 27).

Based on studies that evaluated semen parameters in accordance with the WHO guidelines (28), it can be concluded that the effects of tobacco smoking lead to a more pronounced poor semen quality in healthy men or men from the general population (19, 29–32) than in infertile men or men from infertile couples (33–37). Moreover, it has been shown that tobacco smoking can affect the conventional semen parameters in a dose-dependent way, since the impairment of semen quality was more pronounced in heavy smokers then in moderate or mild smokers, which indicates more severe reproductive consequences with longer or intensive smoking (19, 29, 30, 38, 39).

More than 7,000 chemicals and toxins have been identified in tobacco smoke including polycyclic aromatic hydrocarbons, of which many are proven carcinogens and mutagens (8, 20, 22, 40, 41). The main pharmacological hazardous substances of tobacco – the alkaloids nicotine and its metabolite cotinine - can penetrate the blood-testis barrier and subsequently affect the process of spermatogenesis, reducing sperm concentration, total sperm count,

and sperm motility (19–23). Smoking has been shown to increase genetic and epigenetic aberrations in spermatozoa, including oxidative DNA damage, chromatin packing abnormalities, chromosomal alterations, mutations, polymorphisms, epigenetic alterations such as DNA methylation and dysregulation of mRNA expression, and all of that can influence sperm functions and men's fertility (8, 20, 22, 37, 41–46). Recent research data suggest that genetic and epigenetic changes in spermatozoa that are caused by the genotoxic components of tobacco can be transmitted to offspring (19, 20, 22, 40–43).

Chemical compounds with which smoking might affect the sperm DNA integrity include cigarette smoke components such as cadmium, lead, arsenic, carbon monoxide, nicotine and cotinine (a metabolite of nicotine), which are directly absorbed into the systemic circulation and accumulated in seminal plasma, initiate oxidative stress and dramatically affect seminal parameters, including sperm DNA fragmentation (22, 40–43). Oxidative damage is the most common cause of sperm DNA fragmentation, which can impair sperm functions, leading to a decrease in male fertility (20). Some authors have suggested using the degree of DNA integrity as an additional parameter of semen quality and a prognostic factor of male fertility (42). In 2021, the WHO laboratory guidelines (47) also recommended the analysis of sperm DNA fragmentation as a new functional test for an extended study of sperm quality. In addition to tobacco smoking, other lifestyles are associated with increased sperm DNA fragmentation, including excessive alcohol consumption, obesity, advanced age, urological diseases, type 2 diabetes (42).

The effect of tobacco smoking on semen parameters can be mediated not only by direct toxic effects, but also by changes in hormonal secretion and metabolism (20, 22, 48, 49). Tobacco smoke can induce a dysregulation of the endocrine system, which leads to problems with male fertility. To date, research assessing the correlation between cigarette smoke exposure and reproductive hormones has shown inconsistent results. Some studies revealed significantly higher levels of testosterone and/or free testosterone in smokers compared to non-smokers with a dose-response pattern (13, 32, 38, 49–52). On the contrary, others reported a decrease in testosterone concentrations in tobacco smokers (29, 53) or did not find any differences in the hormonal level between smokers and non-smokers (54, 55). Although many studies have found increased testosterone levels among smokers, the mechanism by which smoking increases testosterone levels remains unclear. The increase in testosterone levels in smokers may be due to a change in the ability of SHBG to bind testosterone (56, 57). Zhao et al. (49) suggested that cotinine (a metabolite of nicotine) can inhibit the testosterone degradation, which leads to the testosterone accumulation in smokers. Moreover, higher total testosterone levels in smokers than in non-smokers were positively associated with number of cigarettes smoked, but with age the positive relationship between smoking and testosterone weakened (58). Interestingly, higher testosterone concentrations in smokers were associated with higher sexual activity, although long-term experience of smoking has a higher risk of erectile dysfunction (20, 59, 60).

Few studies have investigated the relationship between tobacco smoking and pituitary hormones. Differences in FSH and LH levels between non-smokers and smokers were very often insignificant suggesting that there is no effect of tobacco smoking on the hypothalamic-pituitary axis (13, 32, 38, 61). Meanwhile, Blanco-Muñoz with colleagues showed that active smokers had higher LH and testosterone levels, although they were unable to detect any changes in estradiol, inhibin B and FSH levels (62). One recent study found that FSH levels were higher in smokers than in non-smokers, while testosterone levels were lower and LH levels were no different (53). Furthermore, Mitra with colleagues found that serum FSH and LH levels were higher in smokers compared to non-smokers, while testosterone levels decreased significantly with increased smoking (63).

Several studies have documented the damaging effects of tobacco smoking on the antioxidant defense system due to increased oxidative stress (20, 22, 40, 41, 64). Cigarette smoke contains high concentrations of nitric oxide, peroxynitrite, free radicals, and can potentially induce the production of reactive oxygen species (ROS) in the human body. Oxidative stress occurs when the antioxidant system can no longer counteract the ROS formation. Excessive generation of ROS damages the structural and functional parameters of sperm, including polyunsaturated fatty acids, proteins, nucleic acids, which are potential targets of ROS. Antioxidants, including superoxide dismutase, glutathione peroxidase, catalase, play an important defensive role in neutralizing ROS. The trace element zinc is an essential element as more than 300 enzymes require zinc for their function, and plays an important role in functioning the male reproductive system, including sperm physiology and the antioxidant defense mechanism (65–67). Several studies have reported that seminal zinc levels were closely associated with semen parameters in men, so zinc deficiency may be an important risk factor for lowered semen quality (65–69). Zinc has antioxidant properties that counteract the excessive production of ROS, in particular, it is a necessary component of superoxide dismutase, the most active antioxidant enzyme in the semen. Zinc depletion or deficiency (particularly the actual levels of seminal zinc) is proved to be a serious side effect of cigarette smoking (69, 70). Taha with colleagues observed that fertile non-smokers demonstrated higher progressive sperm motility, seminal zinc level, lower levels of sperm DNA fragmentation and seminal ROS compared to fertile smokers (64). Similarly, in infertile couples, it was found that the seminal zinc concentration and semen parameters (sperm concentration, motility and morphology) were lower in smokers than in non-smokers, indicating that seminal zinc may be involved in the mediating effects of tobacco smoking on semen parameters (70). In a large cohort of men from infertile couples, it was observed that sperm progressive motility, abnormal head rates, sperm viability, seminal zinc concentration were significantly reduced, as well as the DNA fragmentation rate was markedly increased in heavy smokers compared with non-smokers (35). When comparing groups of male volunteers, the smoking group compared with non-smokers showed a lower sperm concentration, the proportion of actively motile and morphologically normal sperm. Seminal zinc levels were also reduced in smokers compared to non-smokers, although there was no significant difference in serum zinc levels between smokers and non-smokers (69). The above studies clearly show the negative

impact of tobacco smoking on the seminal zinc level, which may contribute to the pathogenesis of the adverse effects of smoking on semen parameters.

Tobacco smoking appears to negatively affect lipid, glucose and protein metabolism. Studies have shown an adverse effect of tobacco smoking on the lipid profile (lower HDL, higher LDL and triglycerides), while advanced age and higher BMI significantly enhance this relationship (71). Nicotine suppresses appetite and can reduce food intake and body weight, so smokers tend to be thinner and have a lower BMI than non-smokers, at that the lipolytic effect of smoking is attributed to the nicotine component through the release of catecholamines (71). Smoking can be associated with impaired in insulin action and glucose regulation, increasing risk for type 2 diabetes (71). Some men smoke because they consider their habit as a method of weight control, however, there is evidence that a part of heavy smokers has a higher BMI and a larger waist circumference than non-smokers, while the combination of smoking with abdominal fat accumulation increases the risk of metabolic syndrome, type 2 diabetes and cardiovascular diseases (72).

Most of the current researches concerning the effect of tobacco smoking on male reproductive function were carried out on specially selected small groups, in most cases with a history of infertility and/or long-term smoking experience, and very few studies have examined the effect of tobacco smoking in population-based cohorts. At the same time, a larger sample size increases its external validity and allows to control numerous potential confounding variables. Moreover, a large-scale population study can clarify previously unresolved issues, in particular, to identify new relationships due to ethnic origin and traditions. Although the exact associations tobacco smoking with the male fertility parameters are still insufficiently studied, our knowledge about its reproductive effects in Russian residents is even more limited, especially in men of active reproductive age. Thus, the purpose of this study was 1) to investigate whether tobacco smoking is associated with the semen quality parameters (total sperm count, concentration, motility, morphology, DNA integrity), the reproductive hormone and zinc levels, metabolic status in Russian men of reproductive age from the general multi-ethnic population and 2) to find out the ethno-specific effects of smoking when comparing different ethnic groups.

Materials and methods

Subjects

For the present study, 1,371 men were recruited from five Russian cities: Archangelsk, Novosibirsk, Kemerovo, Ulan-Ude, Yakutsk. The city of Archangelsk is located in European North of Russia within the circumpolar area, the cities of Novosibirsk and Kemerovo in Western Siberia; all three cities have a predominantly Slavic population (approximately 90-95%). The cities of Ulan-Ude and Yakutsk are located in Eastern Siberia. Buryats make up 32% of the total population of Ulan-Ude, and Yakut - 43% of the total population of Yakutsk. In all cities, the study design and standardized recruitment protocol were the same, which were described earlier (68, 73, 74). The study included volunteers from the general population, regardless of their fertility, most of the participants were undergraduate or postgraduate students, and university staff. Any man over the age of 17 who wanted to know his reproductive health and endocrine status was invited to participate in the study. All the participants were born or lived for at least 3-5 years in the cities prior to the study. Participants were recruited without restrictions on body weight and BMI. All the participants were informed about the study in various ways, including advertising on the Internet, on television or at special lectures on men's health, with detailed information about the purpose and objectives of the study. Each participant filled in a standardized questionnaire with information on age, place of born, self-identified nationality, family status, some life style characteristics, including smoking consumption. All study subjects were voluntaries and did not received any financial compensation. Since our study population was multi-ethnic and consisted of men of different nationalities or descendants from ethnic mixed marriages, the questionnaire also included questions about the ethnicity of the participant's parents and grandparents. To investigate ethnic differences in the effects of cigarette smoking, three most numerous ethnic groups were selected from our multi-ethnic study population. They were Slavs (n=654), Buryats (n=191) and Yakuts (n=125). Participants were stratified into the ethnic groups, taking into account self-identified ethnicity and ethnicities of their parents and grandparents. The participant was eligible if the ethnicity of himself and his relatives was the same. The data of participants were stored anonymously.

All participants were examined by an andrologist, and some participants were diagnosed with current urogenital disorders, which included clinical varicocele grade II, prostatitis, testicular cysts, hydrocele, and hypospadias. Body weight (kg), height, waist and hip circumference (cm) were measured, body mass index (BMI, kg/m^2) was calculated. Testicular volume (ml) was estimated by a Prader orchidometer and was presented as bitesticular or paired testicular volume (BTV). Age calculated as the difference between year of attendance in study and self-reported year of birth. Each participant was asked about necessity of sexual abstinence for 2-7 days before the examination. Each participant provided both blood and semen sample. The fasting blood sample was drawn in the morning between eight and eleven a.m. before the semen sample was collected. Serum samples were stored at -40°C until an analysis for hormones, metabolites and zinc. Semen samples were collected by masturbation into disposable sterile plastic containers. The participants with the following characteristics as azoospermia, varicocele of II or III grade, orchitis, criptorchidism or the consequences of surgery for cryptorchidism, testis trauma, using drugs, chronic diseases in an acute phase were excluded from the study. Participants, who took anabolic steroids (reported themselves or indicated from the profile of reproductive hormones) or did not provide information about smoking status, as well as men with AZF microdeletions of the Y chromosome (known genetic causes of infertility) were also excluded. Totally, 1,222 participants (89.1% of recruited men) were included in the study. Participants were classified as smokers if they indicated that

they currently smoke daily and reported an approximate number of cigarettes smoked per day, while non-smokers indicated that they had never smoked. Current smokers were further stratified into two groups, depending on the amount of cigarettes smoked daily: moderate (≤ 10 cigarettes/day) and heavy (> 10 cigarettes/day).

Semen analysis

Sperm concentration (million/ml), semen volume (ml), total sperm count (million/ejaculate), a proportion of morphologically normal (%) and progressively motile sperm (%), the teratozoospermia index (TZI) were analyzed according to the WHO laboratory manual for the examination and processing of human semen (28). Semen analysis was described earlier (68, 73, 74). The semen samples were kept at 37^0C for 1 hr. for liquefaction. Ejaculate volume was estimated by weighing the collection container and subtracting the weight of the empty preweighed container, assuming that 1 ml of ejaculate weighs 1 g. To determine the sperm concentration, 100 µl of well-mixed ejaculate was diluted in 400 µl of a solution (5% NaHCO3; 0.35% formaldehyde; 0.025% trypan blue in distilled water). Staining was carried out for 1 hr. at room temperature, after which the samples were stored in the refrigerator at a temperature of +40°C for subsequent counting. Sperm concentration was assessed using the Goryaev's hemocytometer under light microscope (magnification ×400). Total sperm count was calculated by multiplying the individual's sperm concentration by the semen volume.

A proportion of progressive motility sperm was estimated in native ejaculate using an automatic sperm analyzer SFA-500 (Biola, Russia). The sperm motility measurements were carried out three times for each sample and mean value was calculated. To assess sperm morphology, ejaculate smears were prepared, fixed by methanol and stained by using commercially available kits Diff-Quick (Abris plus, Russia) according to the manufacturer manual. Two hundred spermatozoa in each sample were examined for morphology with an optical microscope (Axio Skop.A1, Carl Zeiss, Germany) at ×1000 magnification with oil-immersion and the sperm morphological anomalies were listed according to the WHO laboratory manual (28). To determine the TZI, the total number of morphological defects determined was divided by the number of morphological abnormal sperm. Sperm morphology evaluations were done in duplicates in random and blind order and we reported here the percentage of sperm assessed as morphologically normal (%).

Serum reproductive hormones and metabolites assay

Serum hormone concentrations were determined by enzyme immunoassay using commercially available kits Steroid IFA-Testosterone-01, Gonadotropin IFA-LH, Gonadotropin IFA-FSH (Alkor Bio, Russia), Estradiol-IFA (Xema, Russia) and Inhibin B Gen II ELISA (Beckman Coulter, USA). The ranges of evaluated concentrations for total testosterone (T), estradiol (E_2), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and inhibin B (InhB) were 0.2-50 nmol/L, 0.1-20 nmol/L, 2.0-100 mIU/mL, 20-90 mIU/mL, and 12-105 pg/ml respectively. The sensitivities for T, E_2, FSH, LH, InhB were 0.2 nmol/ml, 0.025 nmol/L, 0.25 mIU/mL, 0.25 mIU/mL, 2.6 pg/ml, respectively. The intra- and interassay coefficients of variation were as follows: for T < 8.0%; E_2 < 8.0%; FSH < 8.0%; LH < 8.0%, InhB ≤ 6.8%.

Serum concentrations of triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), glucose and uric acid were determined by enzymatic colorimetric method using commercially available kits (Vector Best, Russia). According to this test system, the normal values of serum metabolite concentrations in adults were TG up to 1.70 mmol/l; TC up to 5.20 mmol/l; HDL-C for men in the range of 0.90-1.80 mmol/l; glucose 4.0-6.1 mmol/l; uric acid for men 200-420 µmol/l. Serum concentrations of low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula (LDL-C = TC - HDL-C - TG/2.2).

Serum and seminal plasma zinc assay

Seminal plasma was obtained by centrifugation of the cellular elements and was stored at -40°C. Serum and seminal zinc was determined by spectrophotometry and direct colorimetry without deproteinization using commercially available kits (Vital Development Corporation, Russia) as previously described (68). The seminal plasma diluted with 1:100 of deionized water before the analyses, the serum samples used without dilution. The total seminal zinc content was calculated by multiplying the individual's seminal zinc concentration by the semen volume. The detection limit for zinc concentration was one µmol/l; the lineal range of zinc concentrations was 6.0 - 61.2 µmol/l; coefficient of variation was ≤ 5%. According to this test system, the normal values of serum zinc concentrations were 7.0-23.0 µmol/l in adults. Lower reference limit for seminal zinc content is 2.4 µmol/ejaculate (28).

All hormones, metabolites and zinc were assayed using photometer Multiskan Ascent (Thermo Electron Corporation, Finland). Spectral range 340-850 nm. Operating wavelengths for TG, glucose, uric acid and zinc – 540 nm; for TC, HDL-C – 492 nm; for all hormones – 450 nm.

Sperm DNA fragmentation assay

The sperm DNA fragmentation was assessed using the Sperm Chromatin Structure Assay (SCSA) using flow cytometry as previously described (75). Immediately after ejaculate collection, semen aliquot (300 µL) was frozen and stores at -40°C until analysis. To analyze sperm DNA fragmentation, the sample diluted in TNE-buffer (0.01MTris, 1 mM EDTA, 0.15 MNaCl, pH 7.4) to the sperm concentration one million/ml and then 200 µl of acid buffer (0.1% Triton-X-100, 0.15M NaCl, 0.08 N HCl, pH 1.2) was added to 100 µl diluted ejaculate. After incubation for 30 s, 600 µl of dye solution containing 6 mg/L acridine orange, 0.2 M Na_2HPO_4, 1 mM EDTA (disodium), 0.15 M NaCl, 0.1 M citric acid (pH 6.0) was added. Sperm with red and green fluorescence were counted by

fluorescence cytometer Guava Easy CyteMini (Guava, USA). Each sample was evaluated thrice (5000 cells in each estimation) and the mean value was used. Extent of DNA damage was expressed as the DNA fragmentation index (DFI%), which is the ratio of red (sperm with fragmented DNA) to total fluorescence. DFI% <15% is a normal percentage of sperm without chromatin damage, 15%≤ DFI% <27% is considerable chromatin damage and DFI% ≥27% is severe chromatin damage (76). The DNA fragmentation index was evaluated in randomly selected subjects from our entire study population (n=247, 20.2%), of which 200 participants were Slavs (145 non-smokers and 55 smokers).

Statistical analysis

Statistical analysis of the data obtained was performed using the statistical package "STATISTICA" (version 8.0). The Kolmogorov-Smirnov test for normality was used. In the entire study population, all the studied parameters were not normally distributed. Descriptive statistics in the tables and in the text is presented as mean (SD) as well as median with 5th and 95th percentiles. Kruskal-Wallis ANOVA test was used to identify differences in age and anthropometric parameters between groups with different smoking status in the entire study population or between non-smokers and smokers in each ethnic group. Analysis of covariance (ANCOVA) was used to identify differences in semen, hormonal, metabolic and zinc parameters between groups with different smoking status or between groups of smokers and non-smokers in each ethnic group. The semen parameters, including DFI% and seminal zinc, were adjusted for the period of sexual abstinence, while serum hormonal, metabolic and zinc parameters were adjusted for age and BMI. Duncan's test was used to determine statistical significance of the differences between the groups. A p value < 0.05 was regarded as statistically significant.

Results

General characteristics of the entire study population

Of the 1,222 participants, 654 (53.5%) men were Slavs (Russians, Belarusians, Ukrainians); 191 (15.6%) - Buryats; 125 (10.2%) - Yakuts; 25 (2.1%) were other ethnic origin; 227 (18.6%) were descendants from ethnic mixed marriages. The median age of men was 23 years, and the vast majority of participants (81.5%) were of reproductive age (18-30 years). Only 37.7% of the participants in our study population were married, and 16.5% of them had at least one or two children. Demographic and anthropometric parameters of the study subjects are summarized in Table 1. The entire study population was characterized by a normal BMI (median 23.4 kg/m^2), although a part of the study population was overweight (25.3%) or obese (8.6%). More than one third of the men (36.8%) were cigarette smokers on daily basis (Table 1). According to the WHO laboratory manual (28), the median semen parameters of the participants were within the values accepted for normozoospermia, including the sperm concentration, motility and morphology (Table 1). The median sperm concentration was 52.49 million/ml; the lowered semen quality was detected in 39.2% of participants in accordance with the WHO, i.e. with sperm concentration less than 15 million/ml, progressive sperm motility less than 32% and normal morphology less than 4%. The median levels of LH, FSH, testosterone and metabolic parameters in our participants corresponded to normal reference ranges for men from European countries and the USA (77–79). The median concentrations of serum and seminal zinc were within the generally accepted normal values for Russian men (80). The median content of seminal zinc was 4.20 µmol/ejaculate, which is almost twice the WHO recommended lower reference value of 2.4 µmol/ejaculate (28).

Comparison of anthropometric, semen, hormonal, and metabolic parameters between groups of participants with different cigarette smoking status

Using our group of non-smokers as a reference group, we found no statistically significant differences between non-smokers and both groups of smoker in height, BTV, serum levels of LH, FSH, testosterone, total cholesterol, LDL-C, fasting glucose (Table 1). The medians of age, body weight, waist and hip circumference, BMI in heavy smokers were significantly higher than in non-smokers ($p<0.05$, Table 1). The smoking intensity (the number of cigarettes smoked per day) was 2.7 times higher in heavy smokers than in moderate smokers ($p<0.05$, Table 1).

Current smokers of both consumption categories had reduced semen volume, total sperm count, sperm concentration and the inhibin B level compared to non-smokers, while only heavy smokers had lower sperm progressive motility and higher TZI and DFI% compared to non-smokers ($p<0.05$, Table 1). In general, the heavy smokers had worse semen quality than moderate smokers or nonsmokers. The group of heavy smokers was also characterized by an impaired metabolic profile: the serum TG and uric acid levels were significantly higher, and the HDL-C level was significantly lower compared with the group of non-smokers ($p<0.05$, Table 1). In addition, the serum zinc concentration was significantly higher, while the seminal zinc concentration and content were significantly lower in heavy smokers compared with non-smokers ($p<0.05$, Table 1). On the contrary, the group of moderate smokers was very close to non-smokers in many respects, with the exception of most semen parameters and the inhibin B level (Table 1).

Comparison of smokers and non-smokers by anthropometric, seminal, hormonal, zinc and metabolic parameters in different ethnic groups

Three of the most numerous ethnic groups were selected from the entire studied population: Slavs, Buryats and Yakuts. The ethnic differences in anthropometric, semen and reproductive hormone

TABLE 1 Anthropometric, semen, hormonal, and metabolic parameters of participants with different smoking status.

Parameters	Smoking status			Total (n=1222)
	Non-smokers (n=772)	Moderate smokers cigarettes/day ≤10 (n=274)	Heavy smokers cigarettes/day >10 (n=176)	
Age, years	24.9(7.0) [a] 23.0(18.0-39.0)	24.4(6.4) [a] 22.0(18.0-37.0)	27.7(8.0) [b] 26.0(18.0-43.0)	*25.2 (7.1)* *23.0 (18.0-39.0)*
Body weight, kg	75.4(13.6) [a] 73.6(57.2-102.0)	74.2(14.3) [a] 72.0(55.0-101.0)	78.5(14.9) [b] 76.3(59.5-106.0)	*75.5(14.0)* *74.0(56.5-102.0)*
Height, cm	177.6(7.2) [a] 177.5(166.0-189.0)	176.4(7.0) [a] 176.5(165.5-188.0)	176.6(6.7) [a] 176.0(165.0-187.5)	*177.2(7.1)* *177.0(166.0-188.5)*
Waist circumference, cm	83.5(10.2) [a] 82.0(70.0-103.0)	84.1(11.7) [a] 82.0(69.0-105.0)	87.9(12.4) [b] 87.0(70.0-107.0)	*84.2(11.0)* *82.0(70.0-105.0)*
Hip circumference, cm	97.9(7.9) [a] 97.0(87.0-112.0)	97.2(8.4) [a] 96.0(85.0-111.0)	99.2(8.5) [b] 98.0(87.0-114.0)	*98.0(8.1)* *97.0(86.0-112.0)*
BMI, kg/m^2	23.8(3.8) [a] 23.3(18.8-31.2)	23.8(4.3) [a] 23.0(18.3-31.8)	25.2(4.6) [b] 24.5(19.1-32.0)	*24.0(4.0)* *23.4(18.7-31.4)*
BTV, ml	39.7(8.6) [a] 40.0(27.0-54.0)	38.8(7.4) [a] 40.0(28.0-50.0)	40.7(9.2) [a] 40.0(29.0-56.0)	*39.7(8.4)* *40.0(28.0-54.0)*
Smoking, cigarettes/day	0	6.6(3.0) [a] 7.0(1.3-10.0)	17.5(4.7) [b] 17.5(12.0-25.0)	*11.0(6.5)* *10.0(2.0-20.0)*
Semen volume, ml	3.6(1.6) [a] 3.4(1.5-6.5)	3.3(1.6) [b] 3.0(1.2-5.9)	3.3(1.6) [b] 3.0(1.1-6.3)	*3.5(1.6)* *3.3(1.3-6.4)*
Total sperm count, ×10^6/ejaculate	196.8(188.4) [a] 156.8(26.1-450.5)	157.6(145.6) [b] 125.5(11.1-455.6)	147.2(118.3) [b] 112.7(9.9-410.2)	*176.1(171.5)* *136.4(15.0-444.5)*
Sperm concentration, ×10^6/mL	55.15(40.74) [a] 44.31(10.11-136.93)	48.26(36.31) [b] 41.28(5.38-137.25)	47.36(35.25) [b] 39.75(4.63-117.85)	*52.49(39.16)* *43.00(7.44-131.58)*
Progressive motility, %	46.6(25.9) [a] 44.7(5.9-89.8)	43.6(26.7) [ab] 42.8(2.8-88.1)	39.3(26.0) [b] 36.1(3.2-83.6)	*43.8(26.6)* *41.7(3.3-89.0)*
Normal morphology, %	6.9(3.0) [a] 6.8(2.3-12.0)	6.4(3.0) [b] 6.1(1.9-11.5)	6.6(3.0) [ab] 6.8(2.0-11.3)	*6.6(3.0)* *6.5(2.0-11.8)*
TZI	1.48(0.12) [a] 1.47(1.32-1.69)	1.49(0.11) [ab] 1.48(1.32-1.71)	1.50(0.12) [b] 1.49(1.33-1.73)	*1.49(0.12)* *1.48(1.32-1.71)*
DFI, %	8.25(5.37) [a] 6.88(2.74-20.35) (n=180)	10.11(7.95) [a] 7.30(2.72-26.25) (n=36)	14.66(12.72) [b] 8.53(2.54-36.89) (n=29)	*9.28(7.29)* *7.06(2.70-25.74) (n=245)*
LH, mIU/mL	3.48(1.39) [a] 3.23(1.59-6.02)	3.68(1.53) [a] 3.38(1.48-6.33)	3.86(1.56) [a] 3.66(1.42-6.64)	*3.58(1.46)* *3.30(1.55-6.24)*
FSH, mIU/mL	3.91(2.20) [a] 3.55(1.50-7.86)	4.08(2.13) [a] 3.63(1.47-8.21)	4.16(2.78) [a] 3.55(1.39-8.88)	*3.98(2.28)* *3.59(1.47-8.10)*
Testosterone, nmol/L	21.02(7.56) [a] 19.88(10.80-34.54)	20.09(7.13) [a] 19.24(10.12-33.24)	20.66(7.85) [a] 19.55(10.58-37.48)	*20.76(7.51)* *19.68(10.60-34.33)*
Estradiol, nmol/L	0.204(0.064) [ab] 0.195(0.113-0.317)	0.213(0.059) [b] 0.207(0.119-0.330)	0.194(0.049) [a] 0.192(0.122-0.284)	*0.204(0.061)* *0.197(0.115-0.314)*
Inhibin B, pg/mL	184.5(63.8) [a] 174.2(98.3-300.3)	172.7(58.8) [b] 168.7(84.4-274.8)	171.0(72.5) [b] 164.0(61.1-311.5)	*178.4(65.0)* *171.5(83.3-295.6)*
TG, mmol/L	1.06(0.65) [a] 0.87(0.39-2.34)	1.12(0.73) [a] 0.93(0.40-2.73)	1.30(0.78) [b] 1.06(0.43-2.90)	*1.10(0.68)* *0.91(0.39-2.39)*
TC, mmol/L	3.88(0.89) [a] 3.77(2.65-5.46)	3.93(0.90) [a] 3.84(2.75-5.57)	4.00(0.86) [a] 3.89(2.77-5.52)	*3.90(0.88)* *3.80(2.67-5.47)*
HDL-C, mmol/L	1.16(0.34) [a] 1.12(0.70-1.74)	1.14(0.32) [a] 1.11(0.70-1.70)	1.08(0.31) [b] 1.03(0.65-1.56)	*1.15(0.33)* *1.11(0.69-1.73)*

(Continued)

TABLE 1 Continued

Parameters	Smoking status			Total (n=1222)
	Non-smokers (n=772)	Moderate smokers cigarettes/day ≤10 (n=274)	Heavy smokers cigarettes/day >10 (n=176)	
LDL-C, mmol/L	2.24(0.89) [a] 2.11(1.03-3.82)	2.27(0.85) [a] 2.22(1.10-3.90)	2.33(0.81) [a] 2.27(1.19-3.72)	*2.26(0.86)* *2.15(1.07-3.82)*
Fasting glucose, mmol/L	4.8(0.9) [a] 4.7(3.6-6.1)	4.9(1.0) [a] 4.8(3.6-6.5)	5.0(1.0) [a] 4.8(3.6-6.8)	*4.8(0.9)* *4.8(3.6-6.3)*
Uric acid, µmol/L	350(92) [a] 340(225-516)	347(90) [a] 333(225-498)	365(101) [b] 354(246-540)	*352(93)* *340(226-518)*
Serum zinc concentration, µmol/L	22.5(9.0) [a] 20.2(13.7-39.2)	22.0(8.5) [a] 19.7(13.4-40.9)	25.2(11.8) [b] 21.2(14.1-51.3)	*22.8(9.4)* *20.1(13.7-41.3)*
Seminal zinc concentration, µmol/L	1589(1001) [a] 1423(395-3527)	1474(923) [ab] 1233(354-3469)	1392(954) [b] 1133(336-3042)	*1536(980)* *1325(372-3487)*
Seminal zinc content, µmol/ejaculate	5.77(4.65) [a] 4.45(1.07-14.04)	5.03(4.11) [ab] 3.85(0.78-11.99)	4.52(3.53) [b] 3.68(0.85-11.20)	*5.43(4.41)* *4.20(0.94-13.19)*

Results based on raw data. Values are presented as mean (SD) and median (5-95th percentile). BMI, body mass index; BTV, paired testicular volume; TZI, teratozoospermia index; DFI, sperm DNA fragmentation index; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; a, b - comparisons with different superscripts within variable are significant (p<0.05).

parameters in men of these three groups have been described elsewhere (73). Briefly, the higher semen quality was found in Slavs, the average in Buryats and the lowest in Yakuts due to the higher testicular function in Slavs compared to Asians. Some anthropometric indicators (weight, height, waist and hip circumference) were higher for Slavs than for Buryats, and weight and height were higher for Slavs than for Yakuts. In addition to published ethnic data, we included in this study the metabolic and zinc parameters, and smoking consumption. The proportion of male smokers and the smoking intensity (the number of cigarettes smoked per day) differed significantly between ethnic groups (p<0.05, Table 2). The prevalence of cigarette smoking was 32.1% among Slavs, 46.6% among Buryats, 44.8% among Yakuts, the latter two did not differ from each other. The median smoking intensity was the highest among the Slavs, moderate among the Buryats and Yakuts, the latter two did not differ from each other (12.5; 9.0; 6.3 cigarettes per day, respectively).

The effect of cigarette smoking on anthropometric, seminal, hormonal, zinc and metabolic parameters in men of different ethnic groups is presented in Table 2. Two categories of smokers were combined in each ethnic group due to the small number of heavy smokers among the Buryats and Yakuts. In the Buryat group, smokers did not differ from non-smokers in all the parameters studied (Table 2). In the Yakut group, smokers had higher weight, waist circumference, BMI and TG level, as well as lower testosterone and inhibin B levels compared with non-smokers (p<0.05, Table 2). In the Slavic group, smokers had significantly reduced semen volume, total sperm count, sperm concentration, progressive motility, and significantly increased TZI and DFI% compared with non-smokers (p<0.05, Table 2). In smoking Slavic men, the impairment in semen quality was associated with a decrease in the seminal zinc concentration and content (p<0.05, Table 2). In addition, in this ethnic group there was a significant increase in waist circumference, the triglyceride and glucose levels in smokers compared to non-smokers indicating a metabolic disorder (p<0.05, Table 2). Thus, the most pronounced negative effects of cigarette smoking were found in Slavs, weaker negative effects were found in Yakuts, and no negative effects of smoking were found in Buryats.

Discussion

Our study is a rather large-scale observational investigation of the effects of tobacco smoking on semen quality, hormonal, zinc and metabolic parameters in men from Russia. According to our data, the prevalence of smoking among Russian men with a median age of 23 years was 36.8% and coincided with WHO data (14) for the world population (36.7%). In the first part of our study, we studied the effect of cigarette smoking in the multi-ethnic study population, whereas in the second part we focused on the reproductive consequences of smoking in selected ethnic groups. The first part of the study included two categories of cigarette smoking (moderate smokers and heavy smokers) to examine the dose-dependent associations between cigarette smoking and the indicators studied. The most important consequences of cigarette smoking were lower semen parameters, including semen volume, total sperm count, sperm concentration and progressive motility, increased sperm DNA fragmentation and teratozoospermia. Our results on men from the general population are consistent with conclusions of many other studies in infertile men (21, 33–36, 81–83), in men from the general population (32, 51, 54, 84, 85) or in case-control studies (29–31, 38, 39). Although the mechanisms by which smoking may be linked to detrimental effects on semen parameters remain to be fully elucidated, they are due to a direct exposure of the toxic content of cigarette smoke (mutagens, carcinogens, nicotine and its metabolites, heavy metals) (12, 20, 22, 81). An additional way may be associated with the direct effect of tobacco components, including nicotine, on the function of

TABLE 2 Comparison of smokers and non-smokers by anthropometric, seminal, hormonal, zinc and metabolic parameters in different ethnic groups.

Parameters	Buryats (n=191)		Slavs (n=654)		Yakuts (n=125)	
	Non-smokers (n=102)	Smokers (n=89; 46.6%)	Non-smokers (n=444)	Smokers (n=210; 32.1%)	Non-smokers (n=69)	Smokers (n=56; 44.8%)
Age, years	23.6(6.6) 21.0(18.0-35.0)	24.4(6.6) 22.0(18.0-38.0)	25.6(7.5) 23.0 (18.0-40.0)	26.1(7.7) 24.0 (19.0-40.0)	24.5(6.4) 22.0 (18.0-33.0)	26.2(7.0) 26.0 (18.0-39.0)
Body weight, kg	70.0(11.7) 67.0(56.0-88.0)	71.6(15.1) 70.0 (53.0-95.5)	78.1(13.7) 75.0 (60.0-103.2)	78.7(14.3) 77.0(60.0-105.0)	68.4(12.5) 65.0 (50.0-90.0)	74.9(14.7) * 73.0 (55.0-106.0)
Height, cm	175.1(6.6) 174.0(165.0-187.0)	174.4(6.1) 174.0 (165.0-184.0)	179.4(6.9) 180.0 (169.0-190.0)	178.5(6.6) (168.0-190.0)	171.8(172.0) 162.0(181.0-5.6)	173.0(6.7) 173.0 (161.0-182.0)
Waist circumference, cm	81.2(10.7) 78.5(68.0-101.0)	83.1(13.9) 82.0 (67.0-103.0)	84.3(10.2) 82.0 (71.0-104.0)	86.2(11.1) * 85.0 (71.0-105.0)	83.0(11.0) 81.0 (68.0-103.0)	88.5(13.7) * 86.5 (69.0-112.0)
Hip circumference, cm	93.6(6.6) 92.0(84.0-104.0)	94.6(8.3) (94.084.0-106.0)	99.2(8.0) 98.0 (88.0-113.0)	99.3(8.4) 99.5 (87.0-112.0)	96.5(7.0) 96.0 (87.0-108.0)	98.8(8.6) 96.0 (88.0-116.0)
BMI, kg/m^2	22.8(3.7) 21.8(18.4-30.8)	23.6(5.0) 23.0 (17.2-30.6)	24.2(3.8) 23.7 (19.2-31.4)	24.7(4.1) 24.2 (18.9-31.2)	23.2(4.0) 22.9 (17.4-31.1)	25.1(4.9) * 23.8 (18.4-35.3)
BTV, ml	36.2(6.9) 38.3(25.0-50.0)	37.0(5.8) 40.0 (27.0-45.0)	40.9(8.6) 40.0 (27.0-55.0)	41.8(8.8) 40.0 (30.0-60.0)	36.9(9.1) 36.0 (29.0-56.0)	36.6(6.7) 36.0 (28.0-48.0)
Smoking, sigarettes/day	0	9.1(5.4) 9.0 (2.0-20.0)	0	12.7(6.8) 12.5 (2.0-20.0)	0	8.2(5.6) 6.3 (1.5-20.0)
Semen volume, ml	3.2(1.2) 3.0(1.5-5.3)	3.1(1.4) 2.8 (1.3-5.9)	3.8(1.7) 3.7 (1.5-6.6)	3.4(1.8) * 3.0 (1.1-6.6)	3.0(1.1) 3.0 (1.5-5.3)	3.1(1.4) 2.9 (1.1-5.9)
Total sperm count, ×10$^{6/}$ejaculate	159.7(140.0) 124.9(33.6-379.9)	129.7(106.3) 103.9 (14.8-370.3)	217.2(202.2) 178.2 (27.4-462.6)	167.8(139.7) * 136.5 (5.8-455.6)	104.2(85.5) 84.8 (16.6-244.2)	106.1(80.7) 79.6 (8.4-272.4)
Sperm concentration, ×10^6/mL	50.89(38.45) 41.16(14.00-120.00)	42.78(30.87) 38.00 (6.37-97.38)	59.99(43.95) 49.09 (10.38-149.18)	52.40(39.70) * 43.38 (3.94-134.52)	36.34(26.04) 33.13 (5.50-92.13)	35.79(26.78) 30.80 (4.50-96.14)
Progressive motility, %	49.8(25.7) 46.1(10.3-89.9)	46.5(27.9) 46.5 (2.8-88.1)	46.7(26.3) 44.3 (5.8-90.6)	41.0(27.2) * 37.8 (2.9-87.3)	37.2(21.0) 34.0 (8.7-73.2)	38.2(24.5) 37.9 (5.8-90.1)
Normal morphology, %	7.0(2.7) 7.0(2.9-11.9)	6.8(2.6) 6.5 (2.8-11.3)	7.2(3.1) 7.0 (2.6-12.8)	6.8(3.3) 6.8 (1.9-11.8)	4.6(2.3) 4.8 (1.0-8.5)	5.3(2.4) 5.3 (1.3-9.5)
TZI	1.49(0.12) 1.47(1.30-1.66)	1.47(0.10) 1.45 (1.33-1.64)	1.48(0.11) 1.47 (1.32-1.69)	1.50(0.13) * 1.49 (1.31-1.76)	1.52(0.12) 1.52 (1.35-1.73)	1.53(0.11) 1.51 (1.33-1.74)
DFI, %	-	-	8.29(5.53) (n=138) 6.81(2.70-20.99)	13.15 (11.24) * (n=49) 8.63 (2.69-36.89)	-	-
LH, mIU/mL	3.81(1.41) 3.43(2.02-6.05)	4.06(1.59) 3.75 (1.92-6.82)	3.45(1.38) 3.20 (1.61-6.09)	3.60(1.47) 3.33 (1.48-6.40)	3.42(1.40) 3.09 (1.53-6.19)	3.84(1.72) 3.95 (1.25-7.02)
FSH, mIU/mL	4.58(2.28) 4.17 (1.86-8.32)	4.24(1.99) 3.95 (1.56-8.80)	3.69(2.31) 3.32 (1.45-7.86)	3.55(1.87) 3.16 (1.29-6.91)	4.67(1.70) 4.53 (2.00-7.53)	5.50(3.70) 4.73 (1.91-12.06)
Testosterone, nmol/L	19.05(6.19) 17.74 (11.37-31.31)	18.24(5.39) 17.67 (9.71-27.98)	21.21(7.51) 20.29 (10.54-34.54)	21.46(8.28) 20.44 (10.55-37.12)	21.32(7.60) 21.87 (10.80-33.36)	18.83(6.52) * 17.57 (10.52-31.79)
Estradiol, nmol/L	0.235(0.068) 0.220 (0.163-0.351)	0.222(0.048) 0.204 (0.162-0.298)	0.192(0.061) 0.182(0.107-0.312)	0.192(0.058) 0.186 (0.114-0.297)	0.234(0.062) 0.217 (0.162-0.354)	0.220(0.044) 0.212 (0.160-0.293)
Inhibin B, pg/mL	144.7(56.1) 146.2 (43.8-245.6)	155.5(64.0) 153.9 (51.5-258.8)	193.5(64.8) 184.3 (104.2-305.7)	183.5(68.1) 179.9 (88.7-307.3)	172.6(46.3) 163.1 (111.1-271.7)	149.4(49.0) * 156.3 (66.1-227.6)
TG, mmol/L	1.04(0.71) 0.84 (0.38-2.35)	1.04(0.70) 0.85 (0.44-2.25)	1.09(0.68) 0.87 (0.39-2.49)	1.27(0.86) * 1.02 (0.41-3.12)	1.04(0.59) 0.85 (0.44-2.33)	1.30(0.66) * 1.16 (0.43-2.90)

(Continued)

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TABLE 2 Continued

Parameters	Buryats (n=191)		Slavs (n=654)		Yakuts (n=125)	
	Non-smokers (n=102)	Smokers (n=89; 46.6%)	Non-smokers (n=444)	Smokers (n=210; 32.1%)	Non-smokers (n=69)	Smokers (n=56; 44.8%)
TC, mmol/L	3.73(0.81) 3.56 (2.60-5.13)	3.85(0.85) 3.82 (2.65-5.38)	3.87(0.88) 3.79 (2.66-5.48)	3.99(0.91) 3.93 (2.75-5.74)	4.01(1.02) 3.95 (2.67-6.80)	4.04(0.81) 4.02 (2.94-5.35)
HDL-C, mmol/L	1.04(0.26) 1.03 (0.69-1.54)	1.08(0.29) 1.04 (0.70-1.66)	1.18(0.36) 1.14 (0.69-1.75)	1.15(0.32) 1.12 (0.66-1.69)	1.16(0.33) 1.11 (0.68-1.82)	1.12(0.35) 1.08 (0.68-1.69)
LDL-C, mmol/L	2.21(0.72) 2.07 (1.25-3.57)	2.29(0.81) 2.26 (1.13-3.86)	2.20(0.89) 2.12 (0.99-3.77)	2.27(0.85) 2.22 (1.08-3.76)	2.38(1.04) 2.15 (1.15-4.91)	2.34(0.76) 2.29 (1.23-3.76)
Fasting glucose, mmol/L	4.9(0.9) 4.8 (3.7-6.6)	4.9(6.4) 0.9 (4.8 -3.5)	4.8(1.0) 4.7 (3.6-6.2)	5.0(0.9) * 4.8 (3.7-6.8)	4.6(0.7) 4.5 (3.3-5.7)	4.9(1.4) 4.9 (3.3-8.0)
Uric acid, µmol/L	346(73) 340(229-470)	344(78) 333 (238-495)	355(91) 345 (225-526)	369(102) 355 (240-539)	333(112) 319 (197-476)	345(105) 332 (208-512)
Serum zinc concentration, µmol/L	19.6(7.5) 17.4(12.9-31.5)	21.3(10.8) 17.5 (12.8-49.5)	23.9(9.9) 20.9 (14.8-42.5)	25.6(11.2) 21.6 (14.9-50.0)	20.5(6.9) 18.6 (12.5-35.3)	21.3(7.1) 19.2 (14.0-32.2)
Seminal zinc concentration, µmol/L	1419(869) 1181(355-2999)	1522(968) 1159 (354-3925)	1725(1077) 1514 (398-3882)	1443(995) * 1162 (348-3483)	1368(979) 1128 (395-2643)	1531(752) 1440 (460-3139)
Seminal zinc content, µmol/ejaculate	4.41(3.20) 3.69 (0.68-11.22)	4.60(3.89) 3.42 (0.89-11.99)	6.46(5.12) 5.16(1.30-15.49)	5.11(4.39) * 3.76 (0.85-12.03)	4.13(2.96) 3.23(0.81-9.57)	4.94(3.06) 4.19(0.90-10.74)

Results based on raw data. Values are presented as mean (SD) and median (5-95th percentile); BMI, body mass index; BTV, paired testicular volume. TZI, teratozoospermia index; DFI, DNA fragmentation index; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; * - differences between groups of smokers and non-smokers within each ethnic group are significant ($p<0.05$).

accessory glands (seminal vesicles and prostate gland), which alter the semen volume and the functional properties of sperm (12, 19, 20, 81, 86). Moreover, oxygen deficiency produced by cigarette smoking affects spermatogenesis, resulting in impaired sperm production (9, 81).

In our study, we also investigated how smoking affects sperm DNA fragmentation in men. It was shown that DFI% increased by about 75% in heavy smokers compared to non-smokers. These findings are consistent with the results of previous reports (43, 64, 87, 88) and confirm the conclusions of recent reviews (12, 20, 22, 41, 42, 81). Most studies have shown that the negative effect of smoking on sperm DNA can mainly be caused by excessive production of reactive oxygen species (ROS) (20, 22, 39, 43, 81), assuming that the increase in sperm DNA fragmentation in heavy smokers in our study may also be caused by an imbalance between antioxidant and pro-oxidant factors.

In our multi-ethnic study population, tobacco smoking did not cause an imbalance in the LH, FSH, testosterone and estradiol levels involved in the control of spermatogenesis, with the exception of inhibin B. These results are consistent with previously reported (21, 54, 55, 61). The hormonal results of our study are in line with the results of two population-based studies, which did not reveal a significant effect of smoking on the levels of testosterone, estradiol, inhibin B, sex hormone binding globulin, LH and/or FSH in young men (54, 55). However, as a rule, studies show contradictory associations between smoking and reproductive hormones (48, 49), and both decreased (38, 53, 63) and increased (50–52, 56, 58, 62) testosterone, LH and/or FSH levels have been described in smokers as compared to non-smokers.

The results of our study showed that cigarette smoking had an adverse effect on the metabolic health of smokers (an increase in triglycerides and uric acid, as well as a decrease in the HDL-C), which corresponds to the data obtained in other studies (71, 89–91). A meta-analysis conducted by Kauss and co-authors demonstrated that smoking was also associated with a decrease in the levels of apolipoproteins AI and AII and an increase in the level of apolipoprotein B, thereby confirming the negative impact of smoking on lipid metabolism (92). A possible mechanism for how cigarette smoking can alter the lipid profile has been proposed (92, 93). During cigarette smoking, nicotine stimulates the secretion of catecholamines and cortisol, activating adenylcyclase in adipose tissue, which leads to both lipolysis and the release of free fatty acids into the circulation, as well as to an increase in the lipoprotein production, including the HDL concentration. But, on the other hand, the release of catecholamines leads an increase in heart rate, blood pressure and cardiac output and increases the risk of cardiovascular disease. In our study, the combination of an altered lipid profile and increased body weight, waist circumference and BMI, which we found in heavy smokers, may further increase the risk of cardiovascular diseases.

It is considered proven that many of the harmful consequences of cigarette smoking are due to smoking-induced increase in the production of reactive oxygen species, leading to oxidative stress (9, 13, 20, 22, 39, 43, 64, 94, 95). The data obtained in our study correspond to the idea that smoking-induced oxidative stress appears to be the main mediating mechanism of the damaging effects of tobacco on male reproductive function, leading to the reduced semen quality, sperm DNA fragmentation, as well as an imbalance of lipid profile, which ultimately weakens male fertility.

Despite a fairly large amount of data on the impact of cigarette smoking on men's reproductive health, often authors receive conflicting data, the interpretation of which is ambiguous and does not reach consensus. Probably, the magnitude of the negative effect depends on environmental and climatic conditions, ethnic origin and cultural traditions of the population, and, most likely, the effect will manifest itself with pronounced and prolonged exposure. There are also some uncertainties regarding the methodical questions, in particular, the dose-dependent effects of smoking on male reproductive health, which may partially explain the inconsistency of the semen and hormonal results. For example, some researchers classify smokers as those who smoke from 1 to 15 cigarettes per day, while others take into account smoking experience and include in the group of male smokers only those who regularly smoke during the year (23).

In the second part of our study, we tried to find out how smoking affects reproduction-related parameters in various ethnic groups. Men of three ethnic groups - Buryats, Slavs and Yakuts - were selected from our multi-ethnic study population. Our previous studies have demonstrated significant reproductive differences between these ethnic groups on semen quality, hormonal levels, and genetic factors (73, 74). In the present study, we have shown that the proportion of smokers was the highest in the Buryat group and the lowest among the Slavs, the Yakuts occupied an intermediate position, but the smoking intensity (cigarettes per day) was the highest among the Slavs and moderate among the Buryats and Yakuts, who did not differ in this parameter. The data obtained suggest a greater tobacco addiction in Slavs than in both Asian groups. Moreover, the most pronounced negative effects of cigarette smoking were found in men of Slavic ethnicity, weaker in Yakuts, and no negative effects were found in Buryats. The main harmful impact of smoking on reproductive function was noted among Slavs, including semen quality, sperm DNA fragmentation, seminal zinc content and metabolic profile.

In our previous study, we found that semen parameters were close and positively related to seminal zinc content, while seminal zinc deficiency was associated with a decrease in semen quality (68). In this study, we showed that in the entire study population and in a separate Slavic group, smokers had both a lower seminal zinc content and impaired semen parameters, including increased sperm DNA fragmentation, compared with non-smokers. Previously published studies have also indicated the adverse impact of smoking on the seminal zinc level (64–67, 69, 70). Our current zinc data coupled with previous published data suggest that seminal zinc is involved in the effect of cigarette smoking on semen parameters. Zinc is a cofactor for various proteins and part of the antioxidant defense, playing an important role in the inactivation of excess reactive oxygen species (ROS) in seminal plasma, as reported by recent reviews (96, 97). It seems obvious that cigarette smoking reduces the seminal zinc level due to increased ROS production and leads to depletion of the semen antioxidant defense.

The ethno-specific effects of cigarette smoking identified in this study may be associated with higher cigarette consumption and, consequently, with more harmful effect of nicotine and other tobacco compounds in the Slavic group compared to both Asian groups, in which smoking did not lead to severe damage. Moreover, taking into account the smoking-induced oxidative stress as the main damaging mechanism, it can be assumed that the ethno-specific effects of smoking may be due to a different genetic background and are connected with genetic differences in the activity of antioxidant enzymes. The seminal plasma possesses an antioxidant system capable of counteracting the harmful effects of ROS, but smoking alters the balance between total antioxidant capacity and ROS production, increasing lipid peroxidation and reducing antioxidant activity, including seminal zinc, vitamin C, and enzymes (superoxide dismutase containing zinc, glutathione peroxidase, catalase) and others antioxidants (20, 95–97). In our previous study, we compared the level of lipid peroxidation and antioxidants in the seminal plasma of young Buryats and Slavs (98). The results showed a more active functioning of the antioxidant system in the Buryats compared to the Slavs. In the present study, a decrease in the seminal zinc level of smoking Slavs compared to non-smokers is an additional evidence of a decline in semen antioxidant defense. Our study highlighted the role of the genetic background in the reproductive effects of smoking, presumably genes involved in antioxidant defense. One study showed that the combination of smoking and one functional polymorphism of the antioxidant gene NRF2 (erythroid 2-related factor 2) led to a significant decrease in semen quality, a fact that is in line with our above assumption (99).

Further research is needed to elucidate the ethno-specific molecular pathways by which cigarette smoking can affect spermatogenesis, which could also explain the conflicting data on the detrimental effects of cigarette smoking on semen quality. Returning to the underlying lifestyle causes of adverse temporal trends in the male reproductive potential, which were first reported more than half a century ago, we can assume, based on the facts obtained in this study, a significant contribution of tobacco smoking to this phenomenon, taking into account its ethno specific features.

A strength of the study is the rather large cohort of men with detailed demographic characteristics, ethnic origin, lifestyle factors, including smoking, past and current diseases for each participant. In the study, we evaluated a wide range of 30 anthropometric, seminal, hormonal, zinc and metabolic parameters in order to more comprehensively analyze the reproductive consequences of cigarette smoking. In addition, we included in the study young men from the general population, who were quite similar in social status; used a standardized recruitment protocol and questionnaire; all samples were collected and processed by the same scientific team using the same laboratory methods, equipment and supplies. The large study population allowed us to adjust the data for known confounders when comparing groups. Given the wide diversity of nationalities in Russia, we took this opportunity to clarify the ethno-specific impact of cigarette smoking on reproduction-related indicators, which can deepen existing knowledge about the reproductive consequences of smoking and have obvious advantages when integrating the results into clinical practice and research.

The study has some limitations. Our study is retrospective and observational in nature, which did not allow us to draw exhaustive conclusions about mediating mechanisms. In addition, cigarette smoking status was self-reported, resulting in risk of bias. It was

difficult to accurately determine the time frame for starting smoking, since the participants, as a rule, gradually and intermittently became involved in tobacco addiction. Furthermore, this study did not analyze antioxidants and antioxidant enzymes other than zinc to determine the extent of damage of the antioxidant system, especially in heavy smokers. Finally, the number of Buryat and Yakut smokers, as well as their data on sperm DNA fragmentation, have been limited and should be expanded in future studies.

Conclusion

Our study showed the negative impact of cigarette smoking habits on male reproductive potential in adult Russian men from the general multi-ethnic population. In smokers, who smoked more than 10 cigarettes a day, we observed reduced semen volume, total sperm count, sperm concentration, progressive motility, seminal zinc levels, increased sperm DNA fragmentation and teratozoospermia, as well as metabolic imbalance. When investigating the effects of smoking in the selected ethnic groups, the most pronounced negative effects were found in Slavs, weaker effects in Yakuts, and no effects in Buryats. Reduced semen quality and seminal zinc content, increased sperm DNA fragmentation and teratozoospermia, serum levels of triglycerides and fasting glucose were detected in Slavic smokers, while Yakut smokers demonstrated only a slight decrease in testosterone and inhibin B levels as well as an increase in triglycerides. The data obtained indicate the ethno-specific effect of cigarette smoking on the male reproductive potential and suggest that ethnic differences may be due to a genetic background, presumably genes regulating the antioxidant system, taking into account smoking-induced oxidative stress as the main mediating mechanism.

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 Research Institute of Clinical and Experimental Lymphology – Branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences (RICEL- Branch of IC&G SB RAS). 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

LO: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing. MK: Formal Analysis, Investigation, Writing – original draft. AO: Conceptualization, Data curation, Methodology, Resources, Software, Writing – original draft.

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Conflict of interest

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

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Huitao Li,
Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, China

REVIEWED BY
Xiang Xiao,
Hangzhou Medical College, China
Siwen Wu,
The State University of New Jersey, United States
Annabel Mao,
Wenzhou Medical University, China

*CORRESPONDENCE
Sergio Minucci
sergio.minucci@unicampania.it

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A short-term high-fat diet alters rat testicular activity and blood-testis barrier integrity through the SIRT1/NRF2/MAPKs signaling pathways

Sara Falvo[1], Sergio Minucci[2]*, Alessandra Santillo[1], Rosalba Senese[1], Gabriella Chieffi Baccari[1] and Massimo Venditti[2]

[1]Dipartimento di Scienze e Tecnologie Ambientali, Biologiche e Farmaceutiche, Università degli Studi della Campania 'Luigi Vanvitelli', Caserta, Italy, [2]Dipartimento di Medicina Sperimentale, Sez. Fisiologia Umana e Funzioni Biologiche Integrate, Università degli Studi della Campania 'Luigi Vanvitelli', Napoli, Italy

Background: Overweight/obesity are metabolic disorder resulting from behavioral, environmental, and heritable causes. WHO estimates that 50% of adults and 30% of children and adolescents are overweight or obese, and, in parallel, an ongoing decline in sperm quality and male fertility has been described. Numerous studies demonstrated the intimate association between overweight/obesity and reproductive dysfunction due to a highly intricate network of causes not yet completely understood. This study expands the knowledge on the impact of a short-term high-fat diet (st-HFD) on rat testicular activity, specifically on steroidogenesis and spermatogenesis, focusing on the involved molecular mechanisms related to mitochondrial dynamics, blood-testis barrier (BTB) integrity, and SIRT1/NRF2/MAPKs pathways.

Methods: Ten adult Male Wistar rats were divided into two groups of five and treated with a standard diet or an HFD for five weeks. At the end of the treatment, rats were anesthetized and sacrificed by decapitation. Blood was collected for serum sex hormone assay; one testis was stored at -80ÅãC for western blot analysis, and the other, was fixed for histological and immunofluorescence analysis.

Results: Five weeks of HFD results in reduced steroidogenesis, increased apoptosis of spermatogenic cells, and altered spermatogenesis, as highlighted by reduced protein levels ofmeiotic and post-meiotic markers. Further, we evidenced the compromission of the BTB integrity, as revealed by the downregulation of structural proteins (N-Cadherin, ZO-1, occludin, connexin 43, and VANGL2) other than the phosphorylation of regulative kinases (Src and FAK). At the molecular level, the impairment of mitochondrial dynamics (fission, fusion, andbiogenesis), and the dysregulation of the SIRT1/NRF2/MAPKs signaling pathways, were evidenced. Interestingly, no change was observed in the levels of pro-inflammatory markers (TNFα, NF-kB, and IL-6).

Conclusions: The combined data led us to confirm that overweight is a less severe state than obesity. Furthermore, understanding the molecular mechanisms behind the association between metabolic disorders and male fertility could improve the possibility of identifying novel targets to prevent and treat fertility disorders related to overweight/obesity.

KEYWORDS

overweight, steroidogenesis, spermatogenesis, meiosis, mitochondria, inflammation

1 Introduction

An intimate connection between balanced nutrition and the preservation of a good state of human health exists, in fact a salubrious diet is associated with a reduction in morbidity and premature mortality (1–3). Many studies reported that, especially in industrialized countries, a considerable percentage of non-communicable diseases (obesity, diabetes, cardiovascular disorders, and even some types of cancer) are correlated, directly or indirectly, to the consumption of unhealthy food, particularly those with the high trans-fatty acids and low essential nutrients content (vitamins, minerals, and proteins) (4–6). It has been estimated that obesity and overweight, syndromes characterized by the accumulation of excessive fatty tissue in the body, affect more than 1.9 billion adults worldwide, rising from epidemic to pandemic states (7). Such high prevalence, accompanied by severe social and economic consequences, makes obesity/overweight one of the major global health issues (8). It is important to note that being overweight may be considered a preclinical condition less severe than obesity, since the excessive accumulation of body fat increases, in turn, the risk of chronic diseases (9). The most used parameter to define obesity is the body mass index (BMI), calculated as a person's weight (in kilograms) divided by the square of his/her height (in meters) (10). Conversely, more accurate but less used indexes, such as waist circumference and weight gain, may provide more reliable and individualized parameters to define the consequence of excessive body fat accumulation on the development of chronic disease (11). Obesity rates have significant impacts on personal and public health; however, overweight status is often trivialized as a mere body image issue (12, 13).

Besides the well-known comorbidities associated with obesity, including dyslipidemia, type 2 diabetes, and hypertension, a growing body of evidence is now focusing on its correlation with human infertility, as evidenced by the numerous papers published on this topic in recent years and, in particular, on the positive correlation between growing BMI and sub-infertility (14–16). Alteration of the hormonal milieu is one of the most evident effects of obesity. In overweight or obese men, excess body fat accumulation can increase the production of serum sex hormone-binding globulin. This glycoprotein, produced by the liver, binds to testosterone (T) and inhibits its biological action; this, along with increased aromatase (ARO) activity, leads to a decreased T/estradiol (E_2) ratio; estrogen increases and, inhibiting Leydig and Sertoli cell function, further impairs T production and the process of spermatogenesis (17–20).

Moreover, obesity has also been defined as a "systemic oxidative stress state", in which an imbalance between reactive oxygen species (ROS) production and antioxidant capacity occurs, leading to oxidative stress. This, ultimately, damages cellular components deleterious for male germ cells (GC), and particularly for spermatozoa (SPZ), as their plasma membrane contains high levels of polyunsaturated fatty acids, and their DNA, once damaged, cannot be repaired due to lack of the cytoplasmic enzymatic systems involved in DNA repair (17, 21–23). Several studies reported that, compared to normal-weight men, obese ones have a higher chance of oligozoospermia, asthenozoospermia, and an increased rate of fragmented DNA in sperm (24–28). Furthermore, in a meta-analysis, Campbell et al. (29) described that male obesity negatively impacts the success of assisted reproductive technology (ART). Interestingly, while changes in sex hormone levels may contribute to obesity-induced male sub-infertility, data from ART indicate that they may not be the only cause; in fact, obesity in men is associated with decreased pregnancy rates and increased pregnancy loss in couples subjected to ART, but, following intracytoplasmic sperm injection, the fertilization rate is considerably improved, indicating that obesity may alter sperm maturation, capacitation, and their ability to bind and fertilize the egg with still unknown mechanisms (29–31). In this regard, one of the most common tools to study obesity and its related comorbidities, including infertility, is the use of animal models, especially mice and rats, fed with a high-fat diet (HFD). The duration of the HFD is crucial; in a recent review, de Moura e Dias et al. (32) summarized the time-dependent effects of HFD in provoking obesity, assessing that at least 3 weeks of HFD are sufficient to obtain satisfactory results. However, to strengthen the phenotypic and metabolic characteristics of obesity, a longer intervention period (from 10 to 12 weeks) is necessary. Coherently, most of the studies focused on the impact of obesity on testicular activity, used a long-term HFD (10-14 or longer weeks of treatment) (33–36), while just a few papers used a different approach, with a short-term HFD (st-HFD), that is correlated to an overweight condition (37–39).

This may be interesting to obtain parameters to be used to monitor the progression of infertility related to being overweight,

even at the early stages before it progresses to obesity, which is considered a real "pathological state". In previous studies, we demonstrated that a 5-weeks st-HFD induced an increase in body weight and serum cholesterol and triglyceride levels, as well as alterations in testis and epididymis, i.e., induced oxidative stress, increased autophagy, apoptosis, and mitochondrial damage (40–42). Here, using the same rats fed with a st-HFD, we evaluated additional parameters of testicular activity, such as steroidogenesis and spermatogenesis, with special attention to the involved mechanisms related to mitochondrial dynamics, and blood-testis barrier (BTB) integrity. Undoubtedly, these key regulators are essential in the spermatogenic process, which guarantees the formation of high-quality gametes (43, 44); on the other hand, testicular cells mitochondria and BTB are two of the main targets highly sensitive to the non-physiological conditions, and particularly in a prooxidant milieu, induced either by environmental (such as the exposure to pollutants) (45–49), and pathological (like diabetes and obesity) (50–52) factors. Finally, because many reports demonstrated the association of SIRT1/NRF2/MAPKs pathways with testicular function altered by obesity (33, 53–55), we verified whether the abovementioned pathways may also be involved in the molecular mechanisms underlying the diet-induced testicular dysfunction obtained via a st-HFD.

2 Methods

2.1 Animals and tissue collection

Male Wistar rats (250–300 g, aged eight weeks) were kept in one per cage in a temperature-controlled room at 28°C (thermoneutrality for rats) under a 12-h light/12-h dark cycle. Before the beginning of the study, water, and a commercial mash (Charles River Laboratories, Calco, Italy) were available *ad libitum*. At the start of the study (day 0), and after seven days of acclimatization to thermoneutrality, the rats were divided into two groups of five and treated as follows:

- The first group of rats (n = 5, C) received a standard diet (total metabolizable percentage of energy: 60.4 carbohydrates, 29 proteins, 10.6 fat J/J; 15.88 kJ gross energy/g; Muscedola, Milan, Italy) for five weeks;
- The second group of rats (n = 5, st-HFD) received a HFD (280 g diet supplemented with 395 g of lyophilized lamb meat (Liomellin, Milan, Italy), 120 g cellulose (Sigma-Aldrich, St. Louis, MO, USA), 20 g mineral mix (ICN Biomedical, Solon, OH, USA), 7 g vitamin mix (ICN), and 200 g low-salt butter (Lurpak, Denmark). Approximate fatty acid profile of this diet was: 45% saturated (SFA), 45% MUFA, 10% PUFA. total metabolizable percentage of energy: 21 carbohydrates, 29 proteins, 50 fat J/J; 19.85 kJ gross energy/g) for five weeks.

At the end of the treatment, rats were anesthetized with intraperitoneal injection of chloral hydrate (40mg/100g body weight), sacrificed for decapitation. The trunk blood was collected and the serum was separated and stored at -20°C for later sex hormone determination. The testes were dissected out, one testis was rapidly immersed in liquid nitrogen and stored at -80°C for western blot (WB) analysis, and the other was fixed in Bouin' solution for histological analysis. This study is reported in accordance with ARRIVE guidelines. Animal care and experiments were conducted in accord with the guidelines of the Ethics Committee of the University of Campania "Luigi Vanvitelli" and the Italian Minister of Health (Permit Number: 704/2016-PR of the 15/07/2016; Project Number: 83700.1 of the 03/05/2015). Every effort was made to minimize animal pain and suffering.

2.2 Determination of serum T and E_2 levels

Sex steroid levels were determined in serum from control and st-HFD rats using T (#DKO002; DiaMetra, Milan, Italy) and E_2 (#DKO003; DiaMetra, Milan, Italy) enzyme immunoassay kits. The sensitivities were 32 pg/mL for T and 15 pg/mL for E_2.

2.3 Protein extraction and WB analysis

Total testicular proteins were extracted from control (n = 5) and st-HFD (n = 5) rats as described in Venditti et al. (56). Forty micrograms of total protein extracts were separated into SDS-PAGE (9 or 15% polyacrylamide) and treated as described in Venditti et al. (57). The membranes were incubated overnight at 4°C with primary antibodies, listed in Table S1. The concentration of proteins was quantified using ImageJ software (version 1.53 t; National Institutes of Health, Bethesda, USA). Each WB was performed in triplicate.

2.4 Histology and immunofliorescence (IF) analysis

For hematoxylin/eosin staining and immunolocalization analysis, 5 µm testis sections were dewaxed, rehydrated, and processed as previously described (58, 59). For details on the used antibodies, see Table S1. The cells' nuclei were marked with Vectashield + DAPI (Vector Laboratories, Peterborought, UK) and then observed under an optical microscope (Leica DM 5000 B + CTR 5000; Leica Microsystems, Wetzlar, Germany) with UV lamp, 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 IF signals and Proliferating Cell Nuclear Antigen (PCNA)/Synaptonemal complex protein 3 (SYCP3) positive cells were performed with 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.

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2.5 TUNEL assay

The apoptotic cells were identified in paraffin sections through the DeadEnd™ Fluorometric TUNEL System (#G3250; Promega Corp., Madison, WI, USA) following the manufacturer's protocol, with little modifications. Briefly, before the incubation with TdT enzyme and nucleotide mix, sections were blocked with 5% BSA and normal goat serum diluted 1:5 in PBS and then treated with PNA lectin, to mark the acrosome. Finally, the nuclei of the cells were counterstained with Vectashield + DAPI. The sections were observed with the same microscope described in Section 2.4. 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.

2.6 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 ± standard error mean (SEM).

3 Results

3.1 Effect of st-HFD on testicular steroidogenesis

Serum T levels in st-HFD rats were significantly reduced by about 28% compared to the controls ($p < 0.01$); by contrast no differences in E_2 levels between the two groups were evidenced (Figure 1A).

To better evaluate the effect of st-HFD on steroidogenesis, the protein levels of steroidogenic acute regulatory protein (StAR), and 3β-Hydroxysteroid dehydrogenase (3β-HSD), two enzymes involved in T biosynthesis, were analyzed (Figure 1B). WB analysis confirmed that st-HFD altered testicular steroidogenesis, as a decrease in StAR ($p < 0.05$; Figures 1B, C) and 3β-HSD ($p < 0.01$; Figures 1B, D) protein levels, as compared to the control, was observed. In addition, the protein level of ARO, the enzyme converting T into E_2, was also evaluated, however, results showed no difference between the two groups (Figures 1B, E)

The effects of st-HFD on steroidogenesis were further confirmed by an IF staining of StAR and 3β-HSD, which is shown in Figure 1F. The signals specifically localized into the interstitial Leydig cells (LC; asterisks; Figure 1F insets); however, fluorescence intensity analysis showed a weaker signal in st-HFD animals ($p < 0.01$; Figures 1G, H) as compared to the control.

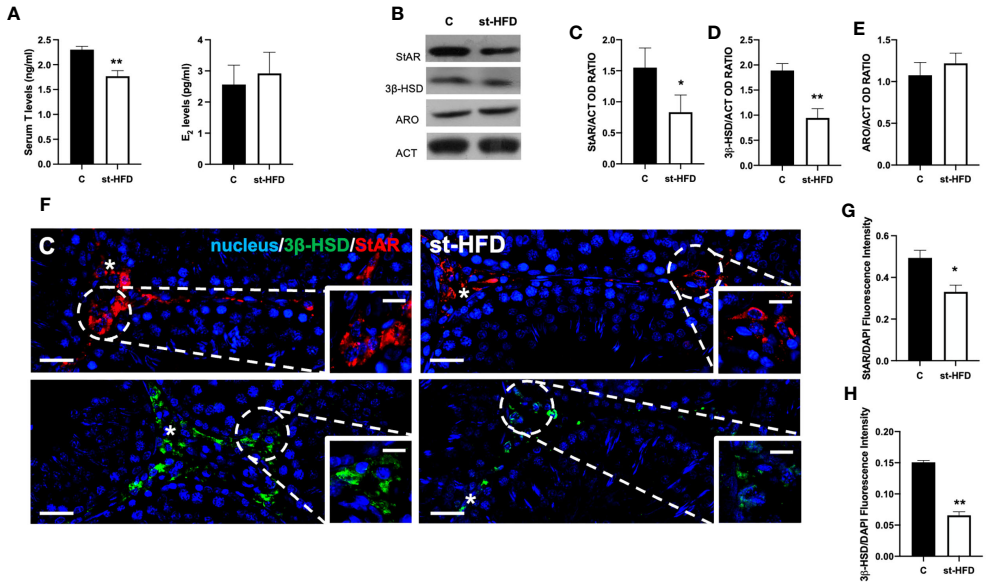

FIGURE 1
Steroidogenesis analysis of controls and st-HFD fed rat testis. **(A)** T and E_2 serum levels and **(B)** WB analysis of testicular StAR, 3β-HSD and ARO protein levels. **(C-E)** Histograms showing StAR, 3β-HSD, and ARO relative protein levels. **(F)** Testicular StAR (red) and 3β-HSD (green) immunolocalization. Slides were counterstained 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. Asterisks: LC. **(G, H)** Histogram showing the quantification of StAR and 3β-HSD fluorescence signal intensity, respectively. All values are expressed as means ± SEM from 5 animals in each group. *$p < 0.05$; **$p < 0.01$.

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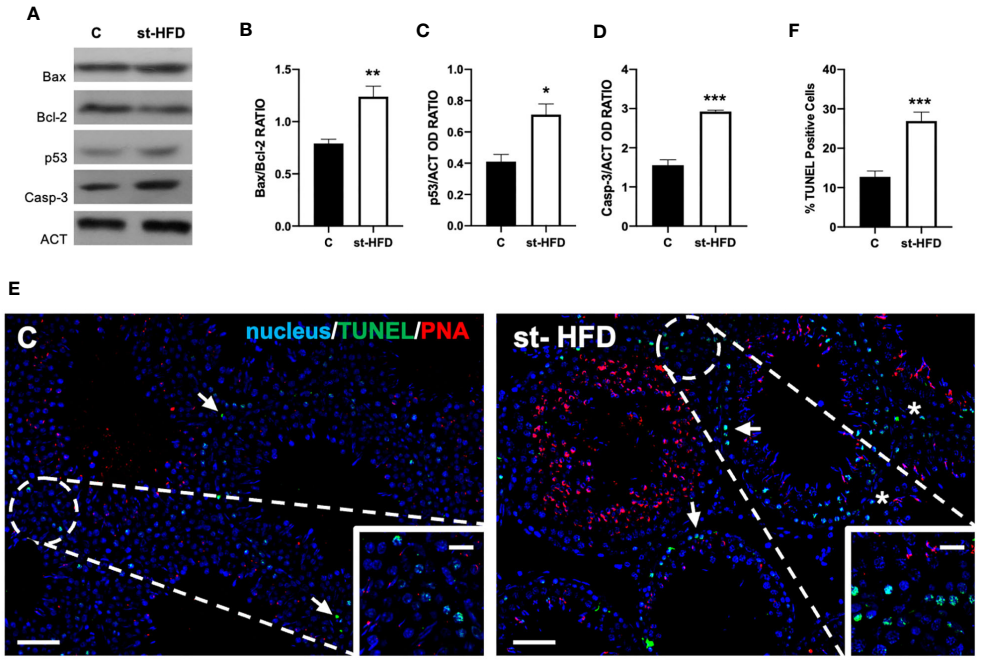

FIGURE 2
Apoptosis rate analysis of control and st-HFD fed rat testis. **(A)** WB analysis of testicular Bax, Bcl-2, p53, and Caspase-3. **(B-D)** Histograms showing the Bax/Bcl-2 ratio, p53, and Caspase-3 relative protein levels. **(E)** Determination of apoptotic cells through the detection of TUNEL-positive cells (green). Slides were counterstained with PNA lectin (red) and with DAPI-fluorescent nuclear staining (blue). The images were captured at x10 magnification (scale bars= 20 μm) and x20 (scale bars= 10 μm) for the insets. Arrows: SPG; Asterisks: LC. **(F)** Histogram showing the % of TUNEL-positive cells. All the values are expressed as means ± SEM from 5 animals in each group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.2 Effect of st-HFD on apoptosis

Figure 2 shows the effect of st-HFD on the apoptotic rate of germ and somatic cells. WB analysis revealed an increase in Bax/Bcl-2 ratio ($p < 0.01$; Figures 2A, B), p53 ($p < 0.05$; Figures 2A, C), and Caspase-3 ($p < 0.001$; Figures 2A, D) protein levels in the st-HFD group as compared to the control.

In support of these data, a TUNEL assay was performed (Figure 2E). Data showed the presence of dispersed apoptotic cells in the control group, especially spermatogonia (SPG; arrows and insets; Figure 2E). st-HFD induced an increase of 165% in the number of TUNEL-positive cells ($p < 0.001$; Figures 2E, F), particularly of SPG, as well as scattered apoptotic LC in the interstitial compartment, as related to the control.

3.3 Effect of st-HFD on spermatogenesis

Testis from control exhibited well-organized germinal and interstitial compartment, showing GC in all differentiation stages and with mature SPZ filling tubular lumina (rhombus) as well as LC and regular blood vessels in the interstitium (asterisk; Figure 3A). The histological organization of the testes from st-HFD rats was not dissimilar from that of controls; however, it appeared clear the reduced diameter of the tubules. Indeed, the analysis of three morphometric parameters further supported this observation since the diameter of the tubules ($p < 0.001$) and the thickness of epithelium ($p < 0.05$) were lower in st-HFD group than in the control, while no differences in the % of tubular lumens occupied by SPZ were detected (Table 1). In addition, although there were no changes in the frequency of the stages characterizing the rat seminiferous epithelium (data not shown), alterations in the different phases of the acrosome biogenesis, highlighted by the PNA lectin staining, were seen (Figure 3B).

At molecular level, to evaluate the effects of st-HFD on spermatogenesis, protein levels of PCNA, phospho-histone H3 (p-H3), SYCP3, and protamine 2 (PRM2) were investigated (Figures 4A-E). The st-HFD provoked a significant increase ($p < 0.05$) in PCNA (Figures 4A, B), and p-H3 (Figures 4A, C), and a decrease ($p < 0.05$) in SYCP3 (Figures 4A, D) and PRM2 (Figures 4A, E) protein levels as related to the controls.

Concomitantly, labeling of PCNA and SYCP3 was performed (Figure 4F). Data showed a PCNA (green panel) specific localization in the SPG (arrows) and spermatocytes (SPC; arrowheads) in the testis of both groups; however, in st-HFD an increase approximately of 51% in PCNA positive cells ($p < 0.05$; Figure 4G) was observed. As for SYCP3, it localized in the SPC nucleus (arrowheads; Figure 4E), and the % of SYCP3 positive cells decreased by 53% in st-HFD group as compared to the control ($p < 0.01$; Figure 4H).

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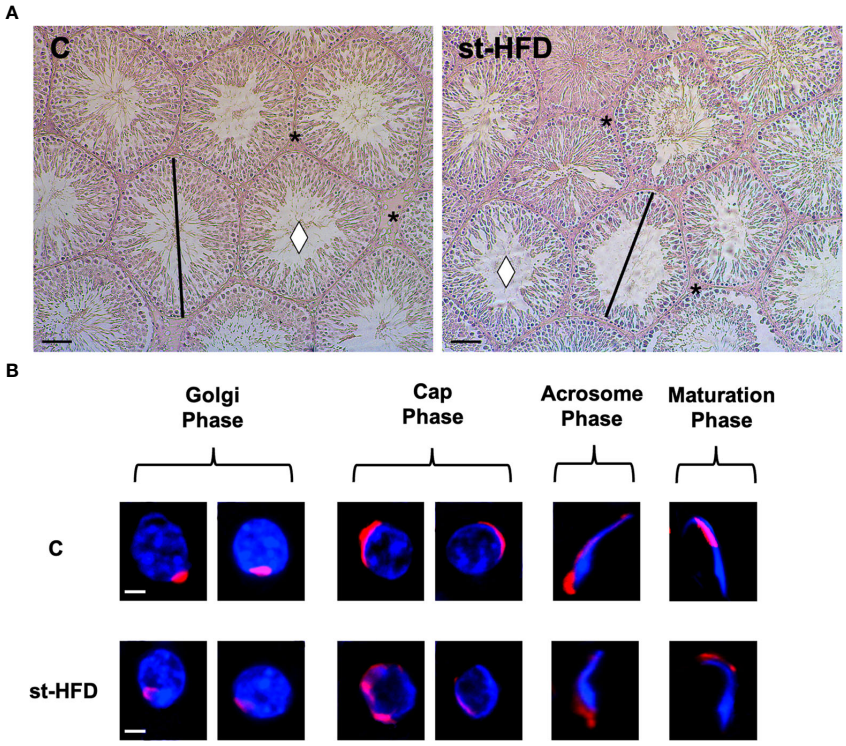

FIGURE 3
Histological analysis of control and st.HFD fed rat testis. **(A)** Hematoxylin-eosin staining of rat testicular paraffin-embedded sections. The images were captured at x20 magnification (scale bars= 40 µm). Rhombus: tubules lumen; asterisks: interstitial compartment. **(B)** PNA lectin acrosome staining (red) showing the different phases of acrosome biogenesis. The images were captured at x40 magnification (scale bars= 10 µm).

3.4 Effect of st-HFD on biogenesis and mitochondria dynamics

To evaluate the effects of st-HFD on mitochondrial biogenesis, peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α), nuclear respiratory factor 1 (NRF1), and mitochondrial transcription factor A (TFAM) were employed as markers. We found a significant decrease in the expression levels of PGC-1α ($p < 0.01$; Figures 5A, B), NRF1 ($p < 0.01$; Figures 5A, C), and TFAM ($p < 0.05$; Figures 5A, D) in the testis of st-HFD rats as compared to controls.

Mitofusin (MFN2) and Optic atrophy 1 (OPA1) were employed as markers of mitochondrial fusion; Dynamin-Related Protein 1 (DRP1) was used as a marker of the fission process. Testes from st-HFD rats exhibited a slight, significant decrease in MFN2 ($p < 0.05$; Figures 5A, E), OPA1 ($p < 0.05$; Figures 5A, F), and DRP1 ($p < 0.05$; Figure 5A, G) protein levels as compared to control animals.

TABLE 1 Effect of st-HFD on testicular morphometric parameters.

Groups	C	st-HFD
Tubules Diameter (µm)	225,32± 2,17	171,49 ± 6,38**
Epithelium Thickness (µm)	43,2 ± 1,13	30,51 ± 2,4*
Empty Lumen (%)	36 ± 2,3	39 ± 1,5

Evaluation of testicular morphometric parameters of control and st-HFD fed rat testis. All the values are expressed as means ± SEM from 5 animals in each group. *$p < 0.05$; **$p < 0.01$.

IF staining was performed for TFAM (Figure 5H), MFN2, and DRP1 (Figure 5J). In the control testis, TFAM localized in the cytoplasm of SPG (arrows), SPC (arrowhead), and in the residual cytoplasm of elongating spermatids (SPT; dotted arrows). Additionally, a clear signal in the interstitial LC was also observed (insets). In the st-HFD-treated group, TFAM localized in the same cell types abovementioned (Figure 5H), but a weaker immunofluorescent signal was observed ($p < 0.05$; Figure 5I). Similarly, DRP1 also localized in the cytoplasm of SPG (arrow), SPC (arrowheads), in eltongating SPT (dotted arrows), as well as in LC (insets); interestingly, MFN2 signal appeared dotted-shaped and diffused in all the cell types composing the seminiferous epithelium. The analysis of MFN2 (Figure 5K) and DRP1 (Figure 5L) fluorescent signals showed a comparable pattern, statistically significant, as observed for the protein level.

3.5 Effect of st-HFD on BTB integrity markers

st-HFD produced substantial alterations in the BTB at both structural and regulatory proteins, compared to control groups (Figures 6-8). Indeed, st-HFD resulted in a significant reduction in the protein levels of N-Cadherin (N-CAD; $p < 0.01$; Figures 6A, B), occludin (OCN; $p < 0.001$; Figures 5A, C), zonula occludens-1 (ZO-1; $p < 0.01$; Figures 6A, D), connexin 43 (CX43; $p < 0.01$; Figures 6A, E), and Van Gogh-Like 2 (VANGL2; $p < 0.05$; Figure 6A, F), as well

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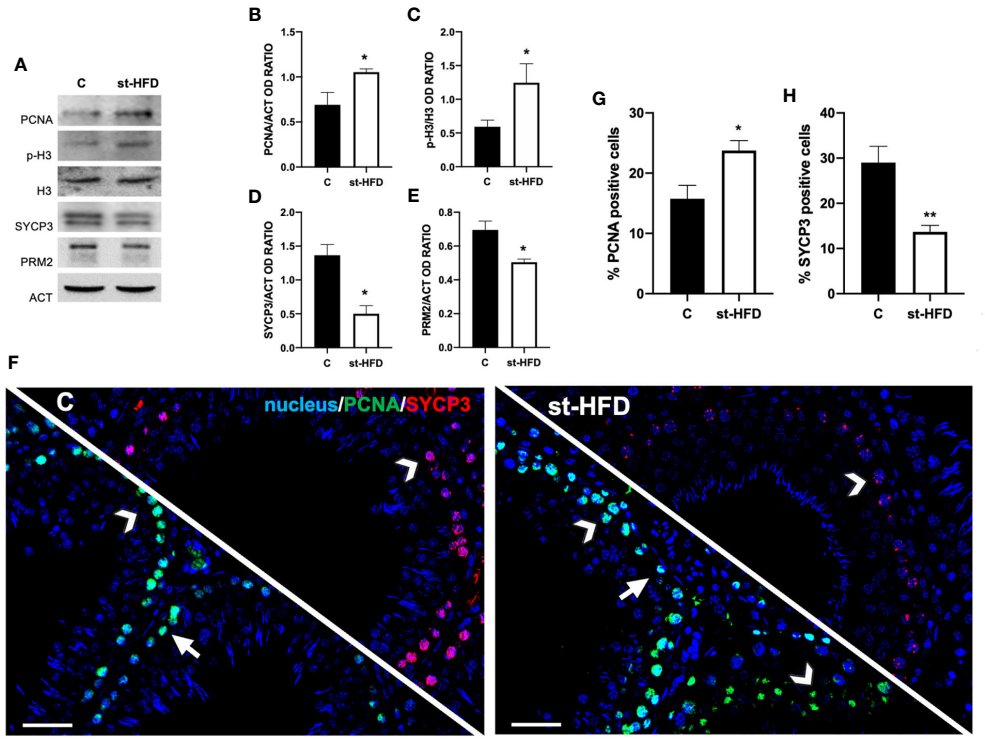

FIGURE 4
Spermatogenesis analysis of control and st-HFD fed rat testis. **(A)** WB analysis of testicular PCNA, p-H3, H3, SYCP3, and PRM2. **(B-E)** Histograms showing the p-H3/H3 ratio, PCNA, SYCP3, and PRM2 relative protein levels. **(F)** Testicular PCNA (green) and SYCP3 (red) immunolocalization. Slides were counterstained with DAPI-fluorescent nuclear staining (blue). The images were captured at x20 magnification (scale bars=20 µm). Arrows: SPG; Arrowheads: SPC. **(G, H)** Histograms showing the % of PCNA and SYCP3 positive cells, respectively. All the values are expressed as means ± SEM from 5 animals in each group. *p < 0.05; **p < 0.01.

as in the phosphorylation status of p-Src ($p < 0.001$; Figures 6A, G), p-FAK-Y397 ($p < 0.01$; Figures 6A, H), and p-FAK-Y407 ($p < 0.05$; Figures 6A, I) as compared to control.

For a more detailed characterization of the effects exerted by st-HFD on N-CAD, OCN, ZO-1 (Figure 7) CX43, and VANGL2 (Figure 8) localization, an IF analysis was carried out. N-CAD, one of the components of cell adhesion complexes (adhesion junctions) in BTB (60), localized both in the basal compartment, at Sertoli cells (SC) interface (striped arrows; Figure 7A), and in their cytoplasmic protrusions of the luminal compartment, associated with the heads of elongating SPT (dotted arrows; Figure 6A). Interestingly, in the testis of st-HFD-treated rats, while N-CAD immunosignal was still present in the basal compartment, in the luminal one it was quite weak, and less intense that of the control group ($p < 0.001$; Figures 7A, B).

OCN (Figure 7C) and ZO-1 (Figure 7E) are integral membrane and adaptor proteins, respectively, that link integral membrane tight junctions (TJ) components to the actin cytoskeleton (61). They specifically localized in the SC cytoplasm (striped arrows; Figures 7C, E; insets) in the two groups; however, the signal intensity decreased in the st-HFD-treated rats ($p < 0.05$; Figures 7D, F) as compared to the control.

CX43 is the principal testicular gap-junction protein, localized between adjacent SC and at the SC-GC interface (62). IF data confirmed this localization pattern; in control, CX43 was detected in the above-mentioned cell types, particularly in SPG (arrows; Figure 8A), SPC (arrowheads; Figure 8A; insets), SC (striped arrows; Figure 8A), and their cytoplasmic protrusions surrounding SPT (dotted arrows; Figure 8A). st-HFD produced a marked decrease of signal intensity in SC and GC, as compared to the control ($p < 0.05$; Figure 8B).

Finally, VANGL2 is a member of the Planar Cell Polarity family, factors that regulate the spatial and temporal expression of actin-regulatory proteins and the polymerization of microtubules at the apical ectoplasmic specialization (ES) and SC-SC and SC-SPT interface levels (63, 64). In the control testis, VANGL2 localized in SPC (arrowheads; Figure 8C), in the SC cytoplasm (striped arrows; Figure 8C; insets), and their protrusions surrounding the SPT/SPZ heads (dotted arrows; Figure 8C). In the st-HFD-treated group, although VANGL2 localized in the above-mentioned cell types (Figure 8C), a weaker immunofluorescent signal was observed ($p < 0.01$; Figure 8D).

3.6 Effect of st-HFD on SIRT1/NRF2/MAPKs pathways

In our previous paper, we assessed that st-HFD induced oxidative stress (42), thus herein we explored the underlying mechanisms, analyzing the SIRT1/NRF2/MAPKs pathways, that are notoriously involved in the cellular response to oxidative stress (65–69). Results

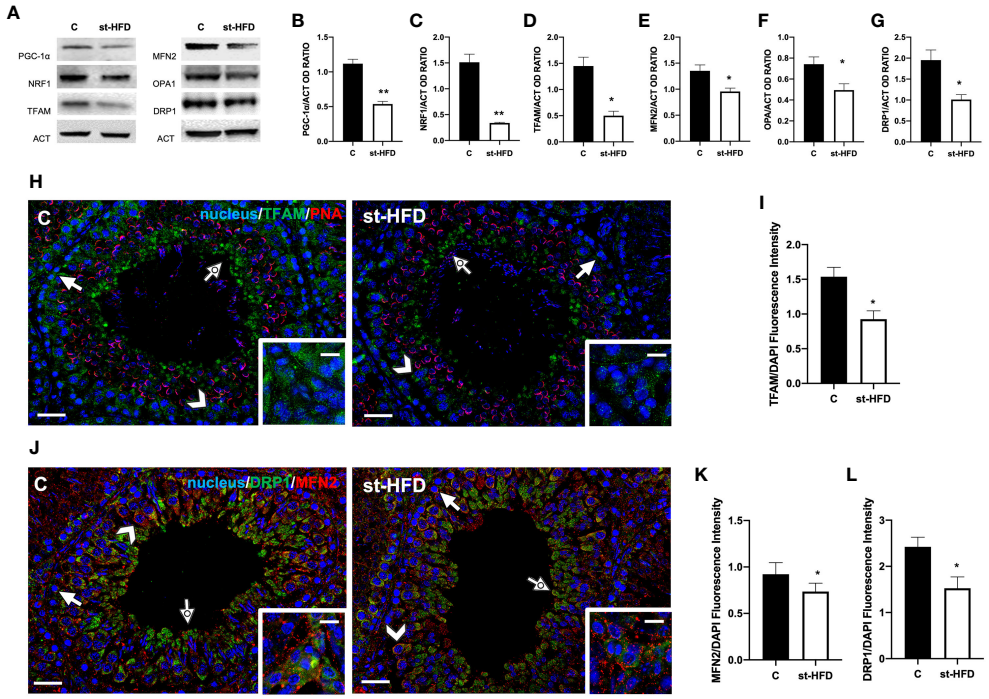

FIGURE 5
Mitochondrial dynamics analysis of control and st-HFD fed rat testis. **(A)** WB analysis of testicular PGC-1α, NRF1, TFAM, MFN2, OPA1, and DRP1. **(B-G)** Histograms showing PGC-1α, NRF1, TFAM, MFN2, OPA1, and DRP1 relative protein levels. **(H)** Testicular TFAM (green) immunolocalization. Slides were counterstained with PNA lectin (red). **(J)**. Testicular DRP1 (green) and MFN (red) immunolocalization. All the slides were counterstained 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. Insets show LC. **(I, K, L)** Histograms showing the quantification of TFAM, MFN2, and DRP1 fluorescence signal intensity, respectively. All the values are expressed as means ± SEM from 5 animals in each group. *p < 0.05; **p < 0.01.

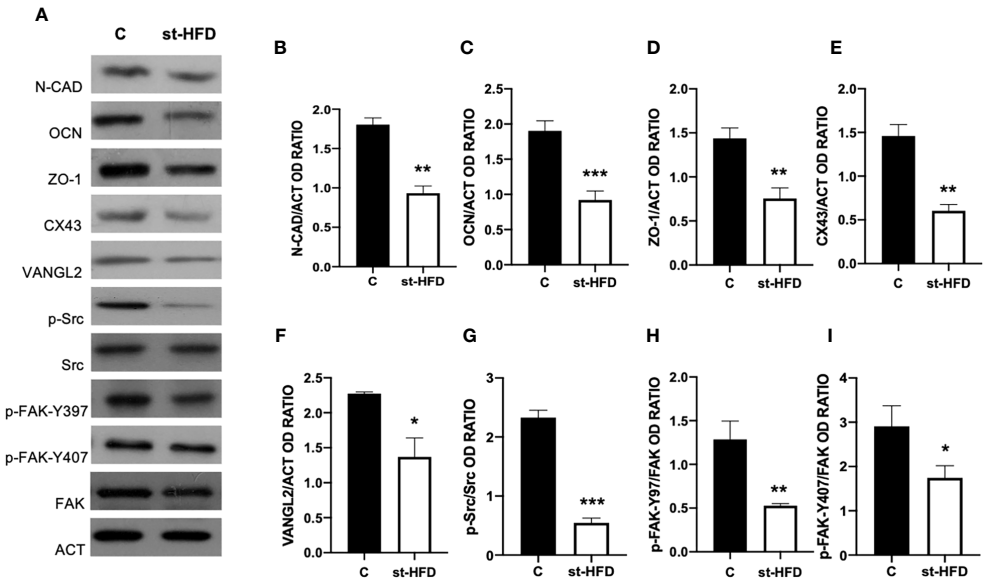

FIGURE 6
BTB markers analysis of control and st-HFD fed rat testis. **(A)** WB analysis of testicular N-CAD, OCN, ZO-1, CX43, VANGL2, p-Src, Src, p-FAK-Y397, p-FAK-Y407, and FAK, in the testes of animals treated with a st-HFD. **(B–I)** Histograms showing N-CAD, OCN, ZO-1, CX43, and VANGL2 relative protein levels, and p-Src/Src, p-FAK-Y39/FAK, and p-FAK-Y407/FAK ratios. All the values are expressed as means ± SEM from 5 animals in each group. *p < 0.05; **p < 0.01; ***p < 0.001.

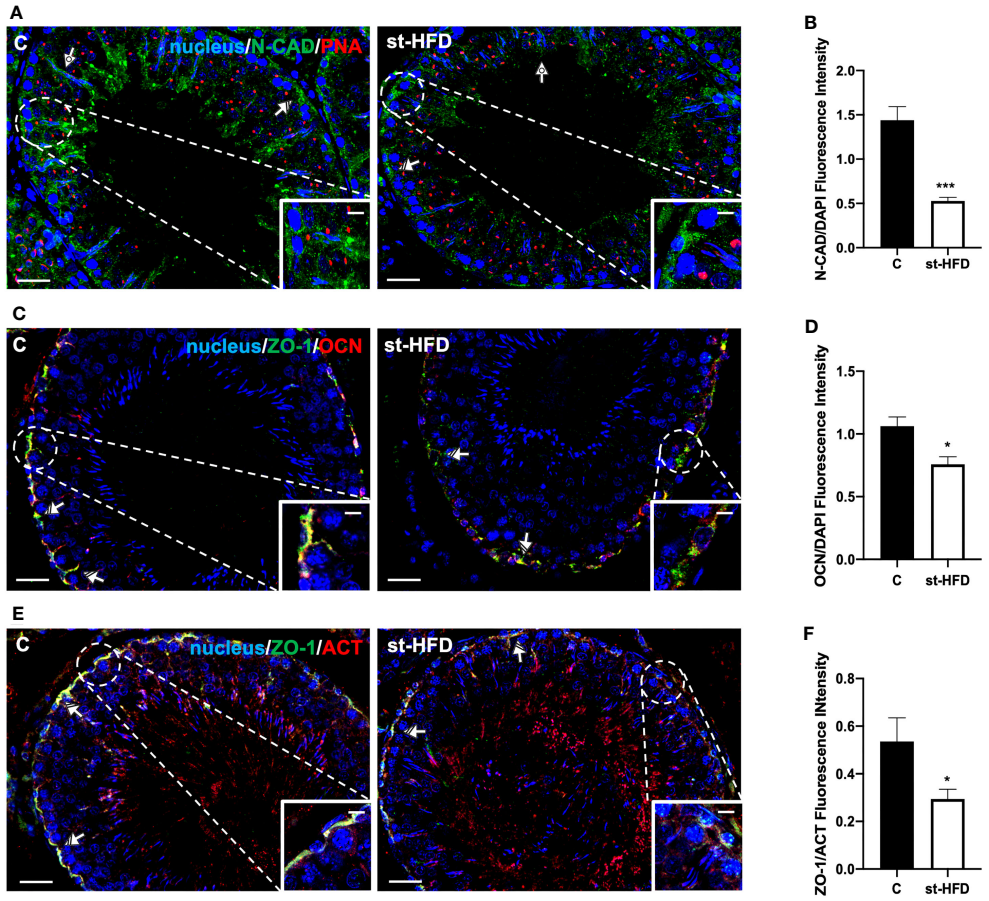

FIGURE 7
IF analysis of N-CAD, ZO-1, and OCN of control and st-HFD fed rat testis. **(A)** Testicular N-CAD (green) immunolocalization. Slides were counterstained with PNA lectin (red) and DAPI-fluorescent nuclear staining (blue). **(C)** Testicular ZO-1 (green) and OCN (red) immunolocalization. **(E)** 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 x20 (scale bars= 20 μm) magnification and x40 (scale bars= 10 μm) for the insets. Striped arrows: SC. Dotted arrows: SPT. **(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 ± SEM from 5 animals in each group. *$p < 0.05$; ***$p < 0.001$.

showed that SIRT1 protein level decreased in st-HFD rat testis as compared to the control ($p < 0.01$; Figures 9A, B); conversely, no differences in FOXO1 levels were observed (Figures 9A, C).

The protein expression of KEAP1 increased in the st-HFD group ($p < 0.05$; Figures 9A, D), while those of NRF2 ($p < 0.01$; Figures 9A, E) and HO-1 ($p < 0.05$; Figures 9A, F) in the testis of st-HFD group were decreased compared with the control. Finally, the phosphorylation status of p38 ($p< 0.01$; Figures 9A, G), JNK ($p < 0.05$; Figures 9A, H), and ERK1/2 ($p < 0.05$; Figures 9A, I) was upregulated in the testis of st-HFD group as compared to the control.

To confirm these data, we performed double immunolabeling on SIRT1 and NRF2 in the two groups. In the control testis, SIRT1 possessed a nuclear localization, especially in SPG (arrows; Figure 9J), SPC (arrowheads; Figure 9J), and SPT (dotted arrow; Figure 9J and insets). On the contrary, although it was present in the same cells, NRF2 sub-localization was cytoplasmic (Figure 9J). In the testis of st-HFD rats, the intensity of both signals was weaker ($p < 0.01$; Figures 9K, L), particularly in the SPG nucleus for SIRT1 (arrows; Figure 9J) and in SPC cytoplasm for NRF2 (arrowheads; Figure 9J).

3.7 Effect of st-HFD on inflammation

To assess whether a st-HFD induced testicular inflammation, several markers, namely NF-κB (Figures 10A, B), β-catenin (β-CAT; Figures 10A, C), TNFα (Figures 10A, D), IL-6 (Figures 10A, E), and IL-1RA (Figures 10A, F) were used. Interestingly, there were no differences between st-HFD and control for any of the selected markers.

4 Discussion

Proper male and female reproductive activity are crucial for the health and survival of the species. This is accomplished by the production and differentiation of good quality gametes that, as for the male counterpart, are based on SPZ with the ability to cross the female genital tract, perform an accurate acrosome reaction, and contribute with an undamaged DNA for fertilization. Such events depend on an extremely intricate and specialized progression, which involves the proliferation (both mitotic and meiotic) of

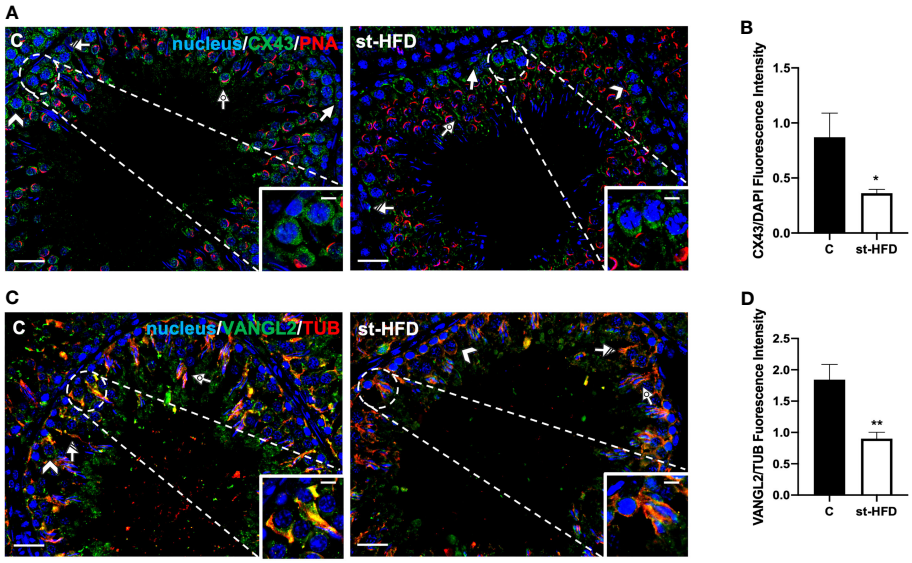

FIGURE 8
IF analysis of CX43, and VANGL2 of control and st-HFD fed rat testis. **(A)** Testicular CX43 (green) immunolocalization. **(C)** Testicular VANGL2 (green) and α-Tubulin (red) immunolocalization. All the slides were counterstained with DAPI-fluorescent nuclear staining (blue). All the images were captured at x20 (scale bars= 20 μm) magnification and x40 (scale bars= 10 μm) 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 ± SEM from 5 animals in each group. *p < 0.05; **p < 0.01.

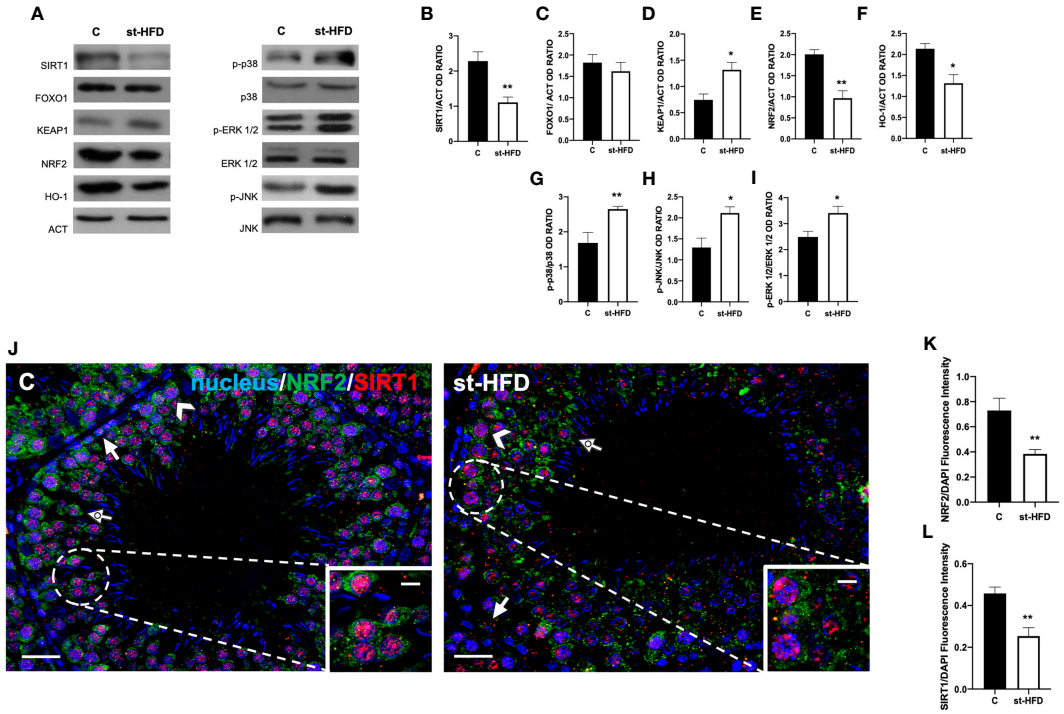

FIGURE 9
SIRT1/NRF2/MAPKs pathways analysis of control and st-HFD fed rat testis. **(A)** WB analysis of testicular SIRT1, FOXO1, KEAP1, NRF2, HO-1, p-p38, p38, p-ERK 1/2, ERK 1/2, p-JNK, and JNK. **(B–I)** Histograms showing SIRT1, FOXO1, KEAP1, NRF2, and HO-1 relative protein levels, and p-p38/p38, p-ERK 1/2/ERK 1/2, and p-JNK/JNK ratios. **(J)** Testicular NRF2 (green) and SIRT1 (red) immunolocalization. Slides were counterstained 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. **(K, L)** Histograms showing the quantification of NRF2 and SIRT1 fluorescence signal intensity, respectively. All the values are expressed as means ± SEM from 5 animals in each group. *p < 0.05; **p < 0.01.

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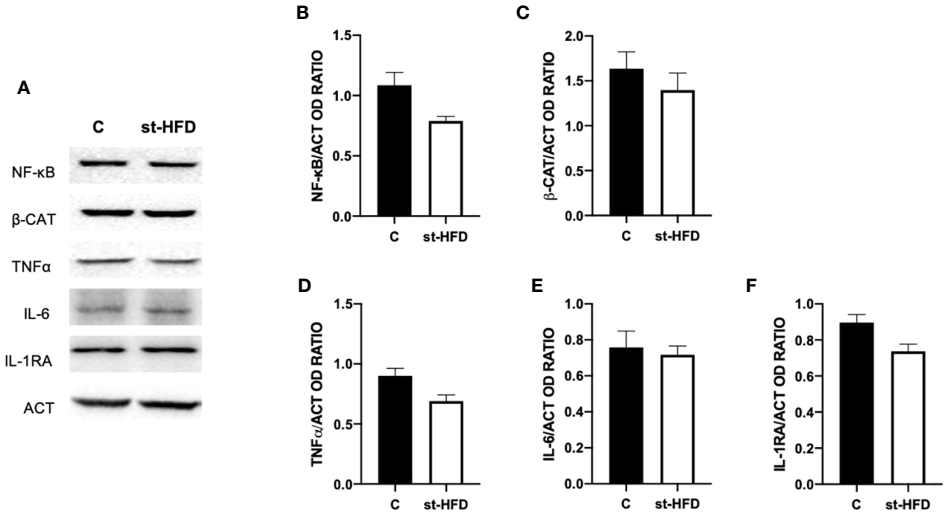

FIGURE 10
Inflammation markers analysis of control and st-HFD fed rat testis. **(A)** WB analysis of testicular NF-κB, β-Catenin, TNFα, IL-6, and IL-1RA. **(B–F)** Histograms showing NF-κB, β-Catenin, TNFα, IL-6, and IL-1RA relative protein levels. All the values are expressed as means ± SEM from 5 animals in each group.

SPG into round SPT and their differentiation into SPZ, with also the contribution, for several aspects, of the somatic Sertoli and Leydig cells. Conversely, the decrease in sperm quality is a worldwide phenomenon, originating from a plethora of factors: genetic, environmental, and behavioral. Among the latter, dietary habits, with the spread of the so-called "Western diet" (characterized by being hypercaloric and nutritionally poor) is one of the most responsible, as a clear, multifunctional association between overweight/obesity and male sub- infertility has been extensively demonstrated (70–72). Indeed, many papers showed a positive correlation, in human and experimental rodent models fed with a long-term HFD, with increasing BMI and the worsening of several aspects related to fertility, as hormonal status (especially T level), sperm count, and motility, as well as the increased rate of oxidative stress and inflammation, increasing the risk of oligozoospermia and azoospermia (73, 74).

This work, with the use of a st-HFD rat model instead of the most usual mice/rats HFD-fed for a prolonged period, takes a different view, aimed to investigate the impact of overweight on testicular activity, since this condition represents the initial stage of the obesogenic process and may assess the status of affected people and direct them to a more correct diet (or other intervention strategies) in an attempt to mitigate its effects.

4.1 st-HFD alters testicular steroidogenesis and spermatogenesis

As expected, we found that the steroidogenesis was compromised in the testis of st-HFD rats. Herein, serum T levels were decreased significantly in the HFD group by about 28%, and our data agree with those by Migliaccio et al. (38), which evidenced a reduction in serum T levels and testicular androgen receptors in rats fed with a st-HFD (for 6 weeks). Nevertheless, the reduction in T levels was evidently less pronounced than that observed in rats fed with HFD for 12 (about 400%) (75), or 20 (about 180%) (35) weeks. On the contrary, we found no difference in ARO protein levels as compared to the control. Of note, a previous paper showed that 16 weeks of HFD induced an increase in serum E_2 levels and testicular ARO expression (76) and, considering that this enzyme converts T into E_2, and that decreased T/E_2 ratio has been related to impaired spermatogenesis (56, 77–79), we highlighted that the disturbance of the hormonal milieu, induced by st-HFD, may be not so severe as that produced by a long term HFD. In addition, reduced T levels, together with the imbalance of oxidative status (42) are among the main causes of the negative impact induced by st-HFD on rat testis. Further, oxidative stress may also be one of the causes inducing LC apoptosis, exacerbating the reduced T bioavailability and, consequently, increasing the number of apoptotic GC. However, the apoptotic rate of testicular cells observed here was less pronounced as compared to that observed in the testis of HFD administered for a longer time (33–36, 80, 81), just confirming that an overweight-like condition provokes less detrimental effect as compared to that of obesity on testicular activity.

Our results showed that st-HFD impacts spermatogenic progression. While the histological organization was similar to controls, a reduced tubular diameter and epithelium thickness were observed. In addition, for the first time, we found significantly lower expression levels of SYCP3, an essential structural component of the synaptonemal complex, and PRM2, a protein associated with histone replacement in haploid cells during spermiogenesis (82). Vice versa, higher levels of PCNA, a nuclear antigen of cell proliferation, and p-H3, a histone protein crucial for chromatin condensation during mitosis/meiosis (83), were detected, together with a higher % of PCNA-positive SPG and I SPC. This last point is of interest, since our data are contrasting with that reported in other papers, in which a reduced number of PCNA-positive cells were observed in the seminiferous tubules of rats

HFD-fed for 8 (36), 12 (84, 85), 18 (34), and 20 (35) weeks. Therefore, a st-HFD appeared to have a major negative effect on meiotic and post-meiotic events, rather than the previous ones. This data was partially supported by the fact that no differences in the frequency of stages characterizing rat seminiferous cycle were observed. This is in contrast with the paper by Komnions and colleagues (86), whose data demonstrated that a long-term HFD altered this value in mice; however, there were slight alterations in the phases of acrosome biogenesis. Further studies are required to clarify the underlying molecular aspects and the impact of a st-HFD on sperm parameters and physiology since proper acrosome formation is fundamental for successful fertilization (87).

4.2 st-HFD alters testicular mitochondrial dynamics via SIRT1 pathway

It is known that self-renewing and proliferating SPG use predominantly glycolysis, while in SPC and SPT, energy is prevalently produced through mitochondrial respiration, for this, fully functional mitochondria are required to complete a successful meiosis (43). Therefore, the altered progression of meiosis in st-HFD testis, as demonstrated by lower SYCP3 and PRM2 levels, could be the result of mitochondria damage, while the increased expression of PCNA and p-H3 in SPG and I SPC may be a compensatory response to the impaired maturation of GC. Bearing in mind the interesting data obtained by Migliaccio et al. (88), reporting that a st-HFD modifies mitochondrial fusion/fission processes in rat liver, we assessed whether the altered steroidogenesis/spermatogenesis in our animal model could also be induced by a consequence in mitochondrial dynamic changes. In particular, we analyzed several proteins involved in three pivotal mitochondrial processes: fusion (that promotes the maintenance of a homogeneous mitochondrial population that can tolerate higher levels of mitochondrial DNA mutations), fission (the division of a mitochondrion into two smaller mitochondria), and biogenesis (89). Our hypothesis on the involvement of mitochondrial damage in impaired spermatogenesis/steroidogenesis is confirmed by a decrease in MFN2 and OPA1 (fusion markers), DRP1 (fission marker), PGC-1α, NRF1, and TFAM (biogenesis marker) protein levels.

In this complex scenario, it should also be considered the multifaceted role played by SIRT1, a NAD^+-dependent deacetylase, for several reasons (90). First, it has a well-recognized role in spermatogenesis, in particular to produce sex hormones by the hypothalamus-pituitary-testis axis (91) and for meiotic and post-meiotic progression (92). Second, SIRT1 is a ROS "sensor", regulating, in oxidative stress conditions, the expression of several redox-related factors, such as FOXOs and NF-κB (90). Third, SIRT1 regulates mitochondrial function and energetic metabolism activating PGC-1α through deacetylation and mediating the induction of several components of the ROS detoxifying system (93). Fourth, testicular SIRT1 downregulation has previously been associated with the insurgence of an oxidative stress status (94) and in HFD-fed mice (53, 95). In view of these considerations, supporting earlier reports, we hypothesize that the effect of a st-HFD on impaired spermatogenesis may be also due to the downregulation of SIRT1 expression/activity and, consequently, of the downstream pathways, including those regulating mitochondrial dynamics.

4.3 st-HFD alters BTB integrity via NRF2/MAPKs pathways

BTB integrity is sensitive to stressful conditions, such as survival factor depletion and oxidative stress, as reported in several papers (96, 97). BTB is a distinctive structure of the testis, dividing the seminiferous epithelium into two compartments: the basal, where SPG and preleptotene SPC reside, and the apical one, which contains all the other cell types. It is composed of several cell junctions, located between adjacent SC, and particular cytoskeleton-based structures (the ES and the tubulobulbar complex), which connect SC to SPT. The BTB is an extremely dynamic structure, which, at stages IX–XI of the rat seminiferous epithelial cycle, is "disrupted" and then "reassembled" to permit the transit of preleptotene/leptotene SPC. This action is mediated by the interplay of various mechanisms that generally regulate fluctuation in the expression, localization, activation, and interactions of structural, scaffolding, and signaling proteins (61). Indeed, all the BTB components work harmoniously through continuous cycles of phosphorylation/de-phosphorylation, endocytosis of membrane proteins, and their recycling to guarantee the accurate moving of GC, and to preserve the immune-privileged microenvironment.

Herein, we confirmed that in the testis of st-HFD-fed rats, the protein levels of ZO-1, OCN, and CX43 were reduced (34). However, to our knowledge, this is the first report showing that a st-HFD affects testicular levels of N-CAD and VANGL2, proteins found at basal and apical ES, respectively, as well as the activation of Src and FAK.

In particular, FAK is a central kinase regulator of BTB dynamics, since its phosphorylation, by Src, at tyrosines 397 and 407, allows it to interact with many other components, including OCN, ZO-1, and Src itself. Once activated, FAK regulates the transit of GC through the seminiferous epithelium, especially maintaining the integrity of the apical ES and SPT adhesion during spermiogenesis until spermiation (98). Thus, as previously observed by other authors in HFD-fed mice for 10 (99), and 16 (100) weeks, we found that also a st-HFD can produce perturbations in BTB components, highlighting that its stability is fundamental for a correct 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.

4.4 st-HFD alters testicular activity via NRF2/MAPKs pathways

Emerging evidence demonstrated that the disturbance of BTB integrity may be due to ROS overproduction, by the downregulation of NRF2 (101) and activation of the MAPKs pathways (102, 103). Worth remembering, in physiological condition, NRF2 levels are maintained low via the repressive action of the protein KEAP1

while, in an oxidative stress environment, NRF2 is released by KEAP1, allowing its translocation into the nucleus, and activating the expression of antioxidant enzymes, including HO-1 and SOD. As for the MAPKs pathways, the increased activity of p38, JNK, and ERK 1/2 leads to OCN ubiquitination and degradation, as well as endocytosis of junction proteins, including N-CAD and CX43 (104–106).

In addition, it has also been reported that p38/JNK work together to activate the mitochondrial apoptotic pathway, via the stimulated expression of pro-apoptotic genes, such as cytochrome c and Caspase-3 (107, 108). Finally, apart from its well-known contribution to cell proliferation, numerous studies revealed that ERK 1/2 is also involved in apoptosis ROS-triggered (109–112). Consistently, our results showed that also a st-HFD induced the inhibition of the NRF2 pathway, as well as the phosphorylation, and thus the activation, of testicular p38, JNK, and ERK 1/2. These results were positively associated with the oxidative stress status and the enhanced apoptosis, while they were negatively correlated with the levels of structural proteins composing the BTB. The combined data suggest that BTB damage and apoptosis may be mediated by the inhibition of NRF2 and the activation of p38, JNK, and ERK 1/2 MAPK pathways, in st-HFD-fed rat testis, as already demonstrated in testicular tissues of type-1 diabetic or obese rodents (99, 113–116).

4.5 st-HFD does not induce testicular inflammation

Finally, for a broader picture of the effect of st-HFD on rat testis, the last analyzed parameter was the protein level of the pro-inflammatory markers NF-κB, β-CAT, TNFα, IL-6, and IL-1RA. However, no differences between st-HFD-treated rats and controls were found, and this point is particularly interesting, since one of the principal manifestations that are evidenced in obesity is the systemic inflammation, that produces altered testicular activity and sperm quality in men (114) and in rodents HFD-fed for a prolonged period (34, 84, 117–119). Thus, although a st-HFD can lead to dysfunction in testicular physiology, the lack of inflammation may be the sign of a less severe influence of overweight on fertility, suggesting that in overweight men there are still possibilities of intervention strategies (restricted diet, exercise, drugs, and others) that may effectively ameliorate testicular activity.

5 Conclusions

This study is one of the few to highlight the effects of a st-HFD on rat testicular activity. We demonstrated that disturbance in the hormonal milieu and the increased oxidative stress enhanced LC and GC apoptosis, reduced meiotic progression, and altered the integrity of BTB. These effects may be related to altered mitochondrial dynamics, and also to dysregulation of the SIRT1/NRF2/MAPKs pathways. However, we highlighted the absence of a claimed inflammation status, as well as the less % of TUNEL-positive cells, the increased % of PCNA-positive cells and no changes in the ARO protein level, as compared to literature papers in which a longer HFD was employed. The combined data led us to confirm that an overweight condition provoked less intense effects than obesity; however, as a limitation of this study, we lack a direct comparison with a long-term HFD, leading us to not completely exclude that these differences could be related to factors other than diet duration. In any case, this report encourages further studies not only to confirm this aspect but also on the development of different strategies to be used in preventing/mitigating the still not-so-severe effects of overweight on male fertility.

Data availability statement

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

Ethics statement

The animal study was approved by Ethics Committee of the University of Campania "Luigi Vanvitelli" and the Italian Minister of Health (Permit Number: 704/2016-PR of the 15/07/2016; Project Number: 83700.1 of the 03/05/2015). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SF: Formal Analysis, Investigation, Visualization, Writing – original draft. SM: Conceptualization, Supervision, Writing – review & editing. AS: Formal Analysis, Investigation, Visualization, Writing – original draft. RS: Methodology, Writing – original draft. GB: Conceptualization, Writing – review & editing. MV: Conceptualization, Formal Analysis, Funding acquisition, Project administration, Visualization, Writing – original draft.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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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.2023.1274035/full#supplementary-material

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C. Yan Cheng,
St. Francis College, United States

REVIEWED BY
Bo Zheng,
Nanjing Medical University, China
Taisen Iguchi,
Graduate University for Advanced Studies (Sokendai), Japan

*CORRESPONDENCE
Chenghua Yang
Chenghua-yang@qq.com
Zhiyong Liu
medlzhy@aliyun.com

†These authors have contributed equally to this work

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RNA sequencing profiles reveals progressively reduced spermatogenesis with progression in adult cryptorchidism

Weihao Sun[1†], Xinhui Zhang[1†], Lei Wang[1†], Guanyu Ren[1], Shuguang Piao[1], Chenghua Yang[1,2*] and Zhiyong Liu[1,2*]

[1]Department of Urology, Changhai Hospital, Second Military Medical University, Shanghai, China, [2]Shanghai Key Laboratory of Cell Engineering, Shanghai, China

Introduction: The fertility of cryptorchidism patients who didn't perform corrective surgery will decrease with age. Herein, we elucidate the histological alterations and underlying molecular mechanism in patients with an increase in the disease duration from 20 to 40 years.

Methods: Testicular tissues were obtained from three patients with cryptorchidism, ranging in age from 22 to 44 years. Three benign paracancerous testicular samples of matched ages were used as controls. The normal and undescended testicular tissues were stained with hematoxylin and eosin (HE) and immunofluorescence and all six testicular samples were subjected to RNA sequencing. RNA sequencing data were subjected to gene set enrichment analysis (GSEA), Kyoto Encyclopedia of Genes and Genomes (KEGG), protein-protein interaction (PPI) network analysis, and Gene Ontology (GO) searches. Real-time reverse transcriptase polymerase chain reaction was used to confirm the DEGs.

Results: The seminiferous tubules' basement membrane thickens with age in healthy testes. As the period of cryptorchidism in the cryptorchid testis extended, the seminiferous tubules significantly atrophy, the number of spermatogenic cells declines, and the amount of interstitial fibrous tissue increases in comparison to normal tissues. The number of germ cells per cross-section of seminiferous tubules was significantly lower in cryptorchidism than in normal testicular tissues, according to immunofluorescence staining, but the number of Sertoli cells remained stable. RNA sequencing analysis identified 1150 differentially expressed genes (DEGs) between cryptorchidism and normal testicular tissues (fold change >2 and $p<0.05$), of which 61 genes were noticeably upregulated and 1089 were significantly downregulated. These genes were predominantly linked to sperm development and differentiation, and fertilization, according to GO analysis. Meiosis pathways were significantly downregulated in cryptorchidism, according to KEGG pathway analysis and GSEA ($P<0.001$). PPI analysis was used to identify the top seven downregulated hub genes (*PLCZ1, AKAP4, IZUMO1, SPAG6, CAPZA3*, and *ROPN1L*), which were then further verified by qPCR.

Discussion: By describing the histological changes and differential gene expression patterns in adult cryptorchid patients of different age groups, we discovered the progression mechanisms of undescended testes in adults with aging and identified seven significantly downregulated hub genes (*PLCZ1, AKAP4, IZUMO1, SPAG6, CAPZA3*, and *ROPN1L*) in cryptorchid testis compared to normal testicular tissues. These genes played a role in the process of spermgenesis and are directly linked to the steady decline in fertility caused by cryptorchidism. Our study provided a better understanding of the molecular mechanisms underlying the loss of spermatogenesis in adult cryptorchidism, and give support for the development of adult cryptorchidism treatments.

KEYWORDS

cryptorchidism, infertility, RNA sequencing, spermatogenesis, testis

Introduction

Cryptorchidism (also known as ndescended testis (UDT)) refers to the failure of one or both testes to migrate through the inguinal canal and into the scrotum due to the abnormal developmental process during fetal life. Cryptorchidism is one of the most common congenital anomalies in male infants, with an incidence rate of 1.6-9% varying across different countries (1). Cryptorchidism is associated with high rates of male infertility, as nearly 10% of males with fertility issues have a history of cryptorchidism and orchidopexy, and 20–27% of male adults affected by azoospermia and 3–8% affected by oligo-terato-asthenospermia were previously diagnosed with testicular maldescent (2). For congenital cryptorchidism, it is recommended to perform corrective surgery within the first 18 months of birth (3, 4).

Therefore, there is a decreasing trend in the average age of patients undergoing UDT surgery. However, studies showed that even if surgery is performed before 6 months of age, 31% of UDT patients still will experience a decrease in sperm count in adulthood (5). In addition to impaired fertility, the risk of malignant tumors has been shown to increase with age at which testicular fixation surgery is performed (6–9). Therefore, postponing surgery for patients with cryptorchidism may have a number of negative consequences. After puberty, patients with undescended testes are now uncommon due to the widespread advice for early orchiopexy within the first year of life. As a results, it is unclear what the progressive process is if the testis is preserved in the undescended state in adulthood. In order to develop appropriate treatments for chronic cryptorchidism, it is important to fully understand the mechanisms underlying persistent cryptorchidism.

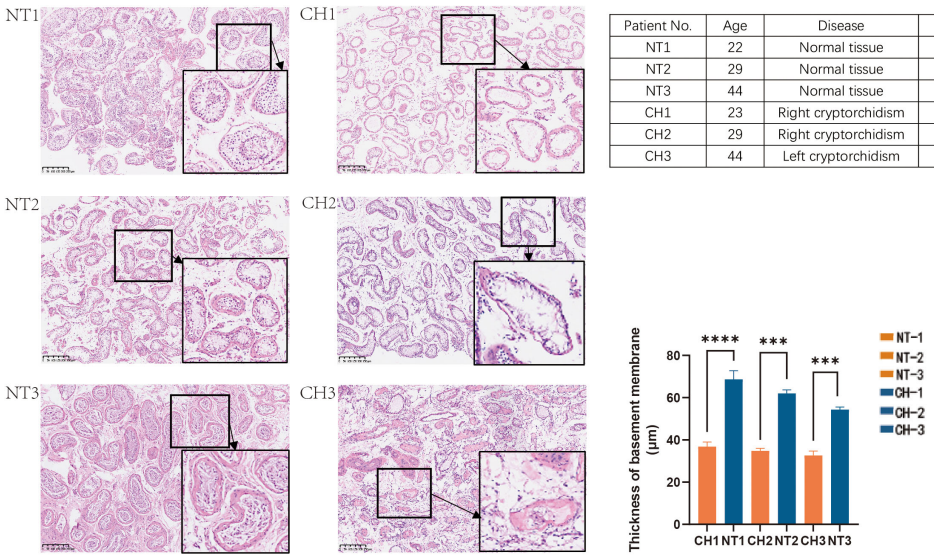
It has been reported that there is a significant reduction in the number of germ cells, and relative increase in the percentage of sertoli cells in patients with cryptorchidism. Testes that remain undescended are associated with progressive loss of germ and Leydig cells (10–12). If cryptorchidism is a progressive disease, the longer the testis remained in the undescended environment, the lesser the number of germ cells and/or Leydig cells (13). However, due to the limited cases that patients take the orchiopexy after adulthood, few studies were carried out to investigate how the cryptorchidism actually progress as the testis remain undescended for longer periods. Moreover, most studies on prolonged cryptorchidism were based on animal models (11, 14–16), which cannot simulate the real human cryptorchidism development and progression. Here, we collected testicular tissue samples from three cryptorchidism patients who underwent orchidopexy at the ages from 22 to 44. Three para-cancerous normal testis tissues were also collected from testicular cancer patients as control with matched ages. By describing the histopathological changes and differential gene expression in each age group, we provided an overview of the development process of adult cryptorchidism with different undescended testis time, and identified the molecular mechanisms that lead to fertility impairment in adult UDT patients, thus provided a research foundation for the future restoration of fertility in adult UDT patients.

Materials and methods

Recruitment of clinical patients

With Changhai Hospital's ethical approval (CHEC2021-086), testicular tissues were obtained from three cryptorchid patients and three testicular cancer patients who underwent orchidopexy. The undescended testis tissues from the cryptorchid patients and the para-cancerous benign testis tissues from testicular cancer patients were collected. Clinical data of cryptorchid patients and testicular cancer patients are shown in Figure 1. The collected tissues were divided into 2 portions, 1 was stored in 4% paraformaldehyde (PFA) immediately, and another 1 was fast frozen with liquid nitrogen and then stored at -80°C until use.

The inclusion criteria for cryptorchidism patients were: (1) Patients who have confirmed cryptorchidism by ultrasound; (2) Patients without reproductive history; (3) Patients aged between 18 and 50 years. The inclusion criteria for testicular cancer patients



Patient No.	Age	Disease	BMI
NT1	22	Normal tissue	25.9
NT2	29	Normal tissue	29.3
NT3	44	Normal tissue	20.7
CH1	23	Right cryptorchidism	22.3
CH2	29	Right cryptorchidism	19.9
CH3	44	Left cryptorchidism	25.4

FIGURE 1
HE staining of the normal and undescended testis in different age groups. NT is for normal testis and CH is for cryptorchidism. Each image's number corresponds to the patient information in the table on the right. (H&E stain, x40, Bar, 250 mm). Data are mean ± SD of three independent experiments. ***P < 0.001, ****P < 0.0001, ANOVA.

were: (1) Patients who have histologically testicular cancer with operations; (2) Patients with reproductive history; (3) Patients without hormonal treatment history (4) Patients aged between 18 and 50.

Exclusion criteria were as follows: (1) Patients with severe diseases including liver, kidney, hematopoietic, and cardiovascular; (2) Patients with testicular injuries; (3) Patients who had received medication within 6 months.

Histological examination

Testicular tissues were fixed in 4% PFA for 24h, embedded in paraffin, and sliced into 6 μm thickness. The sections were stained with Hematoxylin and eosin (H&E) and observed under a microscope.

Immunofluorescence staining

Immunofluorescence staining was performed based on previously established protocol (17). Briefly, slides were first stained with a rabbit anti-*DAZL* antibody (Cat. No. ab215718, Abcam; 1:250); a rabbit anti-*DDX4* antibody (Cat. No. ab270534, Abcam; 1:250); a rabbit anti-SOX9 antibody (Cat. No.AB5335, Millipore;1:250); a rabbit anti-*SYCP3* antibody (Cat. No. ab15093, Abcam;1:250); or a rabbit anti-*STAR* antibody (Cat. No. 12225-1-AP, Proteintech;1:200). After rinsing, tissues were stained by secondary antibodies (a donkey anti-rabbit Alexa 647 (Cat. No.ab150075, Abcam; 1:1000)) at room temperature for 2 h. Nuclei were counterstained by the mounting medium with DAPI (Cat. No. ab285390, Abcam). Images were taken by a confocal microscope (Leica SP-8, Leica Corporation, German).

Whole transcriptome sequencing

A total of 2 μg RNA per sample was used as input material for the RNA sample preparations. RNA-seq library preparation was performed by Novogene (Novogene Corporation Inc., Beijing, China). According to the manufacturer's instructions, total RNA was isolated from tissues with the Qiagen RNeasy Plus Mini Kit (Qiagen). Then RNA quality was determined by 2100 Bioanalyser (Agilent, CA, USA) and quantified using the ND-2000 (NanoDrop Technologies, CA, USA). Only high-quality RNA sample (OD260/280 = 1.8~2.2, OD260/230≥2.0, RIN≥6.5, 28S:18S≥1.0, >1μg) was used to construct sequencing library. RNA-seq transcriptome library was prepared following TruSeqTM RNA sample preparation Kit from Illumina (San Diego, CA, USA) using 1μg of total RNA. Shortly, messenger RNA was first isolated according to polyA selection method by oligo(dT) beads and then fragmented by fragmentation buffer. Secondly double-stranded cDNA was synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA, USA) with random hexamer primers (Illumina). Library quality was assessed on the Agilent Bioanalyzer 2100 system.

RNA-seq analysis

The RNA sequencing data obtained were normalized using the Limma Bioconductor package in the R computing environment. After normalization and batch effect removal, the fluorescence intensities were transformed into a log2 base prior to further analysis. Differential gene expression levels were determined by comparing processed array signals in different samples. Genes with an FDR of <0.05 were considered statistically significant. Pathway and gene ontology analyses were performed using the Ingenuity

IPA program for DEGs. PPI networks were established by Search Tool for the Retrieval of Interacting Genes Database (STRING) based on the combined score > 0.9 and visualized by Cytoscape 3.9.1 (version 4.2.2) to reveal the interactions among proteins of common DEGs.

RNA extraction and quantitative real-time PCR array

Trizol (Cat. No. R0016, Beyotime, Beijing, China) was used to extract RNA from tissue samples according to the manufacturer's instructions. Use NanoDrop ND-1000 to determine the concentration of RNA, and store the extracted RNA in a refrigerator at -80°C. Using 500 ng total RNA as a template, cDNA was synthesized with cDNA Synthesis Kit (Cat. No. R312, Vazyme, Beijing, China). Samples were stored at -20°C and subjected to qPCR using a StepOnePlus Real-Time PCR System (Applied Biosystems). Each qPCR sample was performed in a triplicate and 10 μL reaction containing 2x SYBR Green qPCR Master Mix (Cat. No. R711-02, Vazyme, Beijing, China), 10 nM forward and reverse primers, and 2 μL cDNA. The qPCR protocol was executed for 45 cycles and each cycle consisted of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Using β-actin as internal controls, quantitative PCR analysis was performed to quantify the relative mRNA expression of targeted genes. Define the result of qPCR from the threshold cycle (Ct) and use the 2-△△Ct method to calculate the relative expression level. The primer pairs specific for various genes used in our experiments are listed in Table 1.

Statistical analysis

All experiments were repeated at least three times. Data were presented as mean ± standard deviation. Two-way ANOVA was used for comparison between two groups. Test standard α=0.05, $p<0.05$ was considered statistically significant, $p<0.01$ was very significant, and $p<0.001$ was extremely significant. Statistical analysis were performed using GraphPad Prism (GraphPad, CA, USA).

Results

Histological differences between cryptorchidism and normal testis

First, we looked at the histological traits of patients with cryptorchid testicular tissues. Para-cancer benign tissues that were matched by age were used as controls. According to histological analysis, the seminiferous tubules' basement membrane thickens with age in normal testes (Figure 1). In contrast, the cryptorchid testes' seminiferous tubules markedly atrophied, the amount of spermatogenic cells decreases, and the quantity of interstitial fibrous tissue grows in comparison to normal tissues as the duration of cryptorchidism increased. All of these histological characteristics of cryptorchid individuals showed that, in comparison to normal testicles, cryptorchid testes were extensively injured. (Figure 1).

Next, we performed immunofluorescence staining to evaluate the composition of different cellular content within the testis, including germ cells, Sertoli cells, and spermatid during testicular development in normal and cryptorchid testis (Figure 2A). Deleted in Azoospermia-like (*DAZL*, which is located in the nucleus of spermatogonia but relocated in the cytoplasm during meiosis where it persists in spermatids and germ cells), DEAD-Box Helicase 4 (*DDX4*, specifically expressed in the germ cell lineage), SRY-Box Transcription Factor 9 (*SOX9*, marker of Sertoli cells), and Synaptonemal Complex Protein 3 (*SYCP3*, relevant with meiosis) proteins were stained in testicular tissues to evaluate cellular composition changes involving in spermatogenesis between cryptorchidism and normal testis. We found that cryptorchid testis had lower *DAZL*, *DDX4*, and *SYCP3* positive cells in seminiferous tubule compared with normal testis, consistent with previous observations by other investigators. A small number of germ cells still exist in cryptorchid patients of the 20-year-old group. However, as time progresses, the number of germ cells in cryptorchid patients continues to decrease, indicating that the damage to fertility caused by cryptorchidism accumulates over time. No significant difference in *SOX9*-positive cells were observed between cryptorchid and normal testis, suggesting that cryptorchidism markedly inhibited spermatogenesis and induced the arrest of spermatogonia without apparently affecting the Sertoli

TABLE 1 Bases sequences of primers.

Primer	Forward (5′-3′)	Reverse (5′-3′)
SPAG6	AAGAGCCAAAGCAGCAGTCT	AATGGACAGGCAGGTGTAATG
CAPZA3	GCCACCAACACTGCCAAAAA	AGTCGCCCATTACATTGTGGT
AKAP4	GCGTACTCTGATACTACAATGATGT	CAGGGTGGACACATCGACAA
AKAP3	CCCAGGACTGGAAAATGGACA	TTGGACGTTTCCCCACCAAA
PLCZ1	CTCTACCATCACCAGAGGCAC	ACCCCTGTTTCCTTGTCTTGA
IZUMO1	GCTCTCGATTTCACGCAACC	GCAGTGTGGCCTCATGCTAA
SPAG6	AAGAGCCAAAGCAGCAGTCT	AATGGACAGGCAGGTGTAATG
ROPN1L	CGCGGGCTATTTTTCAGCTC	GCTTGTGGTGACACTGCTTG
ACTB	ACAGAGCCTCGCCTTTGC	GATATCATCATCCATGGTGAGCTGG

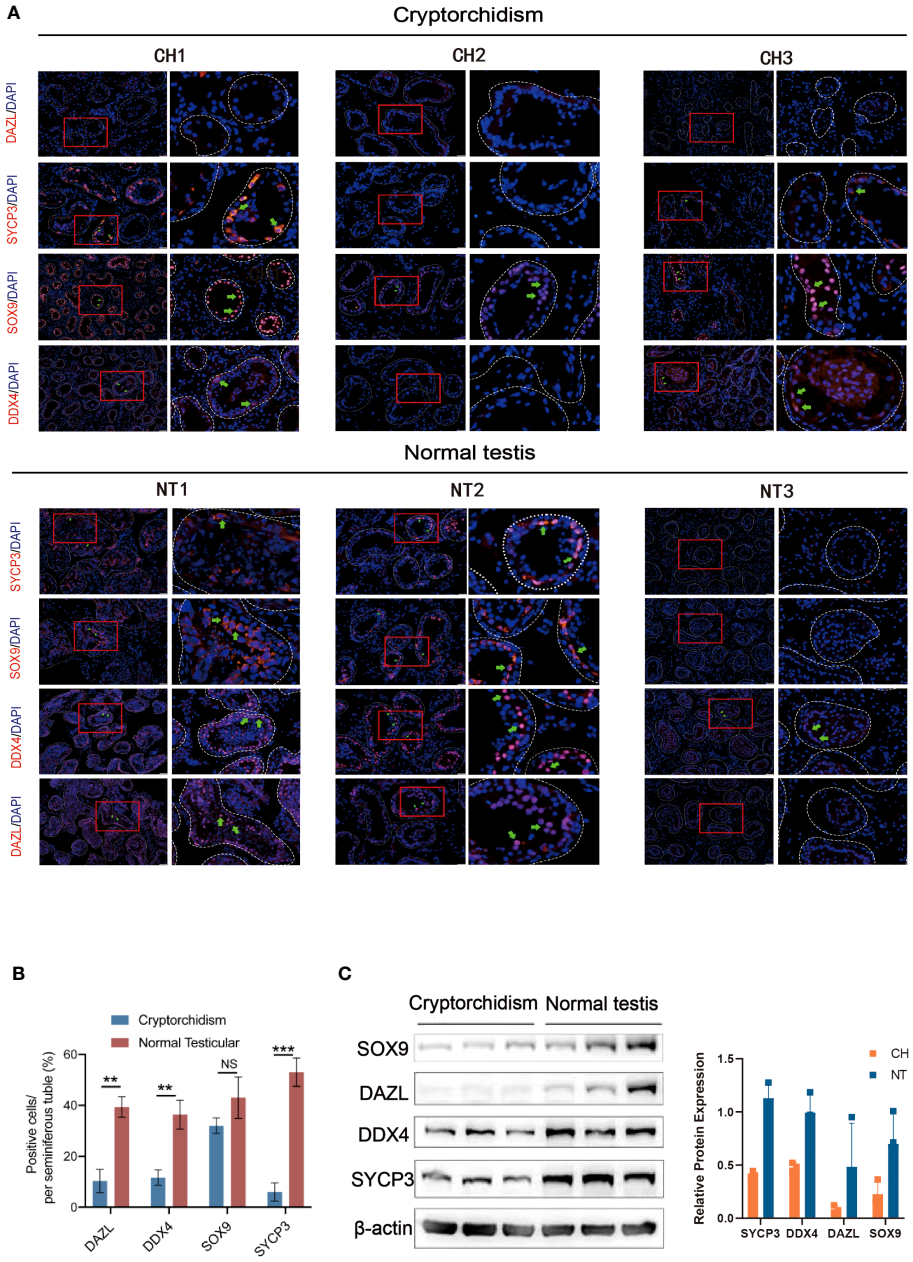


FIGURE 2
The expression of DAZL, DDX4, SOX9 and SYCP3 between cryptorchidism and normal testis. **(A)** IF staining of DAZL, DDX4, SOX9, and SYCP3; **(B)** Quantification of the number of DAZL, DDX4, SOX9 and SYCP3 positive cells per seminiferous tubule. **(C)** Western blot of the expression of SOX9, DAZL, DDX4 and SYCP3. Data are mean ± SD of three independent experiments. **P<0.01, ***P < 0.001, ns P>0.05, ANOVA.

cell. Similar results were observed by examining the expression of *SOX9, DAZL, DDX4* and *SYCP3* in the testis tissues of both groups by western blot (Figure 2B).

Differential expression of genes between the cryptorchidism and normal testis

To elucidate the mechanisms by which cryptorchidism impairs fertility with age increasing, we performed RNA sequencing of cryptorchid and normal testis. We first analyzed the differences in expression levels between cryptorchid and normal testis using venn diagram and volcano plot (Figures 3A, B). The Venn diagram reveals significant differences in the detected genes among different age groups, with only 1613 common genes (Figure 3A). The differential genes between cryptorchid testis and normal testis were screened by fold change and p-value statistical criteria. The top 5 significantly downregulated genes in cryptorchid testis were *PRM2, PRM1, TNP1, HMGB4* and *LELP1*, which also showed a decreasing trend with age in normal testicular tissues, indicating their significant role in male reproduction (Figure 3C). Additionally, the heatmap showed significant differences in gene expression between the two groups

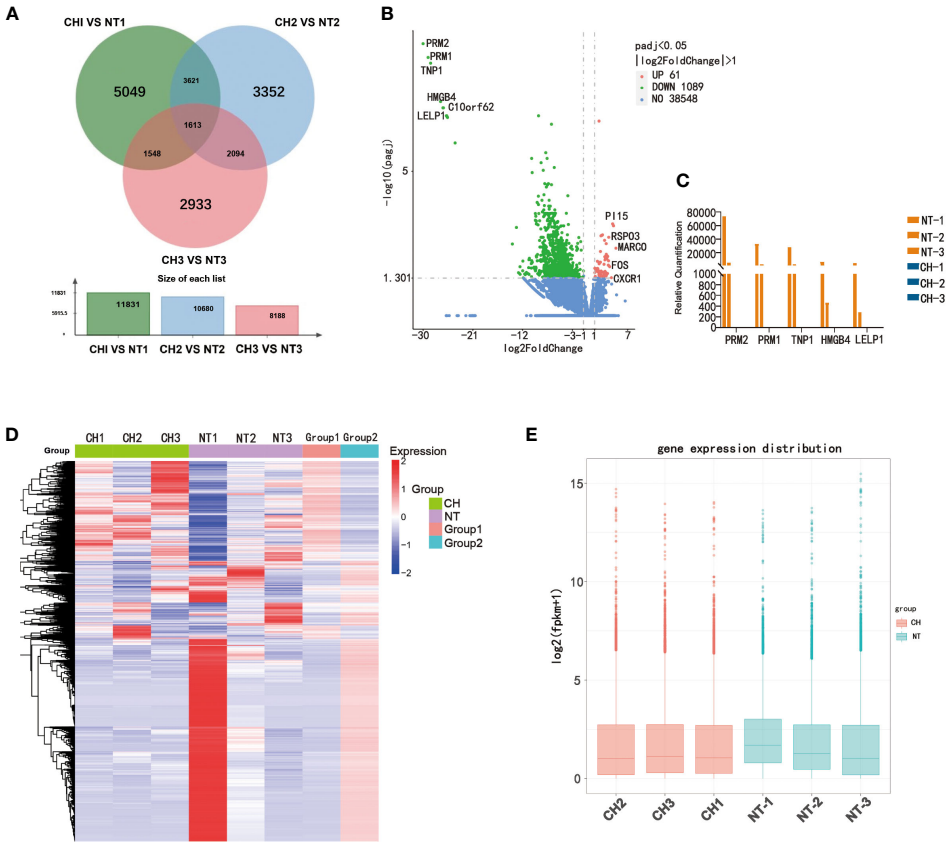


FIGURE 3
Differentially expressed genes between normal testis and cryptorchid testis. **(A)** A Venn diagram showed the intersection of genes acquired from the CH1 *VS* NT1 group (green), CH2 *VS* NT2 group (blue) and CH3 *VS* NT3 group (red). **(B)** The volcano map showed DEGs between cryptorchidism and normal testis. The green and red dots are the downregulated genes and the upregulated genes, respectively with statistical significance (log2 [Fold change] >2 and $p < 0.05$). **(C)** The expression of PRM2, PRM1, TNP1, HMGB4 and LELP1 in different samples. **(D)** Heat map shows the relative expression of DEGs between cryptorchidism and normal testis **(E)** Boxplot, a method of describing data in terms of minimum, first quartile (25%), median (50%), third quartile (75%), and maximum. The abscissa is the sample name, and the ordinate is the signal value of the probe after log2.

(Figure 3D). A box plot was used for visualizing the intensities of expression values of genes in two different groups after normalization, which also showed a significant decrease in gene expression levels in the cryptorchidism group (Figure 3E). These results indicate significant differences in gene expression patterns between cryptorchid and normal testicular tissues, and the expression levels of these DEGs are moderately correlated with age. It suggests that these DEGs may play important roles in male fertility.

Bioinformatics analysis of differentially expressed genes

GO and KEGG analyses were carried out to elucidate the function of the differently expressed genes. According to GO analysis, DEGs play a major role in fertilization, spermatid development and differentiation, among other processes (Figure 4A). According to KEGG pathway analysis, the disrupted genes were prominent in cell cycle, meiosis, and cAMP signaling (Figure 4B). We next looked at the expression of the genes connected to the top six GO biological processes. The expression of these genes was shown to be lowered in cryptorchidism, and the decreased level became more noticeable with age (Figures 4C–H). These genes are mostly involved in sperm motility and germ cell development, which provides underlying molecular mechanism explanations for the histological characteristics of cryptorchidism as shown in Figure 1.

Gene set enrichment analysis (GESA) was also performed to further explore the impacts of alternated genes on biological pathways. The top altered gene sets were shown in Table 2. In addition to oocyte meiosis pathway as identified in GO analysis, motor proteins, apoptosis, Ras signaling pathway, and TGF-β signaling pathway were the top enriched gene sets in cryptorchid testis. oocyte meiosis and motor proteins pathways were down regulated in cryptorchid testis, while apoptosis, Ras signaling pathway, and TGF-β signaling pathway were upregulated in cryptorchid testis (Figure 5). However, "cAMP signaling pathway" did not show significant differences in GSEA analysis. Research has shown that motor proteins are expressed at high levels in the testes and play an important role in telomere movement

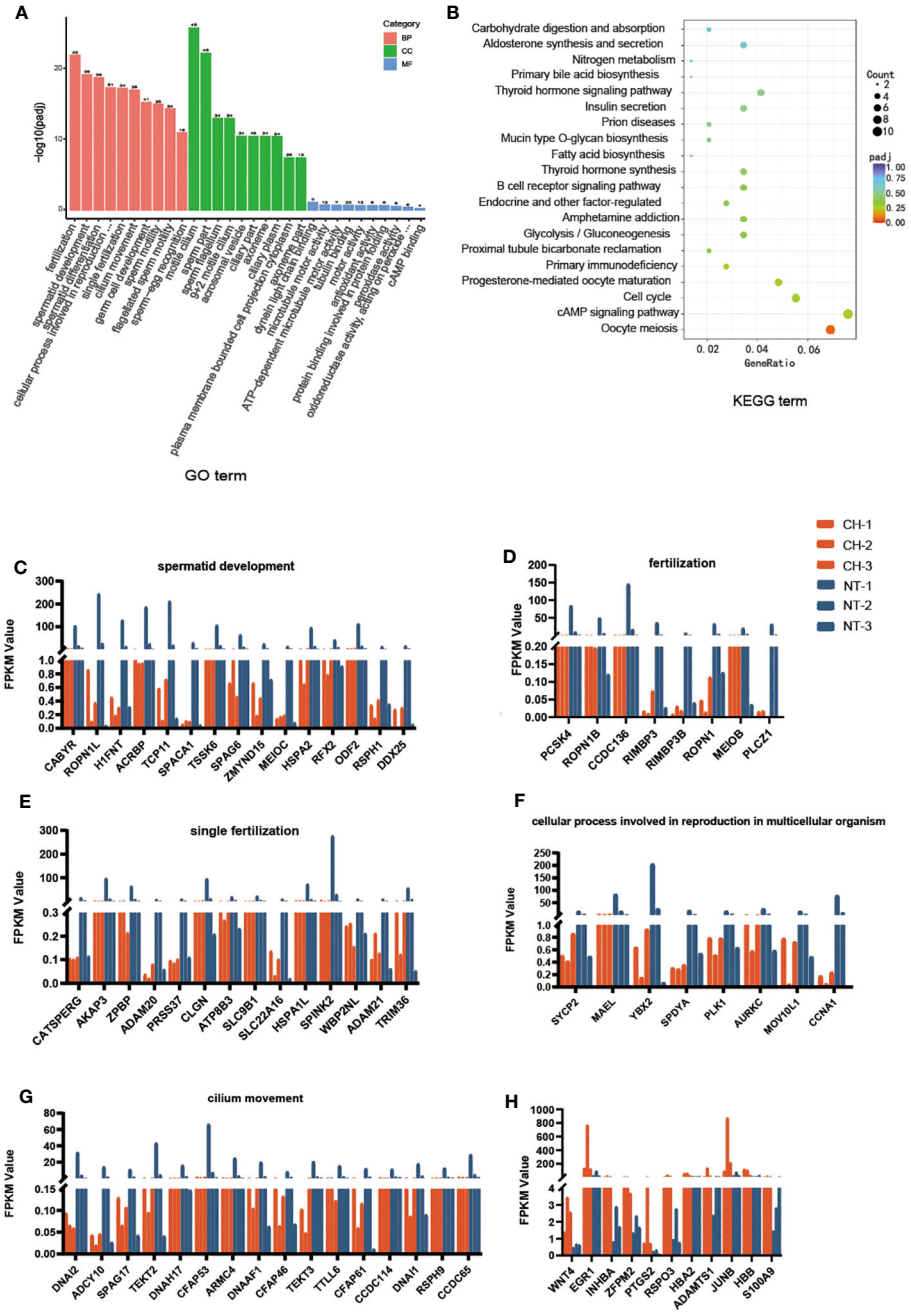


FIGURE 4
Biological chracteristics of the differentially expressed genes. **(A)** GO enrichment annotations of DEGs between cryptorchidism and normal testis. The orange bar is the Biological Process, the green bar is the Cellular component and the blue bar is the Molecular function. **(B)** KEGG pathway enrichment analysis revealing key signaling pathways of DEGs. **(C–H)** The expression of genes within the first six upregulated and downregulated GO biological processes in different samples.

during meiotic division (18). The misplaced position caused by cryptorchidism can lead to thermal stress injury of the testes, activating the apoptotic pathway and resulting in impaired testicular and sperm quality (19). Ras signaling pathway has been studied extensively in the biological context since it regulates the cell cycle and cellular growth, differentiation, metabolism, and senescence. Thus, it appears that Ras signaling is essential for the normal development of mammalian tissues. Therefore, it is not surprising that the dysregulation of this pathway can have profound consequences on testis tissue development of cryptorchidism (20). A previous study has shown that exposure to the antiandrogen flutamide *in utero* can lead to failure of testicular descent in adult rats, and changes in the molecular components of the TGF-β signaling system have been identified (21). The results indicate

TABLE 2 Gene sets enriched in phenotype high.

Gene set name	NES	NOM p-value	FDR q-value
Oocyte meiosis	-1.45567	0.011286	0.064119
Motor proteins	-1.83254	4.15E-07	0.000114
Apoptosis	1.291074	0.019275	0.095915
Ras signaling pathway	1.275003	0.01148	0.064119
TGF-beta signaling pathway	1.53573	0.001258	0.014968

NES, normalized enrichment score; NOM, nominal; FDR, false discovery rate.
Gene sets with NOM p-value <0.05 and FDR q-value <0.25 were considered as significant.

that cryptorchidism leads to alterations in various reproductive pathways, resulting in apoptosis or differentiation blockade of germ cells, ultimately impairing fertility.

Moreover, the STRING online database was used to analyze the protein-protein interactions among the DEGs. The results were extracted and visualized using Cytoscape software. After excluding the isolated nodes, we chose the top 80 hub genes based on degree method scores in the PPI network, as is shown in Figure 6A. Then we selected top 20 hub genes with the highest degree (the number of direct connections that a node has with other nodes) to investigate their effect on the development of cryptorchidism (Figure 6B).Then we found the GO function of these genes are very similar with the first six GO biological process (Figure 6C). So we think these genes may play key roles in the infertility of cryptorchidism with increase in age.

qPCR validation for gene expression

According to the extent of difference between cryptorchidism and normal testis, the expression of seven downregulated genes (*PLCZ1, AKAP4, AKAP3, IZUMO1, SPAG6, CAPZA3*, and *ROPN1L*) were further selected for validation by qPCR (Figure 7). We confirmed that these genes were significantly downregulated in the cryptorchid testis, which is consistent with the RNA sequencing results.

PLCZ1 encoding 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase zeta-1 (*PLCζ*) which is the primary stimulus for egg activation and early embryonic development. If the expression levels of sperm plcζ was reduced, it will lead to male infertility (22).

AKAP4 (A-kinase anchoring protein 4) and *AKAP3* (A-kinase anchoring protein 3) genes are structural proteins of the fibrous

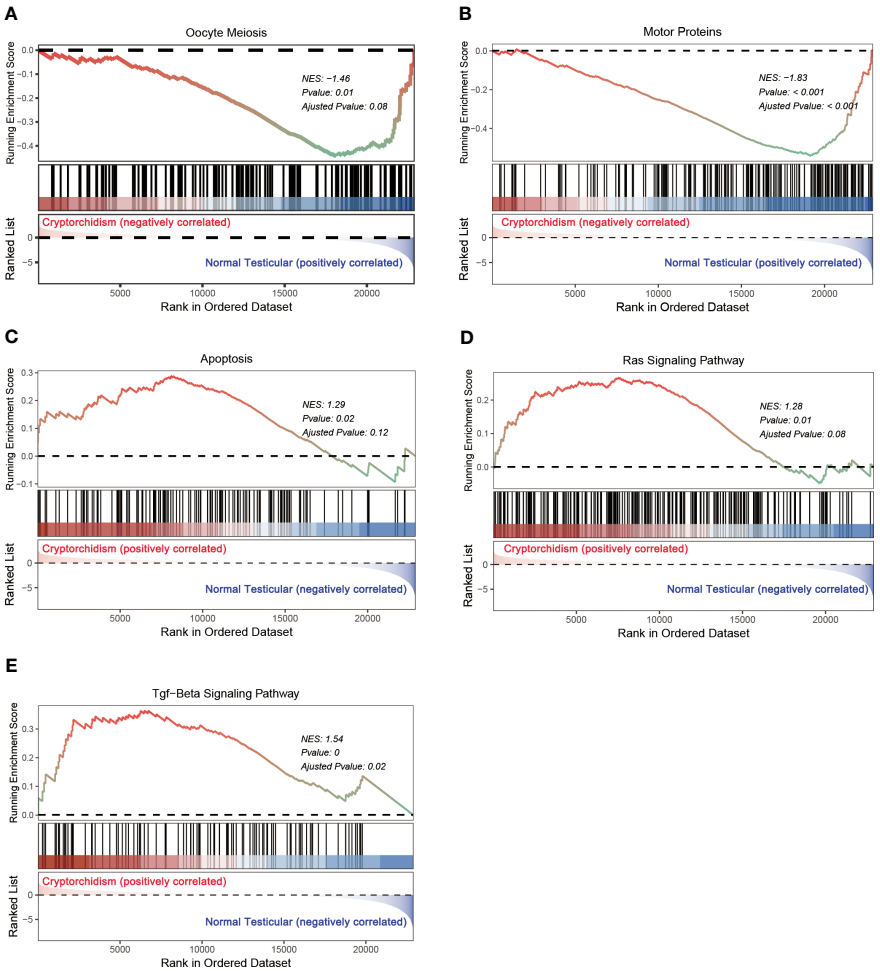


FIGURE 5
GSEA analysis of differential expression genes. **(A–E)** represents the signaling pathway enriched in the DEGs.

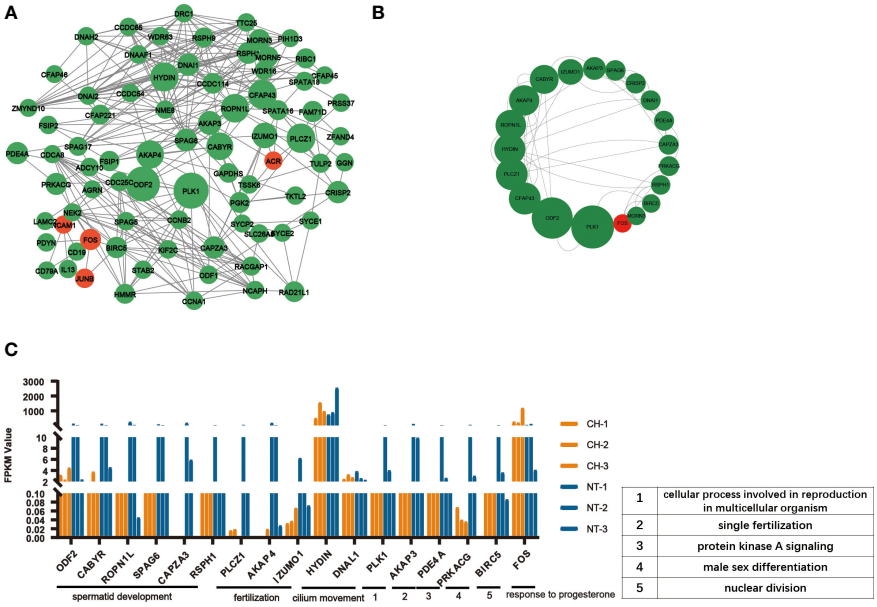


FIGURE 6
PPI network analysis of DEGs. **(A)** Protein-protein interaction network of DEGs. Each node represented a protein. The width of the edges was proportional to the score of protein-protein interaction. **(B)** The top 20 densely-connected modules identified by Betweenness. Red nodes were upregulated ones, green downregulated ones. **(C)** The expression of top 20 hub gens in different samples. The biological processes associated with these hub genes were noted.

sheath which play important roles in sperm formation and motility (23). *AKAP4* is mainly responsible for regulating the signal transduction and metabolic pathways that support sperm motility and capacitation. Oxidative stress will destroy the *AKAP4* which results in the defective sperm function associated with male infertility (24).

IZUMO1 is the only cell surface protein expressed on sperm that is known to be essential for sperm–egg interaction *in vivo* which plays an essential role in recognition or adhesion between the sperm and egg. When sperm and egg bind, the *IZUMO1* protein binds to the JUNO protein on the egg membrane, allowing the sperm to penetrate the egg membrane and enter the egg, thereby initiating fertilization (25).

Spag6 (sperm-associated antigen 6) is essential for sperm flagellar motility which it is important for the maintenance of the structural integrity of mature sperm. Research indicate that if *Spag6* is deficient, their sperm was marked motility defects and was morphologically abnormal with frequent loss of the sperm head and disorganization of flagellar structures, including loss of the central pair of microtubules and disorganization of the outer dense fibers and fibrous sheath (26).

CAPZA3 (Capping Protein Muscle Z-line Alpha 3) localized to the anterior head in intact sperm similar to *IZUMO1* (27). *CAPZA3* plays a role in maintaining polymerized actin during spermiogenesis (28).

ROPN1L (Ropporin 1-like) gene encodes a sperm head-specific protein that plays an important role in sperm development and maturation. Sperm proteins *ROPN1* and *ROPN1L* bind *AKAP3*. The mice deficient in *ROPN1L* (*RLKO*) will reduce sperm motility (29).

Expression of candidate genes was validated by qPCR based on the previously frozen tissue samples. Consequently, the result of RT-qPCR (Figure 7) has a similar expression to the result of RNA sequence. Taking together, these results shed light on potential genes that might be relevant to comprehend human fertility disorders caused by cryptorchidism.

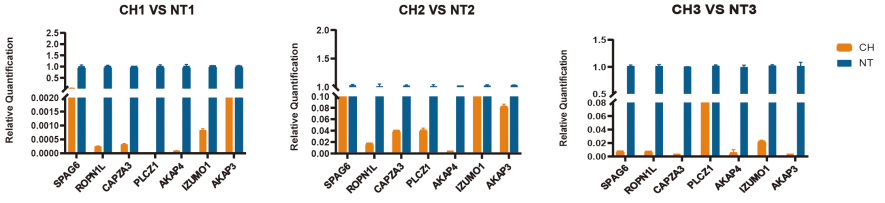


FIGURE 7
Validation of selected top 7 genes by quantitative RT-PCR. The relative expression levels of 7 genes were determined by real-time RT-PCR. All values represent mean and standard deviation. The orange bar is the cryptorchidism and the blue bar is the normal testis.

Discussion

Testes descending into the suitable position of the scrotum is a critical process for the development of reproductive system. Failed testes descending will lead to cryptorchidism which is a high risk factor of fertility. Now newborns who has cryptorchidism usually receive the corrective surgery before 1 year old. However, there are a small portion of patients did not receive the correction surgery even after adulthood due to various reasons. Additionally, as the undescended testis persists longer in the abdomen, the tissue structure of cryptorchidism deteriorates, which causes fertility to gradually drop. For instance, those who did not have corrective surgery, as depicted in Figure 1, exhibited aberrant and atrophic seminiferous tubule shape with missing germinal cells, and the effects of damage progressively aggravated. Additionally, immunofluorescence labeling reveals that the majority of cell populations in adult cryptorchidism decreased with age in comparison to normal testis (Figure 2). However, currently there is a lack of research on adult patients with cryptorchidism who wish to preserve their fertility and avoid malignant transformation.

Hence, identifying the underlying mechanism of progressive cryptorchidism would be useful for early diagnosis and treatment. In this study, we performed RNA sequencing in undescended testes from adult human cryptorchidism aiming to find the key genes involved in the progression of cryptorchidism. We identified 1150 DEGs in undescended testes in comparison to age-matched normal testis, including 61 upregulated and 1089 downregulated genes. Most of the DEGs are downregulated in cryptorchidism, indicating that incorrect positioning leads to the inability of many genes to be expressed in the testes. GO analysis showed that these DEGs are mainly enriched in processes such as fertilization, spermatid development, spermatid differentiation, single fertilization, and cilium movement. These biological processes are closely related to testicular development, spermatogenesis, and germ cell differentiation. Furthermore, KEGG analysis also showed that the oocyte meiosis signaling pathway, cAMP signaling pathway, and cell cycle signaling pathway were affected. GSEA analysis further confirmed that the oocyte meiosis signaling pathway was significantly downregulated in adult cryptorchidism patients, which is essential for spermatogenesis and fertility (30). Furthermore, we found that the histological features and gene expression patterns also change with aging, providing an overview for the aging process in the testis tissues. Additionally, we used PPI analysis to identify top 20 hub genes inside these DEGs (Table 3). The majority of these genes were involved in spermatid development, fertilization, cilium movement, etc., which is similar with the findings from GO, KEGG, and GESA study. In the end, we chose 7 genes and used q-PCR to confirm that their expression was consistent with the findings of RNA-seq.

In conclusion, our study significantly increased our understanding of how adulthood cryptorchidism develops and had some implications for treating adulthood cryptorchidism by providing an overview of the transcriptomic profiles in the development of adulthood cryptorchidism and identifying key genes and associated pathways involved in its progression. Our research also offered hints about changes in spermatogenesis with age. Understanding the functions of the major genes played in the development of cryptorchidism proved to require further research.

TABLE 3 Top 20 hub genes with higher connection with others.

Number	Gene name	Degree
1	PLK1	8926.88
2	ODF2	8402.89
3	CFAP43	5667.07
4	PLCZ1	5665.8
5	HYDIN	5467.25
6	ROPN1L	5303.34
7	AKAP4	5187.02
8	CABYR	4770.95
9	IZUMO1	3818
10	AKAP3	2961.71
11	SPAG6	2335.78
12	CRISP2	2234
13	DNAI1	2194.98
14	PDE4A	2166.01
15	CAPZA3	2148.74
16	PRKACG	2096.11
17	RSPH1	2007.12
18	BIRC5	1727.41
19	MORN3	1704
20	FOS	1674.12

Degree was calculated using CytoNCA which representing the correlation of genes.

Conclusions

In this study, we investigated the histological changes and identified 1150 DEGs in adult cryptorchid patients of different age groups, which are mostly associated with spermatid development and fertilization pathways. We selected seven significantly downregulated genes (*PLCZ1, AKAP4, IZUMO1, SPAG6, CAPZA3*, and *ROPN1L*) in cryptorchid testis, and validated their expression by q-PCR. Our research findings contribute to a better understanding of molecular mechanism associated with the failure of spermatogenesis in adult cryptorchidism and provide evidence for treatment development for adult cryptorchidism.

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 in the article/supplementary material.

Ethics statement

The studies involving humans were approved by Changhai Hospital's ethical approval (CHEC2021-086). 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

WS: Resources, Writing – original draft, Writing – review & editing. XZ: Methodology, Supervision, Writing – original draft, Writing – review & editing. LW: Conceptualization, Formal Analysis, Investigation, Project administration, Validation, Writing – original draft. GR: Data curation, Methodology, Project administration, Writing – original draft. SP: Formal Analysis, Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – original draft. CY: Data curation, Funding acquisition, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. ZL: Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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EDITED BY
Xiang Xiao,
Hangzhou Medical College, China

REVIEWED BY
Huitao Li,
Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, China
Larisa Suturina,
Scientific Center of Family Health Problems and Human Reproduction (RAS), Russia
Sílvia Moreira,
University of Aveiro, Portugal

*CORRESPONDENCE
Huang Liu
k114@163.com

†These authors have contributed equally to the article

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Leydig cell metabolic disorder act as a new mechanism affecting for focal spermatogenesis in Klinefelter syndrome patients: a real world cross-sectional study base on the age

Huang Liu[1*], Zhenhui Zhang[2†], Yong Gao[3†], Hai Lin[1], Zhiyong Zhu[1], Houbin Zheng[1], Wenjing Ye[4], Zefang Luo[1], Zhaohui Qing[5], Xiaolan Xiao[5], Lei Hu[1], Yu Zhou[1] and Xinzong Zhang[1]

[1]Department of Andrology, National Health Commission (NHC) Key Laboratory of Male Reproduction and Genetics, Guangdong Provincial Reproductive Science Institute (Guangdong Provincial Fertility Hospital), Human Sperm Bank of Guangdong Province, Guangzhou, China, [2]Reproductive Medicine Center, Shunde Hospital, Southern Medical University (The First People's Hospital of Shunde), Foshan, China, [3]Department of Reproductive Medicine Center, Guangdong Provincial Key Laboratory of Reproductive Medicine, Guangdong Provincial Clinical Research Center for Objective and Gynecological Diseases, Sun Yat-sen University First Affiliated Hospital, Guangzhou, China, [4]Reproductive Medicine Center, National Health Commission (NHC) Key Laboratory of Male Reproduction and Genetics, Guangdong Provincial Reproductive Science Institute (Guangdong Provincial Fertility Hospital), Human Sperm Bank of Guangdong Province, Guangzhou, China, [5]Department of Anesthesiology, National Health Commission (NHC) Key Laboratory of Male Reproduction and Genetics, Guangdong Provincial Reproductive Science Institute (Guangdong Provincial Fertility Hospital), Human Sperm Bank of Guangdong Province, Guangzhou, China

Background: Klinefelter's syndrome (KS) was once considered infertile due to congenital chromosomal abnormalities, but the presence of focal spermatozoa changed this. The key to predict and promote spermatogenesis is to find targets that regulate focal spermatogenesis.

Objective: To explore the trend of fertility changes in KS patients at different ages and identify potential therapeutic targets.

Methods: Bibliometric analysis was used to collect clinical research data on KS from the Web of Science Core Collection (WoSCC) from 1992 to 2022. A cross-sectional study was conducted on 75 KS patients who underwent microscopic testicular sperm extraction (mTESE) from 2017 to 2022 in the real world. The reproductive hormones, testicular histopathology, androgen receptors, insulin-like factor 3 (INSL3) receptors and sperm recovery rate (SRR) were analyzed.

Results: Male infertility, dysplasia, Sertoli cells, Leydig cells, testosterone and spermatogenesis were the research focuses related to KS. Luteinizing hormone (LH), testosterone, and INSL3 were evaluation indicators of Leydig cell function that fluctuate with age. Testosterone and LH peaked at ages 13-19 and 30-45,

while INSL3 only peaked at ages 13-19. 27 patients (27/75) recovered sperm through mTESE and experienced SRR peaks at the ages of 20, 28, 34, and 37. The SRR of fibrosis patients was 46.15%, fatty degeneration was 7.14%, and melanosis was 40.00%. The INSL3 and androgen receptors were highly expressed and roughly balanced in focal spermatogenesis.

Conclusion: Abnormal metabolism of Leydig cells led to imbalanced expression of INSL3 and androgen receptors, which might be a potential target for spermatogenesis in KS.

KEYWORDS

Klinefelter syndrome, Leydig cell, metabolic disorder, age, microscopic testicular sperm extraction, spermatogenic

1 Introduction

Klinefelter syndrome (KS), the most common sex chromosomal abnormality, was first described by Harry F. Klinefelter (1) in 1942 as a clinical entity presenting with features of primary hypogonadism, and the disorder, confirmed by Jacobs (2) through cytogenetics, was demonstrated to be caused by one or more X chromosomes inherited from the paternal or maternal chromosome. The classical clinical manifestations of KS are small firm testes, eunuchoidism stature, sexual dysfunction, infertility and gynecomastia, sometimes accompanied by hyperglycemia, obesity, osteoporosis, cognitive dysfunction and psychological problems, but the phenotype can vary from the typical one with androgen deficiency to a normally virilized group (3, 4). The prevalence of the syndrome, ranging between 0.1% and 0.2% in newborn male infants, increases to approximately 3% in infertile males and to 10–12% among azoospermic patients, and the incidence tends to increase yearly according to a recent study (4, 5).

Nevertheless, due to the variability of the phenotype or to the lack of typical symptoms in prepuberty, approximately two-thirds of Klinefelter patients are not confirmed during their whole life, and the rest can sometimes be diagnosed mainly because of infertility in the reproductive domain (3). Assisted reproductive technology, especially intracytoplasmic sperm injection (ICSI) combined with microdissection testicular sperm extraction (mTESE), is the core therapy for KS patients who suffer from azoospermia to seize the hope of having their own offspring (6). Unfortunately, there are still many problems in the existing diagnosis and treatment of KS. On the one hand, the failure of generative cell function starts from puberty, but the low diagnostic rate may deprive us of the best timeline for testicular sperm extraction (TESE) and fertility preservation action (3, 7, 8). On the other hand, although the fluctuation of serum hormones such as testosterone, follicle-stimulating hormone and luteinizing hormone is helpful in evaluating spermatogenesis, there are discrepant data and opinions on whether these indexes are capable of accurately predicting the success of mTESE in KS (9, 10). From the aspect of histopathology, KS is characterized by progressive hyalinization of the seminiferous tubules of the testis and loss of spermatogonia, accompanied by the hyperplasia of Leydig cells, which is contradictory to the absence of androgen levels in adult patients with KS (11, 12). The majority of previous studies of the various cell types that exist in the testis have focused on the degeneration of germ cells and Sertoli cells in the field of KS (7, 13–15). Although the disturbed maturation of Leydig cells has attracted further attention in recent research on KS, there is still little conclusive evidence of the pathophysiological mechanism and treatment methods based on Leydig cells (16, 17). An in-depth understanding of the mechanisms of testicular cell failure and its timeline is supposed to help better inform the constant debate regarding the related factors affecting therapeutic outcome in KS and to help further understand the clinical heterogeneity in different age groups of KS.

Taken together, a comprehensive assessment of fertility in KS patients is essential for patient counseling, treatment decision-making and therapeutic benefits. In this study, we preliminarily summarized the emerging trends and the cluster of studies on Leydig cells in patients with KS using a bibliometric analysis of the scientific landscape, which is a qualitative and quantitative tool frequently applied to systematically study the characteristics of global scientific literature and to build the knowledge frame in specific fields (18, 19). The interesting results of the visual landscape include numerous randomized controlled trials (RCTs), in which recruited participants meet the specific inclusion criteria and are monitored with predefined protocols.

Therefore, a real-world study was then conducted to consistently demonstrate whether the results could be extended to an actual clinical environment. In real-world clinical settings, global multidisciplinary clinicians have been actively exploring multiple factors and seeking means to predict the testicular function of patients with KS at different ages. At present, there is a lack of specific predictive methods for the occurrence of focal spermatogenesis in the testicles of KS patients. However, in clinical practice, it has been found that the probability of focal spermatogenesis varies among KS patients at different ages. Leydig cells constitute the microenvironment of spermatogenesis, and we

hypothesized that metabolic function may have potential value in these individuals' focal spermatogenesis, and the sign of Leydig cell metabolism disorder or advanced failure in the testis among different age groups could help better understand the dynamic progression of KS.

2 Method

2.1 Literature retrieval

Relevant bibliometric data on KS and Leydig cells were collected and identified using the Web of Science Core Collection (WoSCC) database for the preliminary review (Figure 1). The WoSCC is a famous scientific database with a high reputation in the global academic community and is regarded as one of the most available sources of the bibliometric analysis of scientific publications worldwide, breaking the barriers of interaction between disciplines, journals and publishers (20–22). To capture current research trends of morphological and functional changes of Leydig cells in KS, we limited the search of relevant documents to the "article title, abstract and keywords" of publications in the WoSCC on the basis of the previous literature retrieval strategy, applying the main search terms of "Klinefelter syndrome" and "Leydig cell" with the operation method "AND" on 17 December 2022. Original articles, editorial materials, meeting abstracts (those including relevant information of patients), notes and review articles in Science Citation Index Expanded (SCIE) were incorporated into our study. To limit the potential impact of irrelevant or duplicate titles, the researchers conducted duplicate detection, and the documents were filtered for relevance and uniqueness. Additionally, only papers in English with raw data of titles, authors, abstracts, keywords, and references from WoSCC were downloaded and saved as plain text files for further data analysis.

2.2 Visual analysis

The preprocessed data were then analyzed using VOSviewer version 1.6.18, Scimago Graphica version 1.0.26 (https://www.graphica.app), R software version 4.2.1 (https://www.r-project.org) with the bibliometrix R package (https://www.bibliometrix.org) and Microsoft Excel Version 2019.

R software and the core R package were applied to fetch the information from the raw files. VOSviewer and SCImage Graphics were then used to plot visual bibliometric maps to present the collaboration relationship between countries, institutions and authors of highly cited literature, the academic influence of authors, and the citation and reference situation. In addition, VOSviewer was also applied to sort out the keywords with high co-occurrence frequency into several clusters and to reveal the research focuses and trends.

2.3 Reproductive hormone characteristics in literature data

In addition, additional sub-analyses were carried out for the ten experimental articles including complete clinical information of patients on the indexes with potential predictive significance in the shape, quantity or function of Leydig cells to investigate the influence of multiple factors on the Leydig cells of patients with KS. Then, according to the literature data, the mean values of testosterone, luteinizing hormone and INSL3 of the subjects sorted at different ages, and the sample size were summarized and statistically analyzed.

2.4 Real-world research

2.4.1 Patient data

After strictly screening from the 68404 infertile males attended at Guangdong Provincial Reproductive Hospital (Guangdong Provincial Institute of Reproductive Sciences) from July 2017 to December 2022, we eventually enrolled 75 KS patients who were hospitalized for testicular incision and microscopic sperm extraction in this study (Figure 2). All patients were operated on after signing the informed consent form. The age of onset, course of disease, drugs used during treatment, and sexual hormone level before and after treatment were recorded in detail, physical examination was performed, and scrotal color ultrasound was reviewed before the operation. This study was approved by the

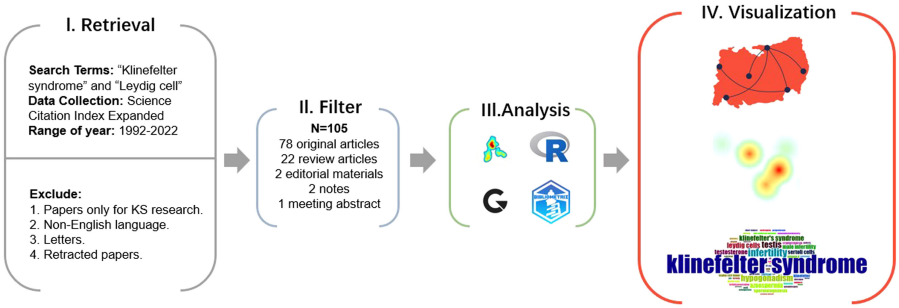

FIGURE 1
Process of bibliometric and visual analysis.

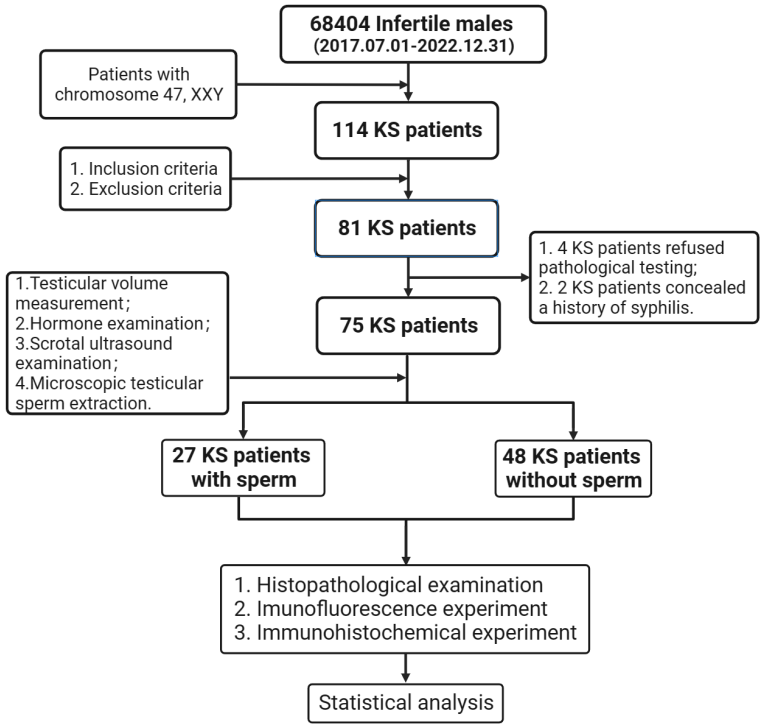

FIGURE 2
Overview of the real world research.

Ethics Committee of Guangdong Provincial Reproductive Science Institute (Guangdong Provincial Fertility Hospital) (Approval No.2021(08)) and had been registered through the Chinese Clinical Trial Registry (https://www.chictr.org.cn/) with the registration number (ChiCTR2200060463).

2.4.2 Inclusion criteria

① According to the karyotype diagnosis of 47, XXY; ② Be able to cooperate with inspection; ③ Willing to accept and able to tolerate mTESE operation; ④ Complete withdrawal from drug treatment for 3 months or more before operation; ⑤ Able to complete sexual intercourse and obtain semen.

2.4.3 Exclusion criteria

① AZF gene deletion; ② Combined with genital tract infection; ③ Combined with systemic diseases; ④ Combined with varicocele; ⑤ Those who still have drug intervention within three months before operation; ⑥ Combined tumor, especially tumor of reproductive organs; ⑦ He has received radiotherapy and chemotherapy. ⑧ Stay up late for a long time or have bad habits such as smoking, alcohol and drug use; ⑨ There are familial hereditary diseases; ⑩ Sexual and ejaculatory dysfunction.

2.4.4 Method

2.4.4.1 Testicular volume

The testicular volume measurement quantum model (size: 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 20, >25 mL) was used to measure the testicular volume, and the testicular volume was matched with the color Doppler ultrasound data (23).

2.4.4.2 Hormone

An automatic chemiluminescence instrument (Roche reagent kit) was used to measure the concentration of sex hormones in peripheral blood, including follicle-stimulating hormone (Roche reagent kit 11775863122), luteinizing hormone (Roche reagent kit 11732234122), testosterone (Roche reagent kit 05200067190) and estradiol (Roche reagent kit 06656021190).

2.4.4.3 Microscopic testicular sperm extraction

All patients were under general anesthesia for tracheal intubation, and a ZEISS S88 ultrahigh-definition operating microscope was used for testicular incision and microscopic sperm extraction. During the operation, the equatorial plane of the testis was taken to cut the white membrane of the testis, expose the tissue in the testis, carefully distinguish the seminiferous tubules, interstitial tissue and capillaries, etc., and the relatively formed seminiferous tubules were extracted and sent to a Nikon inverted microscope to tear up and check whether there was sperm. If no sperm are found in one testicle, cut the other testicle to look for sperm and thoroughly determine whether there are sperm. At the same time, a small amount of testicular tissue was taken for pathological examination during the operation. Photographs and videos were used to record the condition and operation process of testicular tissue during the operation.

2.4.4.4 Scrotal ultrasound examination

All patients were placed in the supine position at room temperature (26 °C), and a 7-14 Hz probe was used to scan both sides of the scrotum longitudinally and transversely. The length,

width and thickness of the testicle were recorded, and the following formula was applied: testicular volume=length × wide diameter × thickness × 0.71 (24).

2.4.4.5 Histopathological examination

The testicular tissue removed under sterile conditions during the operation was placed in Bonn's immersion and sent for histopathological examination on the same day. The condition of spermatogenic cells in the tissue was scored by the Johnson scoring method (25).

2.4.4.6 Immunofluorescence experiment

Paraffin sections of testicular tissue obtained from the mTESE operation were dewaxed and dehydrated and then placed in a microwave oven for antigen repair. Primary antibodies (Anti -INSL3 Rabbit pAb, GB113558-100, Servicebio, 1: 3000) and secondary antibodies (HRP labeled goat anti rabbit secondary antibody, GB23303, Servicebio, 1:200) were added for incubation, and DAPI was added dropwise for staining. Follow the steps below to conduct the experiment: ① Paraffin section dewaxing to water. ② Antigen repair. ③ Blocking endogenous peroxidase. ④ Serum blocking. ⑤ Adding Primary antibodies(Anti -INSL3 Rabbit pAb, GB113558-100, Servicebio, 1: 3000). ⑥ Adding secondary antibody (HRP labeled goat anti rabbit secondary antibody, GB23303, Servicebio, 1:200). ⑦ DAB color rendering. ⑧ Recombinant staining of cell nucleus. ⑨ Dehydration and sealing. ⑩ Microscopic examination. A self-luminescent quenching agent was added, and then the seal was observed by fluorescence microscopy, and images were collected. The ultraviolet excitation wavelength of DAPI is 330-380 nm, the emission wavelength is 420 nm, and it emits blue light; FITC excitation wavelength 465-495 nm, emission wavelength 515-555 nm, green light; CY3 excitation wavelength 510-560, emission wavelength 590 nm, emitting red light. The results showed that the nucleus stained by DAPI was blue under ultraviolet excitation, and the positive expression was red or green light labeled by corresponding fluorescein.

2.4.4.7 Immunohistochemical experiment

Paraffin sections of testicular tissue obtained from mTESE surgery were dewaxed and dehydrated, placed in a microwave oven for antigen repair, blocking endogenous peroxidase, added to serum to seal the tissue and incubated with the first and second antibodies. Finally, DAB staining solution was added to control color development so that the positive cells were stained brown. Finally, hematoxylin solution was used to restain the nucleus so that the nucleus was stained blue, and the dehydrated seal was placed under a white light microscope to interpret the results. The stained nucleus was blue, and the positive expression of DAB was brown.

2.4.5 Statistical analysis

The data were statistically analyzed and plotted with OriginLab Origin 2022 (OriginLab Corporation, USA). The counting data were expressed in the form of n or percentage (%). The chi-square test was used for analysis. The mean difference of the measurement data was expressed in the form of plus or minus standard deviation. The K-S test was used to test the homogeneity of variances. When the variances were homogeneous, the t test was used to compare the intergroup or intragroup differences. When the variances were uneven, the U test was used. AVNOA univariate analysis of variance was used to assess the significance of each influencing factor. When $P<0.05$, the difference was statistically significant. The missing follow-up data was not included in our final analysis.

3 Results

3.1 Bibliometric analysis and visualization

3.1.1 Publications and journals

A total of 105 identified articles met the above retrieval criteria. As shown in Figure 3A, the overall quantity of publications is not large, and the annual publication output is unstable from 1992 to 2022, which indicates a lack of research attention to KS and Leydig cells, but the number of documents issued shows a growing trend in the past decade, with two peaks in 2016 and 2020. Figure 3B depicts the literature types: 78 original articles (74.286%), 22 review articles (20.952%), 2 editorial materials (1.905%), 2 notes (1.905%), and one meeting abstract (0.952%).

According to our analysis, the published papers about KS and Leydig cells from 1992 to 2022 were mainly disseminated in 66 journals, and the top 15 journals with the largest numbers are summarized in Table 1. Journal of Clinical Endocrinology & Metabolism and Andrology were the most productive journals. The impact factors of the top 15 journals in 2022 varied from 3.054 to 7.49, and most of them belong to the United States and the United Kingdom.

The time-based analysis of journal productivity is presented in Figure 3C, where the colors denote the average year of all published papers in a journal. The average rate of quoting each paper in the journals is presented in Figure 3D, visually indicated by the color evolution from purple to yellow. It can be found that the average publication year of Clinical Endocrinology & Metabolism appears earliest with the highest local citation, which indicated that it had realized the significance of Leydig cells to patients earlier than others. In comparison, the number of published papers from andrology and fertility and sterility appeared to increase between 2015 and 2020, and the two journals also achieved remarkable local citation scores.

3.1.2 Countries and authors

A total of 23 countries contributed to the published studies on Leydig cells in KS patients. The distribution of published documents and the connection between countries participating in international cooperation are shown on a world map by Scimago Graphica (Figure 4A), which indicated that collaborative teams were built between them and that the global research centers were predominantly located in Europe and the United States. According to the findings by R, the United States contributed 18 articles among the top ten countries (Figure 4B) active in the relevant studies, and studies from Denmark had the highest number of citations, followed by the United States, Italy, France and Finland. (Figure 4C).

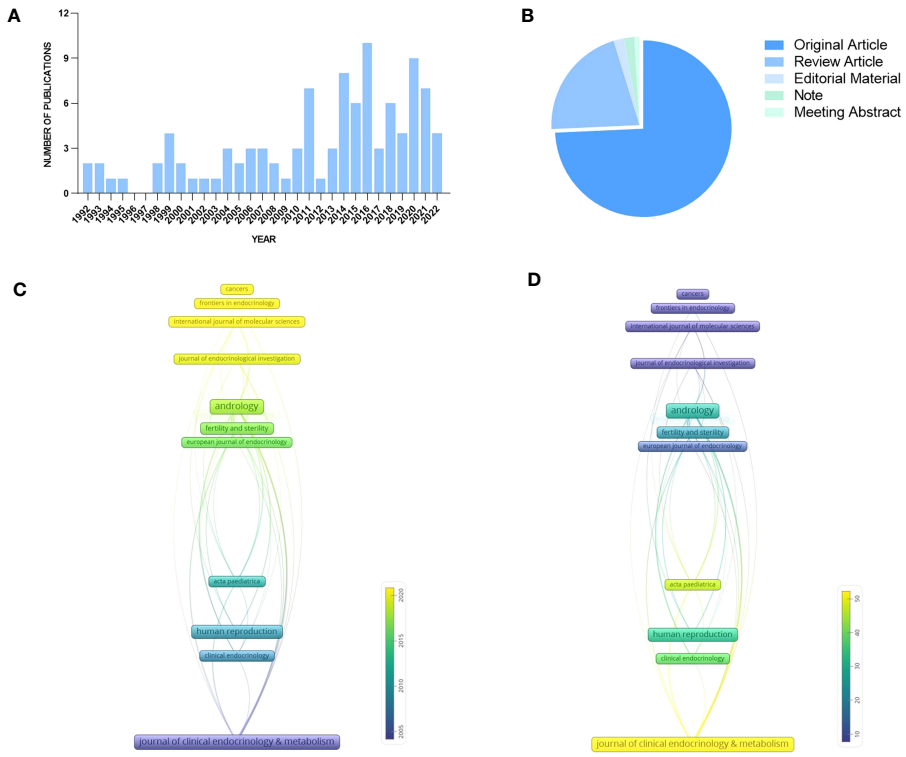

FIGURE 3
Publications and journals for quantitative analysis: **(A)** Publication output. **(B)** Literature types. **(C)** Average year publication of highly productive journals. **(D)** Average citation scores of highly productive journals.

TABLE 1 Journals ranked by numbers of articles in the field of Leydig cells in patients with Klinefelter syndrome (Top 15).

	Journal	Articles	Proportion	Region	Import factor(2022)
1	Journal of Clinical Endocrinology & Metabolism	8	7.407	USA	6.134
2	Andrology	8	7.407	USA	4.456
3	Human Reproduction	6	5.556	United Kingdom	6.353
4	Fertility and Sterility	4	3.704	USA	7.49
5	Acta Pediatrica	3	2.778	United Kingdom	4.056
6	Clinical Endocrinology	3	2.778	United Kingdom	3.523
7	Endocrinology	3	2.778	USA	5.051
8	International Journal of Molecular Sciences	3	2.778	USA	6.208
9	Scientific Reports	3	2.778	United Kingdom	4.996
10	American journal of medical genetics. Part C, Seminars in medical genetics	2	1.852	USA	3.359
11	Asian Journal of Andrology	2	1.852	China	3.054
12	European Journal of Endocrinology	2	1.852	United Kingdom	6.558
13	Cancers	2	1.852	Switzerland	6.575
14	Journal of Endocrinological Investigation	2	1.852	Italy	5.467
15	Frontiers in Endocrinology	2	1.852	USA	6.055

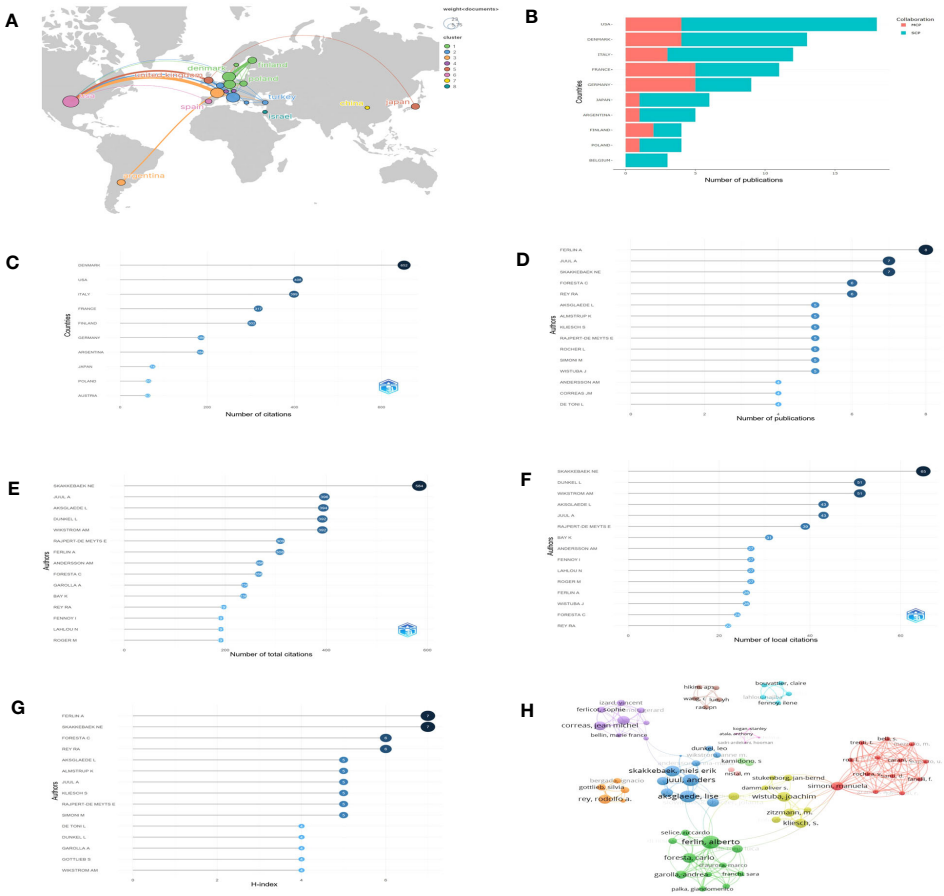

FIGURE 4
Countries and authors for quantitative analysis: **(A)** The network map of countries. **(B)** Numbers of publications of countries (SCP, single country publication; MCP, multiple country publication. **(C)** Total citations of countries. **(D–G)** The top 15 authors in terms of number of publications, total citations, local citations and H-index. **(H)** Collaboration network.

Over 508 authors have conducted research on Leydig cells in KS patients. The top 15 authors in terms of number of publications, total citations, local citations and H-index are shown in our results (Figures 4D–G) to assess their influence. Among them, Ferlin A was the most prolific author with 8 articles, followed by Juul A (7 articles) and Skakkebaek NE (7 articles). Skakkebaek NE was also cited the most in both total and local citations, 584 times and 65 times, respectively. Juul A was the second most frequently cited author in total citations (396 times), followed by Aksglaede L (394 times), Dunkel L (392 times) and Wikstrom AM (392 times). When applying the h-index to evaluate the influence of authors, the ranking was headed by Ferlin A, which was followed by Skakkebaek NE, Foresta C and Rey RA. The distribution among the authors was also studied and presented by a collaboration network (Figure 4H).

3.1.3 Citation and Keywords

To analyze the citations of the documents, we present the top 10 documents with the most citations in Table 2, which shows that there were 7 articles with more than 10 local citations. Among them, "Natural history of seminiferous tubule degeneration in Klinefelter syndrome" (2006), published by Aksglaede L in Human Reproduction Update, had the most local citations and global citations, up to 25 and 187 times, and it was also the center of the citation network of the documents (Figure 5A).

Based on a total of 3700 cited references, Figure 5B shows the cocitation network between cited references with more than five citations, which reveals that references with the strongest citation bursts were Lanfranco F (2004), Aksglaede L (2006) and Wikstrom AM (2004).

A total of 195 keywords among 3049 keywords were identified from 105 documents after we normalized similar keywords such as "Klinefelter syndrome", "XXY syndrome" and "Klinefelter's syndrome" and set the minimum number of occurrences of a keyword to two times to avoid redundancy and synonyms. Figure 5A provides an overview of overall trends and interconnections in the field. Klinefelter syndrome, infertility, testis, testosterone, spermatogenesis, Sertoli cells and Leydig cells were the co-occurring keywords with the highest frequency, which reflected the focus of research. The keywords could be classified into 4 categories: three major clusters (age stratification, testicular cells related to mechanisms of spermatogenesis, and biomarkers such as hormones) and one minor thematic cluster (clinical manifestations), which reflected multiple focuses of relevant research. According to Figures 5B, C, which highlight the networks of "Leydig cells" and "Klinefelter syndrome", respectively, a robust

TABLE 2 Top 10 citation documents on Leydig cells in KS patients.

	Document	Year	Title	Local Citations (LC)	Total Citations (TC)	Ratio (TC/LC)	Normalized LC	Normalized TC
1	26, HUM REPROD UPDATE	2006	Natural history of seminiferous tubule degeneration in Klinefelter syndrome.	25	187	13.37	1.92	1.56
2	27, INT J ANDROL	1995	Quantified testicular histology in boys with sex chromosome abnormalities.	15	51	29.41	1.00	1.00
3	28, J CLIN ENDOCR METAB	2004	Inhibin B and anti-Müllerian hormone, but not testosterone levels, are normal in infants with nonmosaic Klinefelter syndrome	14	113	12.39	1.83	1.89
4	29, J CLIN ENDOCR METAB	2006	Serum insulin-like factor 3 levels during puberty in healthy boys and boys with Klinefelter syndrome	13	76	17.11	1.00	0.63
5	30, J CLIN ENDOCR METAB	2005	Insulin-like factor 3 serum levels in 135 normal men and 85 men with testicular disorders: relationship to the luteinizing hormone-testosterone axis	12	133	9.02	1.50	1.41
6	31, CLIN ENDOCRINOL	2007	Establishment of testicular endocrine function impairment during childhood and puberty in boys with Klinefelter syndrome	11	71	15.49	2.75	1.97
7	32, ACTA PAEDIATR	2011	Clinical and biological parameters in 166 boys, adolescents and adults with nonmosaic Klinefelter syndrome: a Copenhagen experience	11	93	11.83	1.88	1.81
8	11, ANDROLOGY-US	2014	Intratesticular testosterone is increased in men with Klinefelter syndrome and may not be released into the bloodstream owing to altered testicular vascularization– a preliminary report	9	36	25.00	3.43	1.68
9	33, BMC GENOMICS	2015	Deregulation of sertoli and leydig cells function in patients with Klinefelter syndrome as evidenced by testis transcriptome analysis	9	41	21.95	2.00	1.33
10	34, J CLIN ENDOCR METAB	2011	Assessment of Leydig and Sertoli cell functions in infants with nonmosaic Klinefelter syndrome: insulin-like peptide 3 levels are normal and positively correlated with LH levels	8	55	14.55	1.37	1.07

association was found between Leydig cell function and numerous biomarkers, such as luteinizing hormone, testosterone and INSL3, which are mainly involved in the progression of germ cell failure and molecular dynamics regulation related to KS.

Figure 5D displays the same network visualization of the keyword map colored based on the average publication year of keywords to visualize focus transfer in research on Leydig cells in KS patients over time. Figure 5E applies density visualization to present the research depth of keywords, Figures 5F, G indicate that the areas of intensively researched domains in the field are primarily located in the high-frequency keywords mentioned above.

3.1.4 Reproductive hormone characteristics in literature data

The results of the subgroup analyses of literature data showed that the levels of three hormones in KS patients fluctuated with age, in which the changes in serum testosterone and luteinizing hormone were roughly similar, with peaks in the ages of 13-19 and 30-45, respectively. However, INSL3 was relatively stable, with a peak only at the age of 13-19 (Figures 6A–D, Table 3). However, the literature data do not provide much information on whether to obtain sperm, so it is impossible to analyze the sperm acquisition rate at different ages, which will be effectively supplemented in our real-world research.

3.2 Real-world results

3.2.1 Basic characteristics of patients

The 75 patients with KS were all from different cities in China. Since our hospital is under the jurisdiction of Guangdong Province, the majority of patients in Guangdong Province are

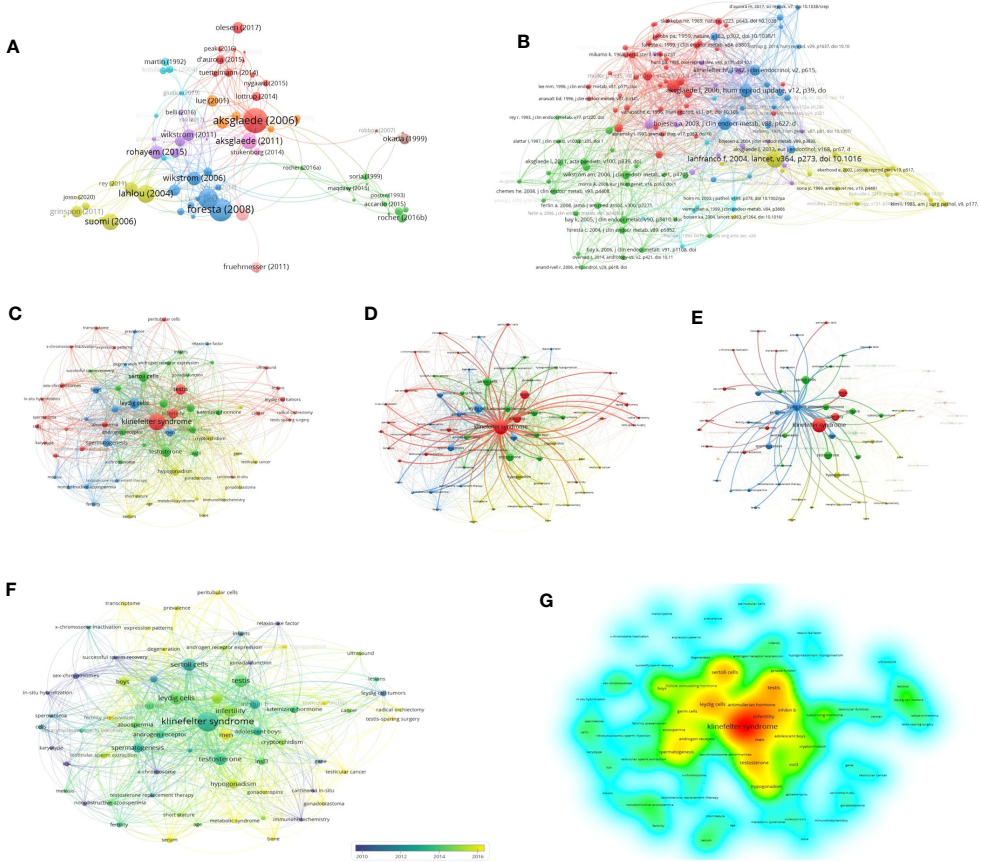

FIGURE 5
Network and keywords for quantitative analysis: **(A)** Citation Network of documents. **(B)** Cocitation Network of references. **(C)** Network of keywords. **(D)** Network of KS. **(E)** Network of Leydig cells. **(F)** Chronological overlay of keywords. **(G)** Density of keywords.

less distributed in other regions. Although it cannot reflect the distribution of all of China, it can also reflect certain unbalanced characteristics (Figure 7A). In Guangdong Province, there are also significant differences in the distribution of KS; in addition, Guangzhou, Dongguan, Yangjiang, Zhongshan and Jieyang are also highly distributed (Figure 7B). We collected the gross domestic product (GDP) of various cities in Guangdong Province in 2021 (Data from http://stats.gd.gov.cn/fsjdgnsczz/content/post_3813633.html), and we found an interesting phenomenon: the distribution of patients with KS seems to have a certain relationship with the distribution of GDP in various cities. The Pearl River Delta region has a relatively high GDP, and the distribution of KS is also relatively dense (Figure 7C). There may be some potential correlation between the production relationship and the occurrence of KS, which is worth further exploration. The age of these patients ranged from 19 to 47 years old, with an average of 29.88 ± 4.25 years old; 39 people had used human chorionic gonadotropin and human menopausal gonadotropin hormone before operation, 25 people had used traditional Chinese patent medicines and simple preparations, and 11 people had not used any medicine; 27 sperm were obtained through testicular microscopic sperm extraction, and 48 sperm were not obtained, with a total sperm capture rate of 36.0%.

3.2.2 Tissue transformation and sperm

The intratesticular conditions of 75 patients with mTESE were photographed and recorded by video. We recorded 75 video clips and nearly 1000 photos. Based on the characteristic metabolic changes in testicular tissue of all patients, we divided the testicular tissue of KS patients into three categories for the first time: fibrosis (Fib), fatification (Fat) and melanosis (Mel). Each category can also be divided into three levels according to the degree of change. Fibrosis includes atrophic (Atr), filamentous (Fil), and hyaline (Hya); fatification includes fat accumulation (Fta), vacuolation (Vac), and melanoid transformation (Met); and melanosis includes nodular (Nod), patchy (Pat), and pectinicity (Pec) (Figures 8A–I).

Referring to the classification criteria and Johnson score, we also compared the testicular tissue status and pathological changes between those who obtained sperm during mTESE and those who did not. The results showed that the Johnson score of those who obtained sperm was significantly higher than that of those who did not obtain sperm (Figure 9A, Table 4). Of the 27 sperm recipients, 12 had fibrosis, 1 had fatification, and 14 had melanosis. Among the 48 patients without sperm acquisition, there were 14 cases of fibrosis, 13 cases of fatification, and 21 cases of melanosis. There was a significant difference between the two groups (Figure 9B)

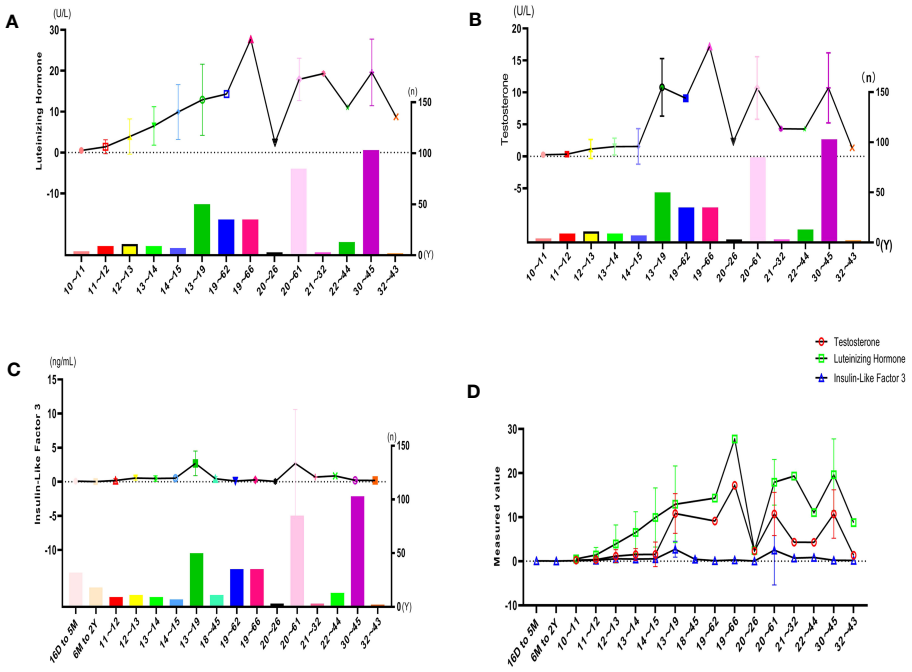

FIGURE 6
Hormone indicators for quantitative analysis: **(A)** LH characteristics of literature. **(B)** T characteristics of the literature. **(C)** INSL3 characteristics of the literature. **(D)** Reproductive hormone characteristics of literature.

(χ^2 = 6.467, p=0.039). The sperm acquisition rate of fibrosis was 46.15%, that of fatification was 7.14%, and that of melanosis was 40.00%. It was difficult for fatification to obtain sperm (Figure 9C).

Based on age as a hierarchical basis and every five years as a stage, we compared the tissue changes and Johnson scores of patients of different age groups, with age divided into five stages: ≤ 20, 21-25, 26-30, 31-35, and ≥ 35 (Figure 9D). Univariate AVNOA analysis revealed that there was no difference in testicular volume, mean Johnson score, or sperm outcome among patients of different ages (Figures 9E–G).

3.2.3 Hormones metabolism and sperm

According to the age sequence, taking the mean value of patients of the same age for analysis of reproductive hormone indicators in patients with KS at different ages, it was found that FSH had a high peak at 26, 32, and 37 years of age, LH had a peak at 19, 26, 33, and 37 years of age, T had a peak at 19, 28, 32, and 37 years of age, and E_2 had a high peak at 19, 20, 36, and 37 years of age. At the ages of 20, 28, 34, and 37, the probability of mTESE acquiring sperm had a peak value (Figures 10A–F).

Based on the principle of obtaining sperm or not, comparing the reproductive hormones of those who obtained sperm with those who did not, the results showed that the LH of those who obtained sperm was significantly lower than that of those who did not obtain sperm, and T was significantly higher than that of those who did not obtain sperm, while there was no significant difference in age, FSH, and E_2 (Figures 10G–K, Table 4).

Age was used as the basis for stratification, and every 5 years was used as a stage. Age is divided into five stages: ≤ 20, 21-25, 26-30, 31-35, and ≥ 35. Single-factor ANOVA revealed that there was no difference in sperm outcome, FSH, LH, and T among patients of different ages, but there was a significant difference in E_2 (Figures 10L–P).

3.2.4 Cells metabolism and sperm

Immunohistochemistry showed that the brown-loving INSL3 receptor was mainly deposited in the seminiferous tubule wall (Figure 11A) and inside the tubule (Figure 11B). The concentration of INSL3 deposition in the seminiferous tubules with sperm (Figure 11C) was significantly higher than that in the seminiferous tubules without sperm (Figure 11D).

Immunofluorescence (single label) labeling showed that the INSL3 receptor in the wall and lumen of the seminiferous tubule where sperm were found (Figures 11E, F) was significantly higher than that in the seminiferous tubule where no sperm were found (Figures 11G, H). Similarly, the androgen receptor in the wall and lumen of the sperm seminiferous tubule (Figures 11I, J) was significantly higher than that in the seminiferous tubule without sperm (Figures 11K, L).

To confirm whether the INSL3 receptor and androgen receptor are expressed in the same cell, we conducted immunofluorescence double labeling tests and observed typical regions at different magnifications of 100×, 200×, 500×, and 1000×. The results showed that the INSL3 receptor and androgen receptor (Figures 11M–O) in the seminiferous tubule of spermatozoa were significantly higher than those in the seminiferous tubule without spermatozoa (Figures 11Q–S), while the INSL receptor between the seminiferous tubule of spermatozoa was significantly lower than

TABLE 3 The Reproductive hormone characteristics of patients in literature data.

Age(D/M/Y)	Sample size	T(nmol/L)				LH(U/L)				INSL3(ng/mL)			
		Mean	Standard deviation	Maximum	Minimum	Mean	Standard deviation	Maximum	Minimum	Mean	Standard deviation	Maximum	Minimum
16 D~5 M	32	NA	NA	NA	NA	NA	NA	NA	NA	0.092	NA	0.222	0.026
6 D~2 Y	18	NA	NA	NA	NA	NA	NA	NA	NA	0.039	NA	0.011	0.11
10~11 Y	4	0.253	0.173	NA	NA	0.5	0.3	NA	NA	NA	NA	NA	NA
11~12 Y	9	0.346	0.223	NA	NA	1.4	1.7	NA	NA	0.2	0.24	NA	NA
12~13 Y	11	1.152	1.481	NA	NA	3.9	4.3	NA	NA	0.55	0.3	NA	NA
13~14 Y	9	1.525	1.315	NA	NA	6.5	4.7	NA	NA	0.48	0.4	NA	NA
14~15 Y	7	1.562	2.775	NA	NA	9.9	6.7	NA	NA	0.54	0.27	NA	NA
13~19 Y	50	10.8	4.5	NA	NA	12.9	8.7	NA	NA	2.7	1.8	NA	NA
20~61 Y	85	10.7	4.9	NA	NA	17.9	5.2	NA	NA	2.5	7.9	NA	NA
22~44 Y	13	4.29	NA	10.81	1.27	2.4	NA	15.6	0	0.06	NA	0.62	0
20~26 Y	3	2.31	NA	3.37	1.27	19.3	NA	19.4	15.5	0.7	NA	0.78	0.12
21~32 Y	3	4.32	NA	4.99	3.69	11	NA	12	9.2	0.86	NA	0.95	0.83
31.5~42.9 Y	2	1.34	NA	2.68	0	8.75	NA	12.8	4.7	0.19	NA	0.31	0.07
19.31~62.35 Y	35	9.1	NA	22.2	0.6	14.3	NA	49.3	0.2	0.12	NA	0.6	0.049
18.97~66.23 Y	35	17.2	NA	27.4	1	27.7	NA	49.2	1.9	0.29	NA	1	0.049
>18 Y	4	NA	NA	NA	NA	NA	NA	NA	NA	0.39	NA	NA	NA
9.9~73.7 Y	82	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
22.35~41.25 Y	103	10.71	5.48	NA	NA	19.6	8.14	NA	NA	0.225	0.0764	NA	NA
18~45 Y	11	NA	NA	NA	NA	NA	NA	NA	NA	0.440	0.35	NA	NA

D, Days; M, Months; Y, Years; T, Testosterone; LH, luteinizing hormone; INSL3, insulin-like factor 3; NA, not available.

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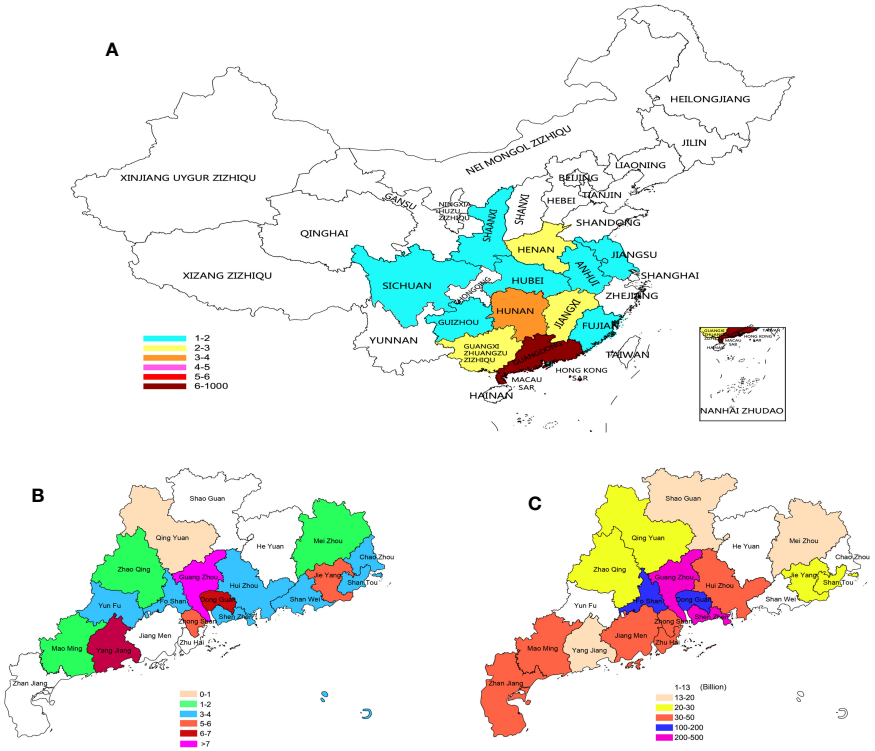

FIGURE 7
Distribution of KS patients: **(A)** Distribution of KS patients in China. **(B)** KS patients in Guangdong. **(C)** GDP of Guangdong cities in 2021.

those in the seminiferous tubule without spermatozoa. After gradual magnification, it was found that the INSL3 receptor and androgen receptor in the sperm seminiferous tubule were roughly the same, and individual INSL3 receptors and androgen receptors might overlap on the same cell (Figure 11P). However, no serious imbalance was found between the two in the sperm seminiferous tubule. Only the INSL3 receptor was found, while the androgen receptor was missing (Figure 11T).

4 Discussion

As the most common sexual chromosome aneuploidy abnormality in men, the cause of KS has always been a complex matter. Although there have been many epidemiological studies since the discovery of KS for more than 60 years, no definitive conclusions have been reached. Currently, there are many opinions that the occurrence of KS is the result of gene mutation, but what causes the mutation? However, this is not yet known. An increasing number of studies have confirmed that changes in the Earth's environment also affect changes in human genetic material, resulting in a large number of chromosomal aneuploidies. As a special type of sex chromosome aneuploidy, the main reason for its occurrence may have a potential relationship with changes in the Earth's environment. For the first time in our study, we analyzed the distribution of birthplaces of KS patients. Although our number of cases is small, they come from various regions in China and exhibit different distribution patterns. In particular, the distribution of patients in Guangdong Province is also very uneven. We compared the gross domestic product (GDP) of the cities where these patients reside in 2021 (Data from http://stats.gd.gov.cn/fsjdgnsczz/content/post_3813633.html) and found that the distribution of KS patients in Guangzhou and Dongguan with higher GDP was also higher. Although we cannot trace whether the GDP of these regions was at a high level when these patients were born, from the perspective of Jieyang and Yangjiang, where the industrialization process is relatively slow, their KS distribution is also higher. At the time of the birth of these patients, these two cities were at the beginning of the industrialization process. Therefore, we speculate that there may be some inherent links between the industrialization process and the occurrence of KS. In future research, we may pay closer attention to the relationship between the occurrence of KS and industrialization.

By means of the bibliometric analysis of 105 documents included and the mapping of the scientific landscape of KS and Leydig cells, we analyzed the countries, journals and authors contributing to this domain and identified some focus topics worth continuous and in-depth exploration for our follow-up studies. From the perspective of the general trend of publications, research on KS and Leydig cells, with erratic growth in annual output, has gradually emerged among academic fields since the 1990s. In particular, the annual publication output reached its peak in the past decade, which suggested that the topic had come into the spotlight with certain research heat and depth over time. The reason for the explosion might be that thanks to the improvement in the diagnostic rate of KS, researchers have an urge to further determine

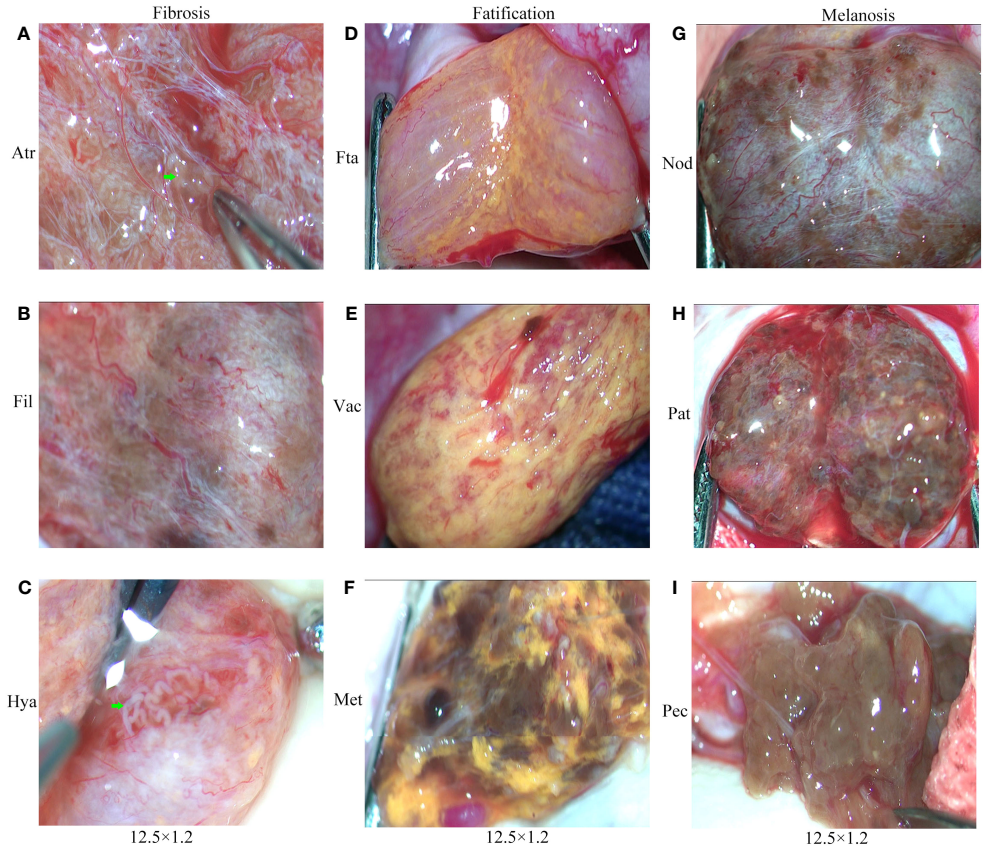

FIGURE 8
Testicular classification of KS patients. **(A)** Atrophy (Atr). **(B)** Filamentous (Fil). **(C)** Hyaline (Hya). **(D)** Fat accumulation (Fta). **(E)** Vacuolation (Vac). **(F)** Melanoid transformation (Met). **(G)** Nodular (Nod). **(H)** Patchy (Pat). **(I)** Pectinicity (Pec).Green arrow: shaped and spermatogenic tubules.

the related internal pathogenesis and mechanisms of germ cell development through pathological histochemistry or burgeoning technologies such as single-cell transcriptomic analysis.

As the geographic visualization and data analysis showed, international cooperation has been primarily conducted in the countries with the largest output of relevant publications, although the regional distribution is dispersed. In the analysis of clustering, temporal and density distribution of keywords in cooperation network, we determined the distribution and internal relationship of hotspots in the field. The keywords with the highest frequency are “KS”, “Leydig cells”, “testosterone”, “inferiority”, “Sertoli cells” and “boys and men”, which indicates that the function of testicular somatic cells is the center of research in this field and that there is a highly connected interaction network between KS and Leydig cells. Whether disordered Leydig cell maturation or later pathological hyperplasia might be associated with testicular failure, ultimately leading to the general clinical characteristic of infertility in KS patients.

Combined with the temporal network clustered by average publication date, we found that the age of KS patients and serological biomarkers have gradually attracted increasing attention from scholars over time. Numerous studies have valued the variation in testosterone, luteinizing hormone (LH) and insulin-like factor 3 (INSL3, regarded as a promising biomarker of Leydig cell maturation) in KS patients of different age cohorts and aimed to use the changing pattern of these indexes as a reflection of the functional status of somatic cells in seminiferous tissue, as well as the prediction of testicular spermatogenesis degeneration (35, 36). For instance, these biomarkers might assist clinicians in choosing the appropriate opportunity for micro sperm extraction and judging the clinical outcome of hormone therapy (37). These might be the most recent topics that suggest future trends in the domain.

Overall, we used the rigorous method of bibliometric analysis to obtain the above valuable information and systematically perused the literature that has a significant impact on the research status and trends in the field. According to the influence of countries and authors, the cited situation of the articles and the frequency of the keywords in the studies within the past three decades (1992-2022), we found that the unstable annual publication output, scattered regional distribution and unsatisfactory scholar cooperation might lead to a lack of an intact theoretical framework in this field, but it has been gradually acknowledged and emphasized that there is a strong link between KS and Leydig cells, which will be one of the core topics for KS. Morphological proliferation, insufficiency of function or disorder of apoptosis regulation in Leydig cells might be tightly related to testicular function at different ages of KS and ultimately show changes in serum markers such as testosterone, LH and INSL3. Eventually, this bibliometric analysis provides

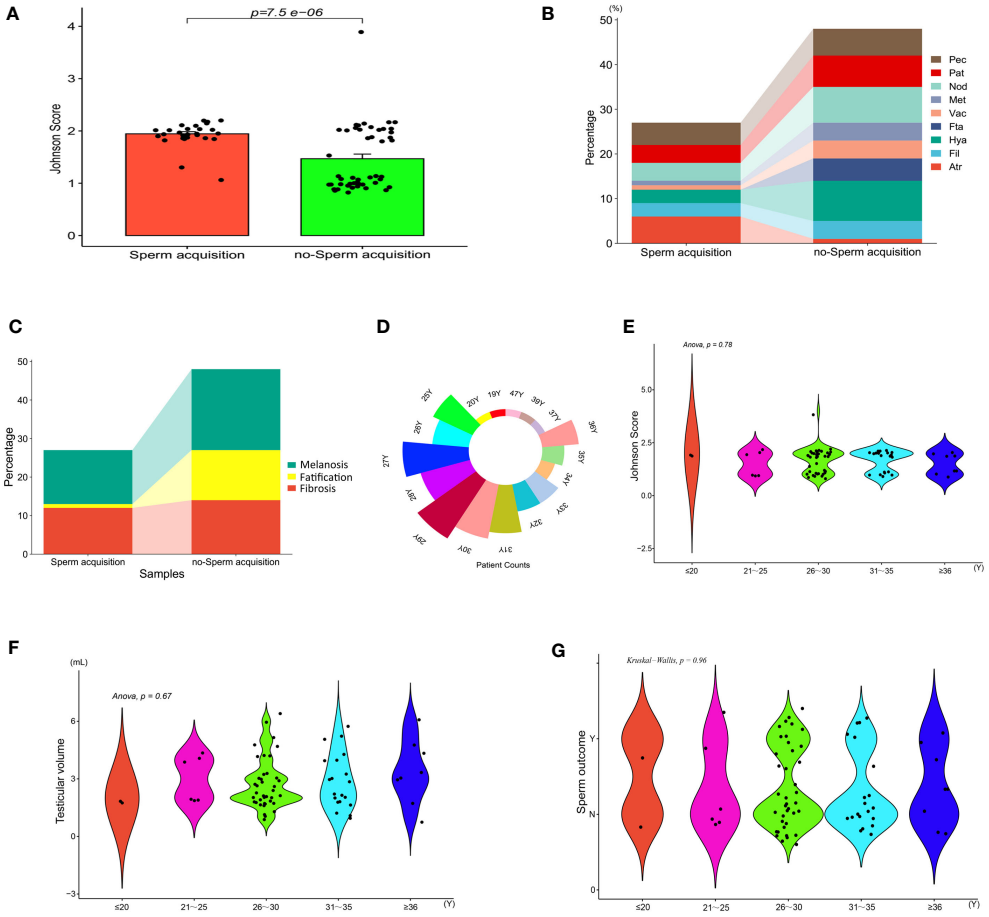

FIGURE 9
Indicators between KS patients. **(A)** Johnson score. **(B)** Testicular tissue classification. **(C)** General classification of testicular tissue. **(D)** Patient count. **(E)** Johnson score of different ages. **(F)** Testicular volume at different ages. **(G)** Sperm outcome of different ages.

suggestions and approaches for the following subgroup analysis and real world research, by which we expect to determine a reasonable and considered method for fertility optimization and preservation of KS patients.

The production and apoptosis of cells are fundamental to the cell cycle. As the basis of human reproduction, sperm also follow the principles of all things. Research shows that the natural production and apoptosis cycle of human sperm is approximately 3 months (38, 39). Bhanmeecho et al. conducted a group study of 55 dogs of different ages and found that the degree of testicular interstitial fibrosis in elderly dogs was significantly higher than that in other age groups. With increasing age, there was a significant difference in the degree of spermatogenic cell degeneration. Age was positively correlated with interstitial fibrosis and positively correlated with the degree of testicular tubular atrophy (40). Therefore, age is considered to be one of the most critical factors regulating spermatogenesis.

Research has found that aging of the gonads of men over the age of 40 can significantly reduce their fertility, including not only changes in sperm quality and sperm function but also a reduction in the ability to combine sperm and testis (41). As the center of the male reproductive system, testicular dysfunction, oxidative stress damage, apoptosis, inflammation, and decreased immune function all contribute to the

TABLE 4 The characteristics of KS patients with and without sperm.

Group	Sample size (n)	Age (Y)	FSH (mIU/mL)	LH (mIU/mL)	T (nmol/L)	E_2 (pmol/L)	Left testicular volume (mL)	Right testicular volume (mL)	Johnson Score
With sperm	27	29.37 ± 3.94	31.43 ± 14.09	17.66 ± 7.19	7.50 ± 4.55	68.44 ± 39.10	3.26 ± 1.40	3.26 ± 1.40	1.94 ± 0.21
Without sperm	48	30.17 ± 4.42	34.95 ± 16.83	21.97 ± 8.92	5.33 ± 34.00	59.93 ± 3.48	2.60 ± 1.23	2.58 ± 1.25	1.47 ± 0.61
p	—	0.439	0.36	0.035	0.023	0.328	0.039	0.035	<0.001

FSH, follicle-stimulating hormone; T, Testosterone; LH, luteinizing hormone; E_2, estradiol.

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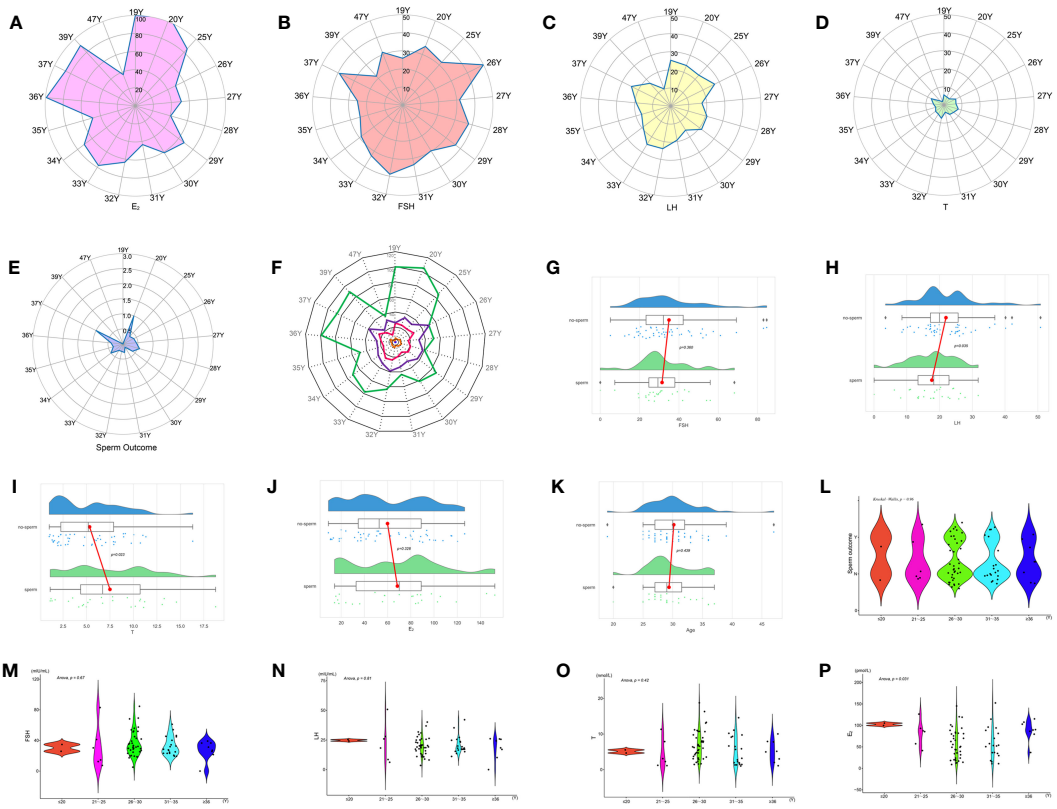

FIGURE 10
Indicators between KS patients of different ages: **(A)** E_2. **(B)** FSH. **(C)** LH. **(D)** T. **(E)** Sperm outcome. **(F)** Comprehensive indicators. **(G)** FSH. **(H)** LH. **(I)** T. **(J)** E_2. **(K)** Age. **(L)** Sperm outcome. **(M)** FSH. **(N)** LH. **(O)** T. **(P)** E_2.

cessation of spermatogenesis (42). However, there were also opposing views. Mularoni et al. found that with age, the number of Leydig cells in the testis decreased, but the peripheral serum androgen concentration did not significantly decrease (43). Research has shown that there are two different types of Leydig cells in mammalian testes: fetal Leydig cells (FLCs) and adult Leydig cells (ALCs), which appear before and after birth, respectively. Among them, FLCs contain a large number of lipid droplets, while ALCs contain a small amount of lipid droplets. When FLCs fail to degrade normally after birth and still exist in the adult testicles, it can affect the function of ALCs in late adolescence (44). Therefore, to clarify the relationship between statue of Leydig cells disorder and spermatogenesis in patients with KS at different ages might be helpful in changing the fertility of patients with KS, We suspect that the proportion of FLCs may be significantly higher than that of ALCs in KS patients with transitional steatosis of the testicular interstitium, resulting in decreased local spermatogenic function, which may also be the main reason for their low mTESE sperm acquisition rate.

Unlike previous scholars, we found significant differences in reproductive hormone indicators among patients with KS at different ages, with FSH having a peak at 26, 32, and 37 years; LH having a peak at 19, 26, 33, and 37 years; T having a peak at 19, 28, 32, and 37 years; and E_2 having a peak at 19, 20, 36, and 39 years. There were also differences in whether there was focal spermatogenesis in the testicles of KS patients at different ages, with the probability of recovering sperm through mTESE reaching a peak at the ages of 20, 28, 34, and 37. The LH of patients with sperm acquisition was significantly lower than that of patients without sperm acquisition, and the T was significantly higher than that of patients without sperm acquisition. There were also significant differences in E_2 among patients of different ages.

This phenomenon is greatly different from the view of other scholars that the younger a KS patient is, the easier it is to obtain sperm (45, 46). Our analysis may have the following reasons: (1) Our small sample size for each age group may cause bias in the results. (2) Our research population was mainly older adults, not adolescents, so the population composition may have some interference with the results. (3) Although we may have the first two types of interference, from the perspective of the probability of mTESE sperm recovery in men of childbearing age, at least the viewpoint of scholars in the early and middle stages of childbearing age is not applicable, which also suggests that the spermatogenesis of men of childbearing age does not develop according to the rule of age, and there may be other regulatory factors in the spermatogenesis of patients of KS childbearing age, and such factors can be interfered with. This may also be a breakthrough in solving the bottleneck in the treatment of KS spermatogenesis and provide some inspiration for our further research.

In the past, the histopathological Johnson score has been considered the gold standard for evaluating testicular spermatogenesis (48). However, with the development of microscopic testicular sperm extraction (mTESE), an increasing number of clinical practices have confirmed the presence of focal spermatogenesis in the testicles of

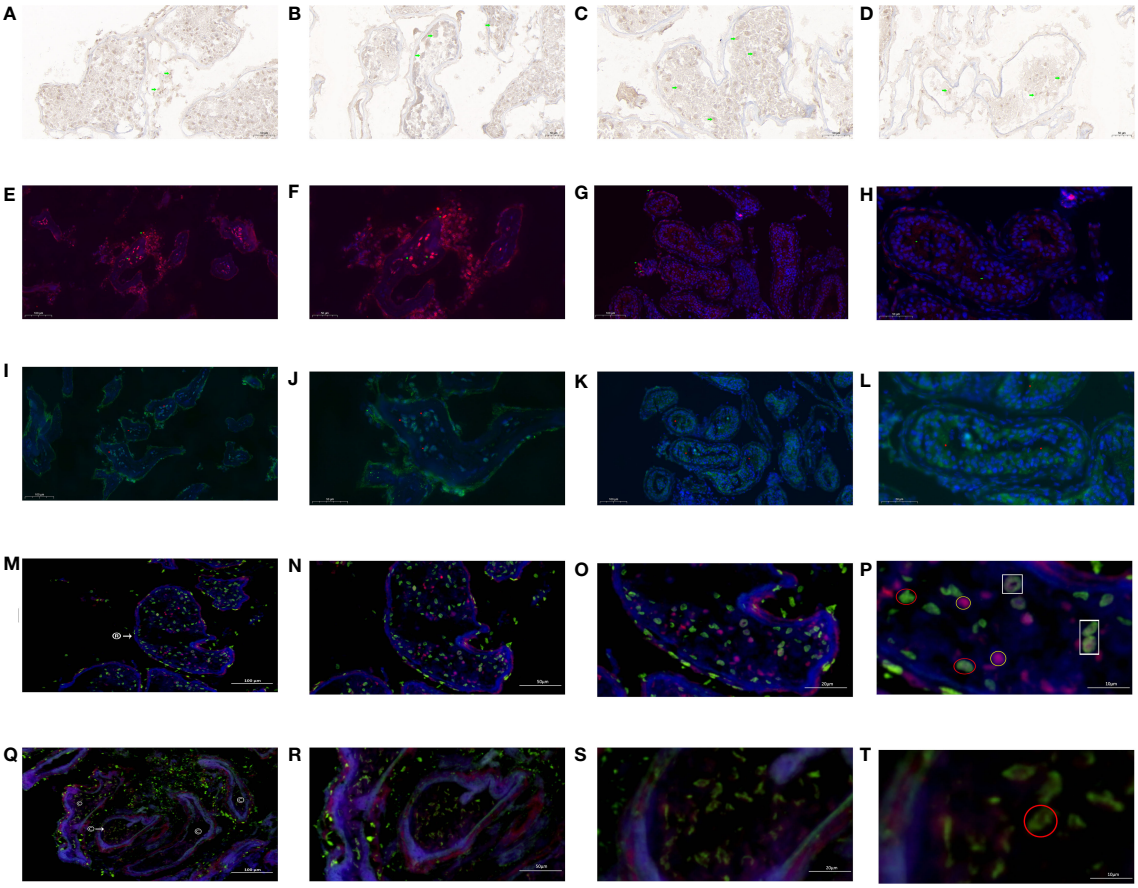

FIGURE 11
INSL3 receptor and androgen receptor in KS patients. **(A)** INSL3 between seminiferous tubules (green mark). **(B)** INSL3 in seminiferous tubules (green mark). **(C)** INSL3 in the seminiferous tubule of spermatozoa (green mark). **(D)** INSL3 in the seminiferous tubule without sperm (green mark). **(E)** INSL3 in the seminiferous tubule of spermatozoa (green mark, red fluorescence, 100×). **(F)** INSL3 in the seminiferous tubule of spermatozoa (green mark, red fluorescence, 200×). **(G)** INSL3 in the seminiferous tubule without sperm (green mark, red fluorescence, 100×). **(H)** INSL3 in the seminiferous tubule without sperm (green mark, red fluorescence, 200×). **(I)** Androgen receptor in the seminiferous tubule of spermatozoa (red mark, green fluorescence, 100×). **(J)** Androgen receptor in the seminiferous tubule of spermatozoa (red mark, green fluorescence, 200×). **(K)** Androgen receptor in the seminiferous tubule without sperm (red mark, green fluorescence, 100×). **(L)** Androgen receptor in the seminiferous tubule without sperm (red mark, green fluorescence, 200×). **(M)** The androgen receptor and INSL3 receptor in the seminiferous tubule of spermatozoa: ®: find the sperm seminiferous tubule. Green fluorescence: INSL3 receptor. Red fluorescence: androgen receptor. 100×. **(N)** The androgen receptor and INSL3 receptor in the seminiferous tubule of spermatozoa: Green fluorescence: INSL3 receptor. Red fluorescence: androgen receptor. 200×. **(O)** The androgen receptor and INSL3 receptor in the seminiferous tubule of spermatozoa: Green fluorescence: INSL3 receptor. Red fluorescence: androgen receptor. 500×. **(P)** The androgen receptor and INSL3 receptor in the seminiferous tubule of spermatozoa. INSL3 receptor: green fluorescence, marked in red circle. Androgen receptor: red fluorescence, marked in yellow circle. Overlapping androgen receptor and INSL3 receptor: marked in white box. 1000×. **(Q)** The androgen receptor and INSL3 receptor in the seminiferous tubule without sperm: ©: the seminiferous tubule without sperm. Green fluorescence: INSL3 receptor. Red fluorescence: androgen receptor. 100×. **(R)** The androgen receptor and INSL3 receptor in the seminiferous tubule without sperm: Green fluorescence: INSL3 receptor. Red fluorescence: androgen receptor. 200×. **(S)** The androgen receptor and INSL3 receptor in the seminiferous tubule without sperm: Green fluorescence: INSL3 receptor. Red fluorescence: androgen receptor. 500×. **(T)** The androgen receptor and INSL3 receptor in the seminiferous tubule without sperm: INSL3 receptor: green fluorescence, marked in red circle. 1000×.

patients with nonobstructive azoospermia (49, 50). In previous studies related to mTESE, we have also found that the sperm recovery rate of patients with nonobstructive azoospermia undergoing mTESE surgery can reach 38.7% (51). KS is a typical nonobstructive azoospermia with congenital chromosomal aneuploidy defects, but spermatogenesis may still exist in its testicles (52, 53). Similar to studies by other scholars, we also found focal spermatogenesis in infertile patients with KS, with a mTESE sperm recovery rate of 36.0%. Although this number is lower than that reported by other scholars, this is accurate data, which may be related to the patient's sample size, age, and previous treatment history. The sample size of this study is small, the age is concentrated in the reproductive age period, and all patients have not received any treatment three months before surgery, or a 3-month washout period is ensured after receiving treatment. Therefore, the mTESE sperm recovery rate in this study will be low.

On the other hand, the Johnson score of testicular tissue in all patients with KS in this study remained between 1-2 points, while only one of 75 patients had a score of 4. According to the Johnson scoring standard, the lower the score, the poorer the development of testicular seminiferous tubules and the lower the rate of spermatogenesis. Therefore, this may also be one of the main factors that caused the sperm acquisition rate in this study to be lower than that of other scholars. Our comparison of Johnson scores between patients with and without sperm also confirms this

phenomenon, and the Johnson scores of patients with sperm are significantly higher than those of patients without sperm.

However, from a single case of histopathological Johnson score, we cannot obtain a valuable predictive conclusion on the presence or absence of focal spermatogenesis from a patient's Johnson score. Therefore, we believe that conducting testicular biopsy in patients with KS before mTESE is meaningless, as it can lead to damage to the testicular microenvironment and is not conducive to the development of focal spermatogenesis.

The hypothalamus-pituitary-testicular gonadal axis is considered to be the center for regulating spermatogenesis (54, 55). Our previous studies have also found that the concentration of reproductive hormones in the testicular environment also plays a key role in regulating spermatogenesis (51). Therefore, reproductive hormones should have a crucial role in spermatogenesis in patients with KS. Due to the presence of X-chromosome aneuploidy, patients with KS are clinically characterized by high levels of follicle stimulating hormone and luteinizing hormone, accompanied by low or normal testosterone. Their follicle-stimulating hormone and luteinizing hormone levels can exceed the normal range by 2-3 times or even higher (56). It has been reported that lowering FSH and LH can promote spermatogenesis in patients with nonobstructive azoospermia (57), but the effect on KS patients is unclear. Therefore, exploring the rules of reproductive hormones in patients with KS has a guiding role in regulating spermatogenesis.

Previous studies have found that Leydig cells appear and can secrete androgens at the 8th week of a male embryo. At the 9th to 14th week of the embryo, Leydig cells turn into differentiated cells. At the 15th to 18th week of the embryo, Leydig cells mature and have a strong androgen secretion ability, forming a peak of androgen secretion during the embryonic period. Thereafter, they gradually degenerate until puberty before increasing and increasing the secretion of androgens again (58–60). Currently, it is believed that there are a large number of LH receptors on the surface of Leydig cells, which, after binding with LH, initiate the transformation process of neutral fatty acids and cholesterol lipids in Leydig cells, promote the conversion of cholesterol into testosterone, keep the epithelium of spermatogenic tubules close to the lumen in a long-term high concentration of testosterone, promote the differentiation of late spermatocytes, and complete the spermatogenesis process (61, 62). There may be a local "short loop" regulatory pattern between the functions of mesenchymal cells and Sertoli cells, and estradiol secreted by Sertoli cells may interfere with the expression of receptors on the surface of mesenchymal cells (63, 64). Therefore, analyzing the function of Leydig cells is crucial for promoting spermatogenesis.

In the subgroup analysis following the bibliometric analysis, we extracted the indicators testosterone, LH, and INSL3 related to Leydig cells and found that the levels of these hormone or hormone-like indicators in patients with KS fluctuated with age, especially when the levels of testosterone, LH, and INSL3 in men with KS were observed to be different at various age groups, and the peaks also occurred at different ages. The change trend of serum testosterone and LH is generally similar, reaching a peak at the ages of 13-19 and 30-45, respectively. Comparatively, INSL3 is relatively stable, peaking only at the age of 13-19, and the peaks of testosterone and INSL3 significantly lag behind those of LH. Correspondingly, in our cross-sectional study of men of childbearing age, we found that the peak ages of testosterone and LH were also similarly reached at the ages of 19, 26-28, 32-33, and 37. The age cohort with higher success rates in sperm extraction was also concentrated at 20 and 37 years old. In the same age group, lower LH and higher testosterone may be associated with positive spermatogenic function. This indicates that the fluctuations of hormones in patients with KS at childbearing age follow a certain rule, which might be related to the development status of testicular spermatogenic tissue, the maturity of somatic cells adjacent to germ cells, and sensitivity to the hormone-stimulating effect at different ages.

Previous studies (9, 32, 35) have shown that during childhood and even early puberty of KS, pituitary-gonadal function is normal, and testicular biopsies of prepubertal KS boys show that the number of germ cells is reduced, but the seminiferous tubules are preserved and Sertoli cells and Leydig cells still work regularly, which indicates that hormone synthesis and secretion may be normal. From the middle of puberty, the seminiferous tubules of patients have rapidly accelerated degradation, with varying degrees of fibrosis, transparency, and interstitial proliferation, as well as maturation disorders and functional deficiencies of Sertoli cells and Leydig cells. However, in this study, LH, FSH, and other hormones in patients with KS still fluctuated during childbearing age and did not directly increase to high levels of gonadotropins, while testosterone levels dropped to low or subnormal levels after an initial increase and were still able to reach a new peak. The reason for these phenomena may be related to the residual spermatogenic tissue in the testis, and it might also be because the stimulation response of LH-testosterone may still exist at a specific childbearing age and stimulates the spermatogenesis of the remaining spermatogenic tissue, which shows that KS at childbearing age still has the potential to produce sperm. In other words, not all somatic cells decline irreversibly with age, and the remaining cells may have the potential to perform compensatory functions, but this needs to be verified in further research. Thus, we believe that the evolution of the morphology and function of somatic cells such as Leydig cells in the testicles of KS of childbearing age and the generated hormone fluctuations are of great significance for research related to the pathogenesis and progression of KS, which also provides a novel explanation for the controversial topic of the relationship between testicular spermatogenic function and hormones.

In our study, we found that LH in sperm recipients was significantly lower than that in nonsperm recipients, and T was significantly higher than that in nonsperm recipients. There were also significant differences in E_2 among patients of different ages. The differences among the three have once again confirmed the relationship between spermatogenesis and the LH——T——E_2 regulatory axis. We believe that LH activation of testicular Leydig cells to secrete sufficient T is the basis for stimulating spermatogenesis, but the balance of transformation between T and E_2 is also a necessary condition for ensuring spermatogenesis, and none of the three is indispensable. We have also confirmed through immunofluorescence single and double labeling that the proportion of androgen receptors and INSL3 receptors is relatively

coordinated in the testicular tissue around the seminiferous tubules with normal spermatogenesis, while in the testicular tissue around the seminiferous tubules without spermatogenesis, there is a serious imbalance in the proportion of androgen receptors and INSL3 receptors. This phenomenon is the first time that it has been discovered. Unlike previous studies, we found that androgen receptors and INSL3 receptors are more intensively distributed in the wall and inside the seminiferous tubules. These two receptors are jointly expressed on the surface of supporting cells. In local areas of transitional proliferation of interstitial tissue, there is a high concentration of androgen receptors, while INSL3 receptors are not widely distributed. These areas are also the areas where interstitial tissue degeneration is most severe, and there is even the possibility of mesenchymal neoplasia. Therefore, we believe that the ratio of INSL3 combined with T may be more suitable as an accurate indicator for predicting spermatogenesis in patients with KS.

5 Conclusion

There are significant differences in LH, T, and E_2 levels between KS patients with focal spermatogenesis at different ages and those without spermatogenesis. The age points of KS patients with easy access to sperm are 20, 28, 34, and 37 years old. The INSL3 receptor and androgen receptor are centrally expressed in the walls and inside the spermatogenic tubules, and a balanced of these metabolites is an important basis for ensuring spermatogenesis. Abnormal metabolism of Leydig cells led to imbalanced expression of INSL3 and androgen receptors, which might be a potential target for spermatogenesis in KS.

5.1 Shortcomings

We found a certain correlation between abnormal lipid metabolism in interstitial cells of KS patients and focal spermatogenesis, but there are still the following shortcomings: (1) Our sample size is small and cannot fully verify the condition of KS patients at each age group. It is also necessary to obtain more detailed information of population data in the future in order to make a more accurate map of the population distribution characteristics of KS patients. (2) Although we have made every effort to provided the fullest presentation of the available data and statistical details to support our conclusion, our results may be disrupted by unavoidable potential biases and statistical limitation, which is supposed to be improved in the future large-scale study. (3) There is lack of validation of animal models and cannot simulate the occurrence of abnormal lipid metabolism in KS patients. (4) We need to further explore the mechanism of abnormal lipid metabolism in KS patients.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Ethics Committee of Guangdong Provincial Reproductive Science Institute (Guangdong Provincial Fertility Hospital) (Approval No.2021(08)). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

HuL: Funding acquisition, Methodology, Project administration, Writing – original draft, Writing – review & editing. ZHZ: Data curation, Formal Analysis, Investigation, Methodology, Software, Writing – original draft. YG: Conceptualization, Investigation, Resources, Validation, Writing – original draft. HaL: Data curation, Formal Analysis, Investigation, Methodology, Resources, Writing – original draft. ZYZ: Conceptualization, Investigation, Methodology, Writing – original draft. HZ: Conceptualization, Investigation, Methodology, Writing – original draft. WY: Writing – original draft. ZL: Conceptualization, Investigation, Methodology, Writing – original draft. ZQ: Conceptualization, Investigation, Methodology, Writing – original draft. QX: Conceptualization, Investigation, Methodology, Writing – original draft. LH: Investigation, Writing – review & editing. YZ: Methodology, Writing – review & editing. XZ: Methodology, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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EDITED BY
Xiang Xiao,
Hangzhou Medical College, China

REVIEWED BY
Huitao Li,
Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, China
Alessandra Santillo,
University of Campania Luigi Vanvitelli, Italy

*CORRESPONDENCE
Hin Ting Wan
wanhinting@hkbu.edu.hk
Chris Kong Chu Wong
ckcwong@hkbu.edu.hk

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PFOS-elicited metabolic perturbation in liver and fatty acid metabolites in testis of adult mice

Wang Ka Lee[1], Thomas Ka Yam Lam[2], Hiu Ching Tang[1], Tsz Chun Ho[1,2], Hin Ting Wan[1]* and Chris Kong Chu Wong[1,2]*

[1]Croucher Institute for Environmental Sciences, Department of Biology, Hong Kong Baptist University, Hong Kong, Hong Kong SAR, China, [2]State Key Laboratory in Environmental and Biological Analysis, Hong Kong Baptist University, Hong Kong, Hong Kong SAR, China

Introduction: Multiple factors can contribute to sub-fecundity, including genetics, lifestyle, and environmental contaminants. PFASs are characterized as "forever chemicals" due to their ubiquitous contamination and their persistence in the environment, wildlife, and humans. Numerous studies have demonstrated that PFAS exposure adversely affects multiple bodily functions, including liver metabolism and gonadal function. It is unclear, however, how the disruption of hepatic fatty acid metabolism affects testicular function.

Methods: In this study, male mice were administered 0.3 and 3 μg/g body weight of PFOS for 21 days.

Results: Our data showed that PFOS exposure caused hepatic steatosis, as evidenced by significant increases in triglyceride levels, expression of ATP-citrate lyase, and fatty acid synthase, as well as fasting insulin levels. PFOS perturbed the expression levels of hepatokines, of which fibroblast growth factor-21 (*Fgf-21*), leukocyte cell-derived chemotaxin-2 (*Lect-2*), and retinol-binding protein-4 (*Rbp-4*) were significantly reduced, whereas angiopoietin-like 4 (*Angptl4*) was noticeably increased. While *Rbp-4 and Fgf-21* are known to contribute to spermatogenesis and testosterone synthesis. In PFOS-exposed groups, testicular ATP, and testosterone decreased significantly with a significant increase in the expression of peroxisome proliferator-activated receptor-coactivator 1α. Mass spectrophotometry imaging revealed the localization of PFOS in testes, along with significant increases in fatty acid metabolites. These included arachidonic acid, dihomo-α-linolenic acid, dihomo-γ-linolenic acid, oxidized ceramide, diacylglycerol, phosphatidylcholine, and phosphatidylethanolamine, which are associated with inflammation and post-testicular causes of infertility.

Discussion: This study revealed potential links between PFOS-elicited changes in hepatic metabolism and their impacts on testicular biology. This study provides insights into alternative targets elicited by PFOS that can be used to develop diagnostic and therapeutic strategies for improving testicular dysfunction.

KEYWORDS

mass spectrophotometry imaging, hepatokines, lipogenesis, testosterone, fecundity

Introduction

Lifestyle, environment, and genetic factors all contribute to subfecundity (1). Increasing evidence suggests that environmental chemical contaminants (e.g., heavy metals and anthropogenic chemicals) disrupt testicular physiology. Among different environmental chemical pollutants, per- and poly-fluoroalkyl substances (PFASs) are one of the prioritized family, known to perturb metabolic and reproductive health (2, 3). PFASs are coined as "forever chemicals" that contaminate public water systems, and linger in the environment, wildlife, and humans (4). The legacy PFAS, including PFOA, PFOS, PFNA, PFHxS share in common the lipophobic C-F chain and hydrophilic functional groups (5). PFASs are used in industrial and consumer products and have unique physicochemical properties, including heat and oil resistance, water repellence, and surfactant properties. They exhibited proteinophilic attraction toward albumin and various fatty acid binding proteins, resulting in their long biological half-lives and bioaccumulation in humans (6, 7). In recent decades, the direct effects of PFOS on testicular physiology have been studied in animal and cell culture models, revealing that it disrupted hormonal signaling in the testicles and perturbed the dynamics of tight junctional protein during spermatogenesis, which significantly affected males' fertility and health (8–10). Even so, little is known about how the systemic impact of PFASs affects testicular function.

Over the past few years, patients seeking reproductive health care increasingly suffer from metabolic disorders, including obesity and insulin resistance (11–13). Since nutritional and hormonal factors influence energy metabolism and reproductive activity, to improve fertility rates, it is crucial to understand the underlying correlation of metabolic syndrome (14). The liver is a primary metabolic tissue that maintains energy and nutrient balance. Further, the liver metabolizes hormones and chemical contaminants, which may be degraded for excretion or bio-activated for more significant toxicity (15). Changes in metabolism affect hormone signals and nutrient flow, which can directly or indirectly affect gonadal function (16). One of the most notable effects of PFOS is the disruption of hepatic liver energy homeostasis, particularly fatty acid metabolism and the signaling of nuclear receptors (17–19). It is unclear how the disruption is related to the perturbing effect of testicular function. It was hypothesized that the disruption in fatty acid metabolism caused by PFASs could affect systemic energy metabolism and fatty acid metabolites in the testes. An integrated approach involving mass-spectrometry imaging, gene expression analysis, and biochemical testing were used in this study to investigate how PFAS affects the mammalian liver and testes. An analysis of the association between PFAS-induced metabolic perturbations and testicular dysfunction was conducted.

Materials and methods

Animals

Male CD-1 mice (8-10 weeks old) were kept in polypropylene cages at 23-24°C and 12 hours of light/dark cycle. The procedure for animal handling was followed according to guidelines and regulations approved by the animal ethics committee (REC/20-21/0234) of Hong Kong Baptist University. Perfluorooctane sulfonate (PFOS, CAS 1763-23-1, Sigma-Aldrich, 98% purity) was dissolved in dimethyl sulfoxide (Sigma-Aldrich) before mixing with corn oil. In the treatment regime, mice with body weights were randomly divided into three groups (control, low, or high-dose PFOS treatment groups) using a random number generator. The animals were provided access to food (LabDiet, 5001, Laboratory Rodent Diet) and water (in glass bottles). The exposed groups received either 0.3 or 3 μg/g of body weight (bw)/day of PFOS for 21 days by oral gavage (Cadence Science). Corn oil was administered to the control group. The low exposure dose is equivalent to human occupational exposure (20). Overnight fasting was performed on day 20. In the next morning, cervical dislocations were performed, and blood samples were drawn. Livers and testes were collected, snap-frozen in liquid nitrogen, and stored at -80°C.

RNA extraction and real-time quantitative PCR (qPCR)

Total RNA was extracted from tissue samples using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. RNA concentration and quality were measured by BioDrop (Biochrom) and then reverse transcribed to cDNA using SuperScript VILO cDNA Synthesis Kit (Applied Biosystems). Gene expression was analyzed by real-time PCR using Fast SYBR™ Green Master Mix (Applied Biosystems) with StepOnePlus PCR system (Life Technologies) using gene-specific primers (Supplementary Table 1A). Relative expression was calculated by normalizing to *actin* using the $2^{-\Delta\Delta Ct}$ method. The specificity of the amplicon was verified using melting curve analysis and agarose gel electrophoresis.

Western blot

Tissues were homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% NP-40) containing Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Tissue homogenates were then sonicated for 8 sec, 5 cycles on ice, followed by centrifugation at 13000 xg, at 4°C for 15min. Protein concentration was determined using the DC Protein Assay Kit II (BioRad). Protein samples were separated by SDS-PAGE and transferred to a PVDF membrane (BioRad). Membranes were blocked with 5% non-fat milk in PBST for 1 hr at room temperature, incubated with primary antibody (Supplementary Table 1B) overnight at 4°C and then incubated with the HRP-conjugated secondary antibody (BioRad) for 1 hr at room temperature. SuperSignal™ West Pico PLUS chemiluminescent substrate (Thermo Scientific) was used to develop the signals.

Testosterone ELISA Kit, blood insulin, glucose *and ATP* determination

Adult male mouse were killed by cervical dislocation. Blood samples were collected and centrifuged at 1000 xg at 4°C for 10 min

to collect sera, which were then stored at -80°C. Testosterone level in serum was measured using testosterone ELISA kit (Cayman Chemical). Briefly, 25 µl of serum sample or standard was mixed with 25 µl of testosterone AChE Tracer and 25 µl of testosterone ELISA antiserum in the mouse anti-rabbit IgG coated-well. The reaction mixture was discarded, and the wells were washed at room temperature on an orbital shaker (Thermo Fisher Scientific). Ellman's Reagent (200µl) was added in each well and incubated for 90 minutes at room temperature in dark on the orbital shaker. Absorbance at 412 nm of each wells were measured using EnSight Multimode Plate Reader (PerkinElmer). The fasting blood glucose levels were measured using an Accu-check Glucometer (Roche, US). Serum was prepared by centrifugation of clotted blood at 1000 xg at 4°C for 10 min. Insulin level was measured with Ultrasensitive Insulin ELISA kit (10-1132-01, Mercodia, Sweden) according to the manufacturer's instruction.

Testis samples were homogenized in luciferase cell culture lysis 5X reagent (Promega) and centrifuged at 13,000 ×g at 4°C for 15 min. The supernatants were collected for ATP measurement, using ATP Determination Kit (Invitrogen) according to the manufacturer's instruction. Luminescence was measured by EnSight Multimode Plate Reader (PerkinElmer). ATP level was then normalized with the protein concentration of each sample using DC Protein Assay Kit II (BioRad).

AFADESI-mass spectrophotometry imaging

Testes were isolated from adult male mice and snap-frozen in liquid nitrogen. The testis was mounted on a cryostat specimen chunk (Thermo Fisher Scientific, U.S.) and sliced at 14 µm in thickness using the Cryostar NX70 (Thermo Fisher Scientific). AFADESI-MSI (air-flow assisted desorption electrospray ionization) was applied to a frozen section mounted on a microscopic slide (Citotest, Jiangsu, China). The images were analyzed using an Orbitrap Exploris™ 120 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) and the AFAI-MSI image platform (Viktor, Beijing, China). A negative ion mode analysis was performed, with signals ranging from 100-1000m/z at a resolution of 60000. A spray solvent mixture of acetonitrile and dimethylformamide (3:1, v/v), was applied at 2 ml/min, with sprayer voltages at -2500V. The X- and Y-direction scanning speeds were 430 mm/s and 150 mm/s. A 140-psi gas flow and 350°C ion transfer tube temperature were used. MSConvert (Nature Biotechnology Commentary) and imzMLConverter (Thermo Fisher Scientific, U.S.) were used to convert MS data to mzML and imzML formats. SCiLSTM Lab (Bremen, Germany) was used to visualize data. The sections fixed in 4% PFA were then stained with hematoxylin.

Statistical analysis

A statistical mean and standard deviation were used to present the data. The GraphPad Prism version 8.0 was used for statistical analyses. Students'-tests were used to evaluate the physiological and gene expression data. A p-value < 0.05 was considered statistically significant.

Results

The exposure regime revealed a significant increase of liver weights and relative liver weights at the high-dose (3µg/g) of PFOS exposure while there was no noticeable effect on the changes of body weights among the control and PFOS treatment groups (Figure 1A). Additionally, hepatic triglyceride levels were found to be significantly increased at the high-dose exposure (Figure 1B). To underpin the underlying process of PFOS-elicited perturbation to hepatic lipid metabolism, western blot analysis of key metabolic enzymes for lipogenesis were conducted. The data revealed significant upregulation in the expression levels of ATP citrate lyase (ACLY), acetyl CoA carboxylase (ACC), phosphorylated ACC, and fatty acid synthase (FASN) at the high-dose PFOS-exposed groups (Figure 1C). The expression of ACLY and the ratio of pACC to ACC (Supplementary Figure 1A) did not change significantly.

As the liver is the primary metabolic tissue, PFOS-induced disruptions in energy metabolism are likely to have a systemic effect. Fasting insulin and glucose levels were measured on day 21 of post-PFOS exposure. A significant increase in serum insulin levels was observed in the high-dose PFOS group, but no significant changes were observed in fasting blood glucose levels (Figure 2A). In western blot analysis, high-dose PFOS treatment significantly reduced hepatic expression levels of insulin receptor (IR) but not the insulin-like growth factor-1 receptor (IGF-1R) (Figure 2B). Considering the liver's role in regulating systemic energy homeostasis, the expression levels of hepatokines, the major liver-to-tissue messengers that responds to perturbed energy metabolism, were investigated. Upon PFOS exposure, there was a dose-dependent reduction in the expression levels of the hepatokines, fibroblast growth factor-21 (*Fgf-21*), leukocyte cell derived chemotaxin-2 (*Lect-2*), retinol binding protein-4 (*Rbp-4*), but a significant induction of angiopoietin-like 4 (*Angptl4*) (Figure 2C). The expression levels of the other measured hepatokines, *Angptl-3*, *Angptl-6*, *Selenop*, and *Smoc-1* showed no noticeable differences (Supplementary Figure 1B).

Due to PFOS' effects on hepatic metabolism and serum insulin levels, it likely perturbed testicular functions. Western blot analysis showed that there were no significant changes in the expression levels of IR and phosphorylated IR in testes (Supplementary Figure 1C). However, PFOS-exposed groups showed a significant dose-dependent decrease of testicular ATP, associated with a significant increase in the expression of peroxisome proliferator-activated receptor γ coactivator 1α, *Pgc1α* (Figure 3A), a transcription coactivator in the regulation of cellular energy metabolism. Additionally, there was a significant reduction in testosterone levels in the high-dose PFOS exposed groups (Figure 3B, left panel). Yet, no significant change in testicular weight and epididymal sperm counts were noted (Figure 3B, right panel). Nonetheless, in testing the expression levels of endocrine and paracrine factors, there were no significant changes in the

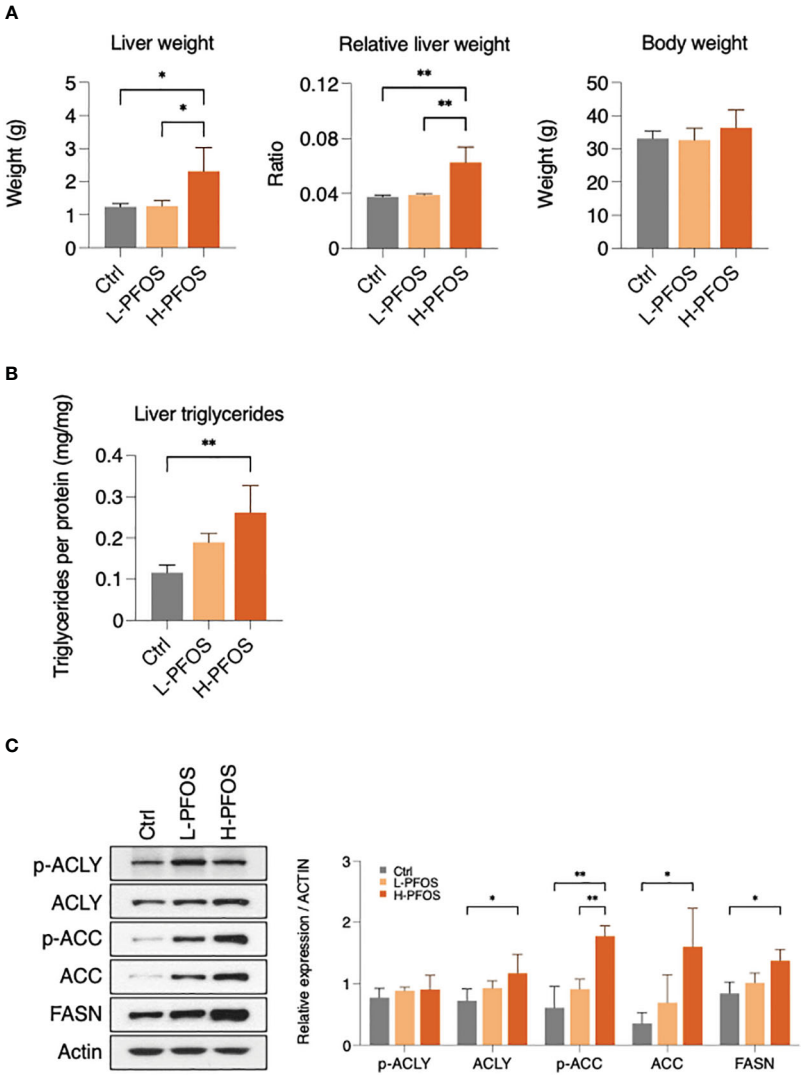

FIGURE 1
Effect of PFOS exposure on liver fat metabolism at day 21. Mice were administered 0.3 µg/g/day (L-PFOS) and 3 µg/g/day (H-PFOS) of PFOS by gavage for 21 days, and the liver was dissected and assessed. **(A)** The absolute and relative liver weights were significantly increased in H-PFOS mice compared to control and L-PFOS mice. **(B)** The triglyceride content was increased in H-PFOS compared to control, and **(C)** the protein expression of lipogenesis enzymes, including ATP citrate synthase (ACLY), phosphorylated ACLY (6X active), acetyl-CoA carboxylase (ACC), phosphorylated ACC (inactive form), and fatty acid synthase (FASN), were significantly upregulated in the PFOS-exposed groups. Actin served as the endogenous control. Graphs show the mean ± S.D. (*p<0.05, **p<0.01).

testicular gene expression of follicle-stimulating hormone receptor (*Fshr*) and luteinizing hormone receptor (*Lhr*) (Supplementary Figure 2A), growth hormone receptor (*Ghr*), insulin-like growth factor (*Igf*) and its receptor, *Igfr* (Supplementary Figure 2B), hepatocyte growth factor (*Hgf*) and its receptor, *Hgf-r* (Supplementary Figure 2C), and the steroidogenic enzymes (*Star, Cyp11a1, Cyp17a1, Hsd-3β, & Hsd-17β*) (Supplementary Figure 3) as compared with the control. Interesting, a significant reduction in the expression levels of Srd5α2 was noted in the high-dose group vs low-dose group.

PFOS is known to perturb fatty acid metabolism via PPARs and fatty acid-mimicry pathways. To unravel the possible impact of PFOS on fatty acid metabolites in testes, we examined PFOS uptake and its perturbation to lipid profiles using MS-imaging. Figure 4A showed a significant increase of PFOS levels in the testes of low- and high-dose exposed mice. MS-imaging data identified significant increases in the levels of the poly-unsaturated fatty acids, eicosa-5, 8, 11-trienoic acid (ETA), eicosa-5, 11, 14-trienoic acid (arachidonic acid, AA), dihomo-α-linolenic acid (DALA), and dihomo-γ-linolenic acid (DGLA) (Figure 4B right panel, Supplementary Table 2), oxidized ceramide (CER) (Figure 4C; Supplementary Table 3), diacylglycerol (DAG) (Figure 4D; Supplementary Table 4), and the phospholipids (phosphatidylcholine & phosphatidylethanolamine) (Figure 4E; Supplementary Table 5). The ion signal of phosphatidylinositol, which is a common and stable component of cell membranes (Figure 4B, left panel), was used for data normalization.

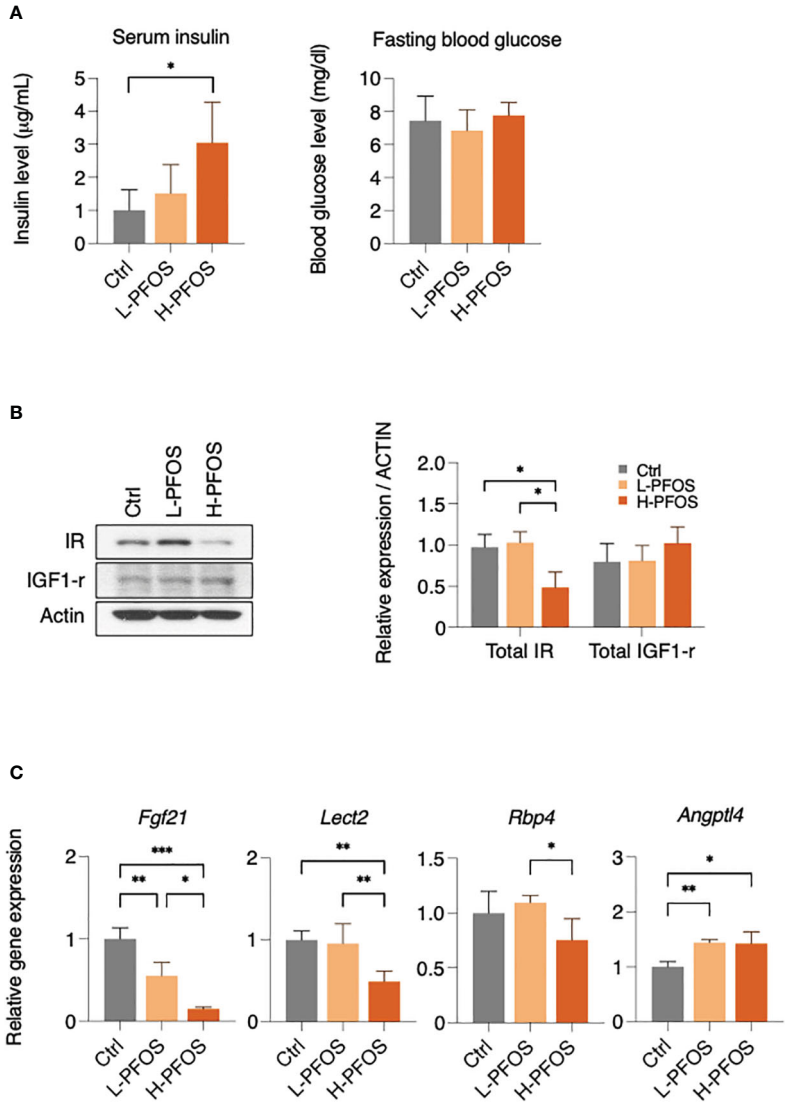

FIGURE 2
PFOS exposure perturb insulin signaling and expression of hepatokines in liver. **(A)** Serum insulin levels were significantly increased in H-PFOS compared to control, with no noticeable changes observed in fasting blood glucose levels. **(B)** The expression of insulin receptor (IR) was significantly downregulated. **(C)** A significant decrease in gene expression of the hepatokines, fibroblast growth factor 21 (*Fgf-21*) and leukocyte cell-derived chemotaxin 2 (*Lect-2*) were noted in PFOS-exposed groups, and a decrease of retinol binding protein 4 (*RBP-4*) was noted among L-PFOS and H-PFOS groups in livers. Conversely, the gene expression of angiopoietin-like 4 (*Angptl-4*) was increased in the H-PFOS compared to control. Actin served as the endogenous control. Graphs show the mean ± S.D. (*p<0.05, **p<0.01, ***p<0.001).

Discussion

With hindsight, the effects of PFOS on both PPAR-dependent and PPAR-independent fatty acid mimicry pathways disrupt multiple metabolic pathways in multiple tissues, resulting in dyslipidemia, insulin resistance, and inflammation (21–24). This study showed that PFOS exposure at the high-dose (3μg/g b.w.) induced hepatic lipid accumulation which exhibited the early sign of non-alcoholic fatty liver disease (NAFLD). The observation is consistent with the previous studies, showing PFOS perturbed lipid metabolism and induced hepatotoxicity (22, 25). The hepatic liver accumulation was associated with the significant increase in the expression levels of ACLY and FASN. A combination of elevated expression of these enzyme activities promoted fatty acid synthesis from cytosolic acetyl-CoA. Despite this, the expression level of phosphorylated ACLY (pACLY) with reported 6-fold higher activity of ACLY (26) remained unchanged. The expression levels of both total ACC (active form) and pACC (inactive form) increased significantly with high-dose PFOS treatment. There was no significant change in the ratio of pACC to ACC, presumably no significant alteration in ACC activity. Nonetheless, it was unanticipated to see a significant increase in pACC with a functional outcome of hepatic lipogenesis in the group receiving high doses of PFOS. In retrospect, AMP-activated protein kinase (AMPK) is the major kinase known to phosphorylate ACC to promote fatty acid oxidation (27). In a recent study, PFOS-induced deranged hepatic metabolism stimulated both lipogenesis and lipid catabolism, as well as an activation of AMPK pathway (28).

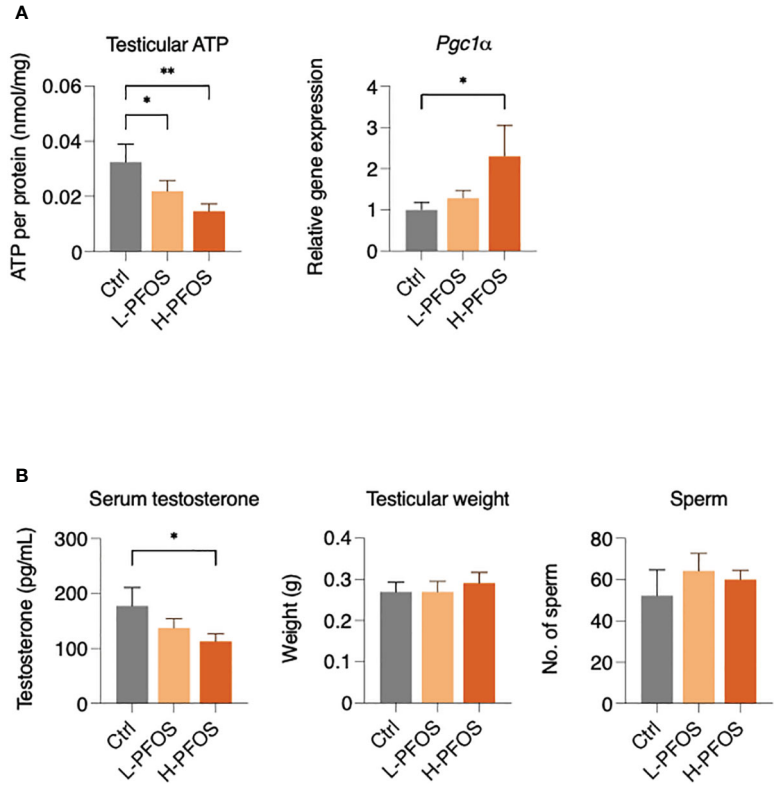

FIGURE 3
Impact of PFOS on testicular activity, energy metabolism and testosterone expression. **(A)** Testicular ATP levels were significantly decreased in PFOS-exposed groups. There was an increase in the gene expression of peroxisome proliferator-activated receptor gamma coactivator 1-α (*Pgc1α*) in H-PFOS groups compared to control. Actin served as the endogenous control. **(B)** Serum testosterone levels were significantly decreased in H-PFOS compared to control with no noticeable changes observed in testis weight and number of epididymal sperm counts. Graphs show the mean ± S.D. (*p<0.05, **p<0.01).

Therefore, in this study the observation of an increased phosphorylation of ACC might be associated with the dysregulation of hepatic metabolism induced by PFOS. The dysregulation was further demonstrated with a significant reduction in hepatic IR expression and a significant increase in blood insulin levels. This shows that PFOS perturbed insulin signaling in the liver and systemically.

Further illustrating the liver's effect on bodily function, the secreted hepatic metabolic factors (hepatokines) were measured. In the PFOS-exposed groups, the expression levels of the four hepatokines (*Fgf-21*, *Lect-2*, *Rbp-4*, and *Angptl4*) were significantly altered. The four hepatokines were found to be associated with the progression of NAFLD (29–33). Apart from being a metabolic regulator, *Fgf-21* was also found to play roles in promoting spermatogenesis, protecting germ cells from diabetes-induced apoptosis (34, 35), and increasing sperm motility (36). *Lect-2* was linked to tissue inflammatory responses (37), coherent with the early sign of PFOS-elicited liver steatosis (22). Another perturbed hepatokine, *Rbp-4* regulated the physiological functions of testosterone receptors in the testicles, including the production of testosterone and spermatogenesis (38). In obese adolescents, *Rbp-4* expression was associated with gonadal functions (39). The decreased expression levels of *Fgf-21* and *Rbp4* imposed negative influence on testicular functions. In fact, fatty liver disease has also been linked to impaired testicular function (40, 41). There were reports indicating an association between NAFLD and low blood levels of testosterone, which caused accumulation of visceral adipose tissues, elevated free fatty acid levels, inflammation, and increased insulin resistance (42, 43). The observations suggest that systemic metabolism and gonadal function are mutually interdependent.

In testes, metabolic perturbation was evidenced with significant reduction in ATP levels and the increased expression of *Pgc-1α*, which was reported to protect against oxidative stress and energy metabolism dysfunction in the testes (44). Previous studies had shown that men with fatty liver disease exhibited lower levels of testosterone and sperm count (41, 45, 46). In this study, our data only showed the reduction of blood testosterone levels at the high-dose group. In our previous studies, a higher dose of PFOS exposure (5 μg/g b.w. for 21 days) in mice, caused significant reduction in testosterone levels, sperm count, and sperm swimming activities (8, 9, 47). To decipher the underlying process of the reduced testosterone levels in the high-dose group, the analysis of the steroidogenic enzymes did not reveal noticeable changes, except a significant reduction of Srd5α2 (steroid 5α-reductase 2, a membrane enzyme catalyzes testosterone to dihydrotestosterone). From a pharmacological perspective, an inhibition of Srd5α2 activity might be linked to an increase in serum testosterone

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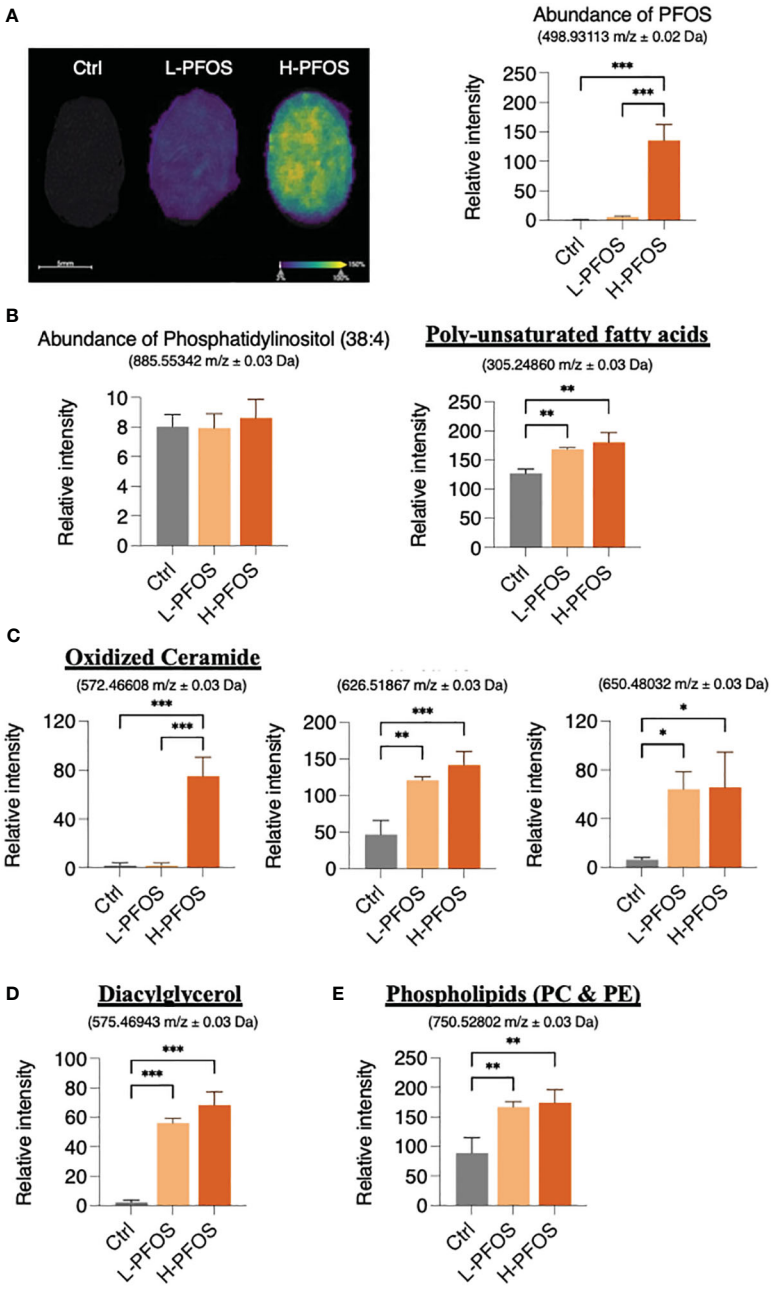

FIGURE 4
AFADESI-Mass Spectrophotometry Imaging of PFOS and metabolite distribution in testis. **(A)** Left panel: Ion image of PFOS distribution in testes with overlayed haematoxylin and eosin staining. Right panel: a corresponding graph shows the relative coneceantration of PFOS in the control, L-PFOS and H-PFOS samples. **(B)** Left panel: The levels of the phosphatidylinositol served for data normalization. Right panel: There was a significant increase in summated levels of the polyunsaturated fatty acids (eicosa-5, 8, 11-trienoic acid, eicosa-5, 11, 14-trienoic acid, dihomo-α-linolenic acid, dihomo-γ-linolenic acid), **(C)** oxidized ceramides (CER), **(D)** diacylglycerol (DAG) and **(E)** phosphatidylcholine (PC) & phosphatidylethanolamine (PE) in PFOS-exposed groups. Graphs show the mean ± S.D. (*p<0.05, **p<0.01, ***p<0.001).

levels (48). This may explain an increase in serum testosterone in the high-dose group of our study. In order to further investigate the impact of PFOS on testicular physiology, we focused on the notorious action of PFOS in perturbing fatty acid homeostasis, which may negatively impact testicular functions (49, 50).

The PFOS exposure increased the testicular levels of the poly-unsaturated fatty acids (ETA and AA), the important metabolites in regulating gonadal functions (51, 52). This observation is related to our previous study showing an increase of hydroxyeicosatetraenoic acids in neonatal testes upon PFOS exposure (49). Additionally, PFOS-induced an increase of the poly-unsaturated fatty acids (DGLA and DGLA), which were reported to be directly associated with the sign of tissue inflammation (53, 54). Moreover, our data showed a significant increase in oxidized ceramides and DAG, the important messengers for spermatogenesis, and apoptosis (55, 56). Furthermore, significant

increases in total phospholipids, especially phosphatidylcholine, and phosphatidylethanolamine, were associated with the post-testicular causes of infertility (57). Overall, the data suggest that PFOS disrupted fatty acid metabolites' homeostasis, altering the fatty acid signaling pathway in testes.

This study aimed to better understand the pleiotropic effects of PFOS on tissue functions. As shown by our data, PFOS affects hepatic lipid metabolism, hepatokine expression, blood insulin, testosterone levels, and testicular fatty acid metabolism. This study provided a mechanistic link between disrupted lipid metabolism and perturbed testicular physiology. These findings may help identify PFOS disregard targets and develop targeted interventions to restore and protect testicular function in mammals exposed to these pollutants. As a result of this study, we have gained a better understanding of the pathology, the molecular mechanisms, and the biochemical changes resulting from PFAS-induced testicular toxicity.

Data availability statement

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

Ethics statement

The procedure for animal handling was followed according to guidelines and regulations approved by the animal ethics committee (REC/20-21/0234) of Hong Kong Baptist University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WL: Data curation, Formal Analysis, Investigation, Writing – review & editing. TL: Formal Analysis, Investigation, Methodology, Software, Writing – review & editing. HT: Investigation, Methodology, Writing – review & editing. TH: Data curation, Investigation, Writing – review & editing. HW: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – review & editing. CW: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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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.2023.1302965/full#supplementary-material

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EDITED BY
Cibele Borges,
Federal University Rural Semi-Arid, Brazil

REVIEWED BY
Ana Flávia Quiarato Lozano,
Universidade Municipal de São Caetano do Sul, Brazil
Leonardo Nascimento,
University of Ribeirão Preto, Brazil

*CORRESPONDENCE
Gang Sun
13309880419@163.com

†These authors have contributed equally to this work

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Correlation between visceral fat metabolism score and erectile dysfunction: a cross-sectional study from NHANES 2001-2004

Lewei Huang[1†], Heqian Liu[2†], Lianqiang Li[1], Shudong Wang[1] and Gang Sun[1*]

[1]General Hospital of Northern Theater Command, Shenyang, Liaoning, China, [2]Wuhu Hospital, East China Normal University (The Second People's Hospital of Wuhu), Wuhu, Anhui, China

Backgrounds: The factors associated with erectile dysfunction (ED) are diverse, and obesity is a significant component. Metabolic Score for Visceral Fat (METS-VF) can assess obesity more accurately than body mass index (BMI). However, the association between METS-VF and ED remains unclear.

Objective: This study aimed to investigate the association between the METS-VF and ED using National Health and Nutrition Examination Survey (NHANES) 2001-2004 data.

Methods: Data were sourced from NHANES 2001-2004. The relationship between METS-VF and ED was analyzed using multivariate logistic regression, followed by subgroup analyses to identify sensitive populations. Nonlinear correlation was evaluated through smoothed curve fitting, and a threshold effect analysis validated the findings. Comparative logistic regression of the Receiver Operating Characteristic (ROC) curve assessed the diagnostic capability of METS-VF against the classical obesity index for ED.

Results: The study enrolled 3625 participants, of whom 961 self-reported ED history and 360 reported severe ED. After adjusting for confounders, METS-VF exhibited a positive association with asthma prevalence (OR= 3.47, 95% CI: 2.83, 14.24). Stratification based on median METS-VF revealed higher ED prevalence in participants with elevated METS-VF (OR= 2.81,95% CI:2.32, 3.41). Nonlinear correlation was observed, with a significant association between METS-VF and ED when METS-VF exceeded 6.63. Subgroup analysis highlighted a stronger correlation in participants aged 50-85 years, Caucasians, hypertensive individuals, diabetics, and those with coronary heart disease. Sensitivity analysis using severe ED as the outcome reaffirmed the nonlinear positive association with METS-VF (OR=3.86, 95% CI:2.80,5.33), particularly when METS-VF surpassed 6.68.

Conclusion: Elevated METS-VF was nonlinearly correlated with increased ED incidence. Individuals with METS-VF above 6.63 should be vigilant about heightened ED risk. Special attention should be given to participants aged 50-85 years, Caucasians, hypertensive individuals, diabetics, and those with coronary heart disease.

KEYWORDS

visceral obesity, METS-VF, NHANES, cross-sectional studies, erectile dysfunction

1 Introduction

Erectile dysfunction (ED) stands as a prevalent affliction among men, progressively impacting a greater number as they age (1). Before reaching the age of 40, ED prevalence ranges from 1% to 10%. Beyond 40 years, the occurrence of ED escalates dramatically to 52% (2), and an alarming 70% of men aged 70 and above contend with ED (3). Notably, Ayta IA underscored an upward trajectory in ED prevalence, projecting that over 322 million men worldwide will grapple with ED by 2025 (4). The elusive nature of ED detection, often reliant on patient self-reporting, underscores the potential for misguided medical intervention, exacerbating the condition and imposing financial strain. As ED's prevalence surges, its socioeconomic burden deepens; current investigations estimate the expenditure on ED prevention and treatment has surpassed $15 billion (4), sans additional concealed expenses. While ED manifestations may not be life-threatening, their repercussions on relationships, mood, and overall quality of life are undeniable (5). Furthermore, despite the common perception linking ED to psychological elements like anxiety and emotional disconnect, it is imperative to acknowledge that in younger patients, erectile dysfunction can signal an underlying organic pathology (6–8).

In addition to well-defined organic pathologies such as vascular and neurological impairments, the etiology of ED is intrinsically intertwined with psychological, hormonal, and environmental factors (9). Numerous comorbidities commonly intersect with ED, encompassing diabetes mellitus, hypertension, hyperlipidemia, obesity, and testosterone deficiency (9, 10). Within the context of obesity's broader implications, it is now apparent that obesity's ramifications extend beyond initiating diabetes, hypertension, and hyperlipidemia, encompassing a heightened susceptibility to ED (11). Previous investigations have demonstrated that obesity can more than double the risk of ED, even when accounting for lifestyle variables (12, 13). In a multicenter inquiry, obesity, defined by waist circumference (WC) and body mass index (BMI), was shown to render individuals twice as prone to developing ED in contrast to non-obese counterparts (BMI < 30 kg/m² and WC < 102 cm). Notably, WC emerged as a superior predictor of ED compared to BMI (14). Recognizing WC's superior sensitivity to obesity compared to BMI (15), it is prudent to establish a metric that better captures the extent of visceral fat.

Recent years have borne the concept of the metabolic score for visceral fat (METS-VF), validated in diverse systemic disorders (16, 17). METS-VF has exhibited enhanced assessment efficacy when contrasted with other established visceral fat metrics (18). Despite these strides, the relationship between METS-VF and ED risk remains inadequately elucidated. Motivated by this gap, our study aims to undertake a cross-sectional analysis, probing the interplay between METS-VF and ED prevalence using data culled from the National Health and Nutrition Examination Survey (NHANES).

2 Materials and methods

2.1 Study population

Utilizing the publicly available NHANES database, a comprehensive cross-sectional survey conducted biennially over the course of nearly two decades, capturing an approximate cohort size of 10,000 individuals per iteration, and overseen by the CDC, data were procured. Our study focused on the 2001 to 2004 dataset because the NHANES workgroup administered the ED questionnaire only during this time frame. Because the ED questionnaire was only administered to adult males over the age of 20, we removed participants under the age of 20 and female participants. To align with our research objectives, a stringent screening process was applied to refine the study population, as illustrated in Figure 1, outlining detailed inclusion and exclusion criteria. This meticulous curation culminated in the enrollment of a final cohort comprising 3625 cases. Within this cohort, 961 cases reported a history of ED, with an additional 360 cases detailing a history of severe ED.

2.2 Data collection and definition

The METS-VF index served as our designated exposure variable, with metabolic score for insulin resistance (METS-IR) calculated as Ln[(2×fasting glucose)+fasting triglycerides)×body mass index]/[Ln(high-density lipoprotein cholesterol)]. The formulation for METS-VF was as follows: METS-VF = 4.466 + 0.011*(Ln(METS-IR))^3 + 3.239*(Ln(WHtr))^3 + 0.319 + 0.594*(Ln(Age)). Assessment of erectile dysfunction (ED) (KIQ400) involved a structured questionnaire. Validated study-specific inquiries included, "Please describe your ability to achieve and sustain an erection adequate for sexual intercourse." Response options encompassed "never," "sometimes," "usually," and "almost always or nearly always." We classified ED instances as respondents indicating "sometimes able" or "never able," serving as our primary outcome measure. For sensitivity analyses, exclusive consideration was given to men who responded "never" in terms of their ability to maintain an erection (19). Prevalence of ED was treated as the outcome of interest. Literature-endorsed potential confounders, potentially impacting the association between the METS-VF index and ED, were consolidated in multivariable adjusted models. The set of covariates embraced age (years), race stratified into Mexican American, White, Black, and Other (20), educational attainment (grouped as less than high school, high school, and beyond high school levels), poverty-to-income ratio (PIR) categorized based on prior research (20), marital status (married or partnered, unmarried), alcohol consumption (derived from questionnaire ALQ101-Had at least 12 alcohol drinks/1 yr? affirmative responses identified individuals as drinkers), physical activity, cholesterol levels (mg/dl), smoking status (in accordance with questionnaire SMQ020-Smoked at least 100 cigarettes in life; affirmative responses categorized participants as smokers), hypertension, diabetes mellitus, coronary heart disease, and asthma (answered affirmatively on the questionnaire). Recognizing the potential impact of both early and advanced prostate cancer on sexual function, participants with prostate cancer were excluded. Dietary factors, comprising energy, fat, sugar, and water intake, were also considered. All participants underwent two 24-hour dietary recalls, with the average consumption from the recalls employed in our analysis.

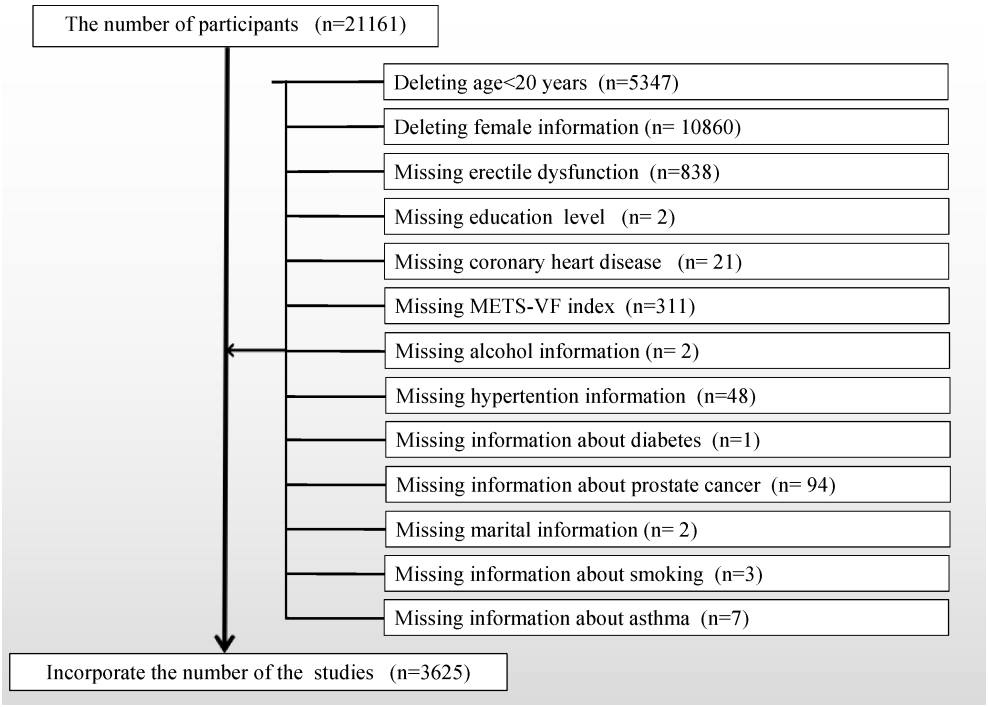

FIGURE 1
Flow chart for participants.

Comprehensive measurement protocols for study variables are accessible to the public at www.cdc.gov/nchs/nhanes/.

2.3 Handling of missing values

Continuous variables with many missing values are converted to categorical variables, and the missing variables are set as a dummy variable group and named "unclear".

2.4 Statistical methods

A significance level of $p<0.05$ was adopted to establish statistical significance. The analyses were conducted using Empower software and R version 4.2.0. Official recommendations from the NHANES website underscored the use of appropriate sampling weights for statistical analyses. Comprehensive guidelines for weight analysis were outlined. New sampling weights were derived by dividing the 2-year weights for each survey cycle by 2 (21). The survey design R package in R was employed to process the provided dataset weights. These weights were further utilized in survey design analyses. For continuous variables, survey-weighted linear regression was employed, while categorical variables underwent survey-weighted chi-square tests. Continuous variables were presented as weighted survey means with corresponding 95% confidence intervals, and categorical variables were expressed as weighted survey proportions alongside their 95% confidence intervals. Consistent with STROBE guidelines, we established three distinct multivariate regression models. Model 1 encompassed no covariate adjustments. Model 2 integrated adjustments for race, education level, and marital status. Model 3 encompassed adjustments for all variables excluding age (as its effect was concurrently captured along with the unadjusted METS-VF index in Model 3). To assess robustness, sensitivity analyses converted the METS-VF from a continuous variable to a bicategorical variable. A linear trend test was applied using the two quartiles of METS-VF as a continuous entity. Further validation was pursued through inverse probability weighting. Addressing the potential nonlinearity of METS-VF in relation to ED, we employed a generalized additive model (GAM) and smooth curve fitting. If nonlinearity patterns emerged, a two-segment linear regression model (segmented regression model) was engaged, fitting each interval and quantifying threshold effects. Finally, the predictive efficacy of WWI, BMI, and WC concerning ED was evaluated via receiver operating characteristic (ROC) curves and area under the curve (AUC) calculations (22).

3 Results

3.1 Baseline characteristics of the participants

Table 1 presents the demographic baseline features of the enrolled participants. The study encompassed 3625 participants, whose weighted attributes were delineated based on ED presence.

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TABLE 1 Baselines characteristics of participants, weighted.

Characteristic	Non-ed formers (n=2664)	Ed formers (n=961)	P-value
Age (years)	41.21 (40.66,41.77)	60.27 (59.29,61.26)	<0.0001
BMI (kg/m^2)	27.91 (27.66,28.15)	29.01 (28.45,29.57)	0.0024
Serum Cholesterol (mg/dl)	201.76 (199.80,203.71)	200.29 (195.54,205.04)	0.562
Waist (cm)	99.10 (98.49,99.71)	105.28 (103.85,106.70)	<0.0001
METS-VF	6.17 (6.15,6.19)	6.65 (6.61,6.68)	<0.0001
Race (%)			0.2586
Mexican American	7.61 (5.81,9.92)	7.01 (4.32,11.18)	
White	78.71 (75.22,81.83)	81.51 (76.51,85.65)	
Black	9.59 (7.62,12.01)	8.41 (6.26,11.22)	
Other Race	4.09 (3.14,5.30)	3.08 (1.95,4.82)	
Education Level (%)			<0.0001
Less than high school	13.33 (12.02,14.76)	28.45 (23.95,33.42)	
High school	27.78 (25.59,30.08)	23.80 (20.55,27.38)	
More than high school	58.89 (56.41,61.34)	47.75 (43.41,52.13)	
Marital Status (%)			<0.0001
Cohabitation	68.59 (65.78,71.27)	77.06 (73.88,79.96)	
Solitude	31.41 (28.73,34.22)	22.94 (20.04,26.12)	
Alcohol (%)			0.0593
Yes	84.41 (80.02,87.98)	81.10 (76.73,84.81)	
No	15.59 (12.02,19.98)	18.90 (15.19,23.27)	
High Blood Pressure (%)			<0.0001
Yes	21.16 (18.85,23.68)	50.17 (46.95,53.40)	
No	78.84 (76.32,81.15)	49.83 (46.60,53.05)	
Diabetes (%)			<0.0001
Yes	3.80 (2.95,4.89)	22.04 (18.25,26.37)	
No	96.20 (95.11,97.05)	77.96 (73.63,81.75)	
Smoked (%)			<0.0001
Yes	54.25 (51.27,57.19)	70.58 (67.08,73.86)	
No	45.75 (42.81,48.73)	29.42 (26.14,32.92)	
Asthma (%)			0.0023
Yes	10.96 (9.67,12.40)	7.34 (5.68,9.44)	
No	89.04 (87.60,90.33)	92.66 (90.56,94.32)	
Coronary Artery Disease (%)			<0.0001
Yes	2.54 (1.93,3.33)	14.83 (11.90,18.34)	
No	97.46 (96.67,98.07)	85.17 (81.66,88.10)	
PIR (%)			<0.0001
< 1.3	14.73 (12.84,16.85)	16.96 (13.92,20.50)	
≥ 1.3 < 3.5	32.04 (29.61,34.57)	40.10 (36.03,44.31)	
≥ 3.5	48.29 (45.07,51.53)	38.12 (33.58,42.87)	
Unclear	4.94 (3.72,6.52)	4.82 (3.26,7.08)	
Total Kcal (%)			<0.0001
Lower	36.90 (34.25,39.63)	58.14 (53.05,63.05)	
Higher	58.15 (55.31,60.94)	37.58 (32.11,43.39)	
Unclear	4.95 (3.98,6.14)	4.28 (2.78,6.54)	
Total Sugar (%)			<0.0001
Lower	39.32 (37.05,41.64)	55.07 (51.45,58.63)	
Higher	51.30 (48.58,54.00)	34.79 (30.22,39.66)	
Unclear	9.38 (7.93,11.07)	10.14 (7.78,13.11)	
Total Water (%)			0.004
Lower	47.31 (43.58,51.07)	54.63 (47.73,61.35)	
Higher	47.74 (43.93,51.57)	41.09 (34.74,47.75)	
Unclear	4.95 (3.98,6.14)	4.28 (2.78,6.54)	
Total Fat (%)			<0.0001
Lower	38.15 (35.17,41.23)	52.49 (48.06,56.88)	
Higher	56.90 (53.59,60.14)	43.23 (38.31,48.29)	
Unclear	4.95 (3.98,6.14)	4.28 (2.78,6.54)	
Testosterone (%)			0.0444
Lower	7.70 (6.63,8.93)	10.84 (8.22,14.16)	
Higher	8.40 (6.87,10.24)	6.54 (4.76,8.93)	
Unclear	83.90 (81.84,85.76)	82.62 (79.06,85.68)	
Estradiol (%)			0.4484
Lower	7.60 (6.53,8.83)	9.47 (7.25,12.27)	
Higher	8.50 (7.01,10.28)	7.91 (5.44,11.36)	
Unclear	83.90 (81.84,85.76)	82.62 (79.06,85.6	

For continuous variables: survey-weighted mean (95% CI), P-value was by survey-weighted linear regression (svyglm).

For categorical variables: survey-weighted percentage (95% CI), P-value was by survey-weighted Chi-square test (svytable).

Notably, substantial distinctions were observed in baseline characteristics, with the exception of race and estrogen levels. Specifically, those afflicted with ED exhibited a propensity toward higher age, blood cholesterol levels, BMI, waist circumference, and METS-VF values, in addition to a higher incidence of hypertension, asthma, diabetes, and coronary heart disease.

3.2 Higher METS-VF indices were associated with higher prevalence of ED

Diverse regression analyses, encompassing distinct adjustments to account for confounding influences on the correlation, illuminated a consistent positive linkage between the METS-VF index and ED across both raw and meticulously adjusted models. Within the fully adjusted model, each incremental unit elevation in the METS-VF index manifested as a substantial 247% surge in ED risk (OR=3.47, 95% CI: 2.83, 14.24). Upon categorizing the METS-VF index into two quartiles, logistic regression highlighted a notable 1.81-fold escalation in ED risk prevalence within the highest group, relative to the lowest METS-VF index category (OR=2.81, 95% CI: 2.32, 3.41). To further substantiate these findings, an inverse probability weighted analysis was performed, post-METS-VF dichotomization. Supplementary Table 1 demonstrates the equalization of baseline attributes between the two groups, following which inverse probability weighted logistic regression unveiled a statistically significant 95% upsurge in ED risk prevalence within the highest METS-VF index stratum, contrasted with the lowest group (OR=1.95, 95% CI: 1.50, 2.54) (Table 2, Supplementary Table 1).

Exploration of the METS-VF index's relationship with ED was extended via generalized additive modeling and smoothed curve fitting. Our findings underscored a nonlinear positive correlation between the METS-VF index and ED (Figure 2). Subsequent application of a likelihood ratio test revealed a discernible threshold effect of METS-VF on ED, with the risk of ED onset exhibiting a sharp increase post-METS-VF index surpassing 6.63 (Table 3).

3.3 Subgroup analysis

Subgroup analyses were performed to assess the robustness of the association between the METS-VF index and ED. Results Age <50 years group (OR=1.33, 95% CI:0.95, 1.88), age 50-85 years group (OR=2.05, 95% CI:1.54, 2.73), Mexican American group (OR=2.71, 95% CI:1.70, 4.33), White group (OR=5.01, 95% CI:3.76, 6.67), black group (OR=2.19, 95% CI:1.46, 3.29), others group (OR=1.76, 95% CI:0.53, 5.84), hypertensive group (OR=3.64, 95% CI:2.56, 5.18),non-hypertensive group (OR=3.38, 95% CI:2.63, 4.25), diabetes group (OR=3.79, 95% CI:1.86,7.71),Non-diabetic group (OR=3.49, 95% CI:2.82, 4.32),Coronary heart disease group (OR=3.82, 95% CI:1.41,10.36),Non-coronary heart disease group (OR=3.47, 95% CI:2.82,4.27) Table 4.

3.4 Sensitivity analysis

For sensitivity analysis, we categorized participants responding as 'never able' to maintain an erection as individuals with more pronounced ED severity. As demonstrated in Table 5, affirmative associations were evident across all models. In Model 3, each additional unit increment in METS-VF exhibited a substantial 286% surge in the risk of ED (OR=3.86, 95% CI: 2.80, 5.33). Findings stemming from the inverse probability weighting technique depicted a 0.65-fold augmentation in the risk of ED prevalence for every elevated METS-VF unit (OR=1.65, 95% CI: 1.10, 2.48). Employing smoothed curve fitting and a generalized additive model, we unraveled a nonlinear positive correlation between METS-VF and more severe ED (Figure 3), with the most favorable inflection point detected at 6.68 (Table 6).

3.5 METS-VF was a stronger predictor of ED than BMI and WC

Conclusively, we delved into the diagnostic potential of METS-VF, BMI, and WC concerning ED. Our analysis unveiled

TABLE 2 Logistic regression analysis between METS-VF index with ED prevalence.

Characteristic	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 3 OR (95% CI)	Model 4 OR (95% CI)
METS-VF	6.10 (5.06, 7.37)	5.94 (4.90, 7.20)	3.47 (2.83, 4.24)	3.21 (2.19, 4.70)★
Categories				
Lower (3.40-6.47)	1	1	1	1
Higher (6.47-7.38)	4.65 (3.93, 5.49)	4.53 (3.80, 5.39)	2.81 (2.32, 3.41)	1.95 (1.50, 2.54)★

Model 1 was adjusted for no covariates;
Model 2 was adjusted for race, marital status and education;
Model 3 was adjusted for covariates in Model 2+diabetes,blood pressure, PIR, total water,total kcal,total sugar, total fat, smoked, physical activity, alcohol use, serum cholesterol, coronary artery disease, asthma, testosterone and estradiol were adjusted.
Model 4:The covariates that need to be adjusted were consistent with those in Model 3.
★ = IPTW analysis only in model 4.

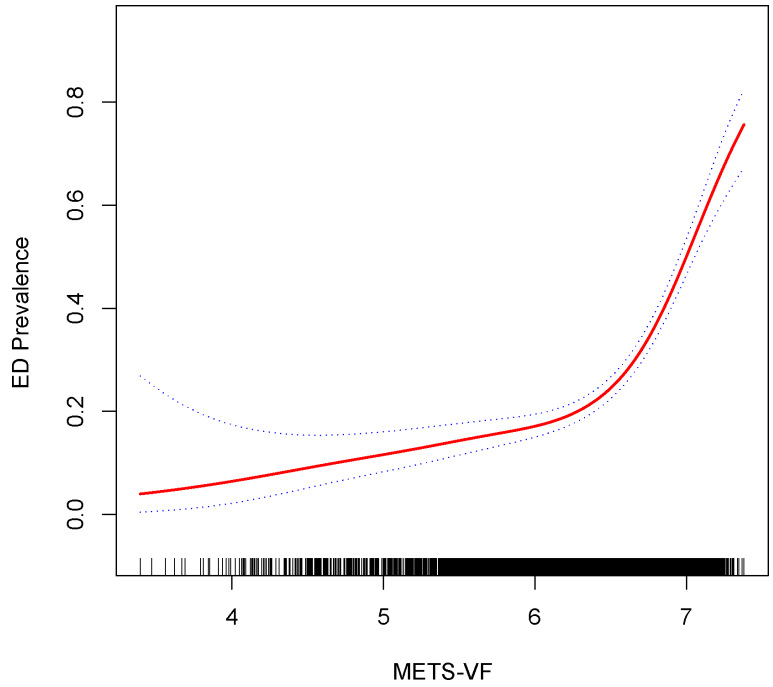

FIGURE 2
Density dose-response relationship between METS-VF index with ED prevalence. The area between the upper and lower dashed lines is represented as 95% CI. Each point shows the magnitude of the index and is connected to form a continuous line. Adjusted for all covariates except effect modifier.

noteworthy findings – the AUC values for METS-VF distinctly surpassed those of BMI and WC, applicable to both ED and more severe ED cases (Figures 4, 5).

4 Discussion

To our knowledge, this marks the inaugural cross-sectional investigation to gauge the connection between METS-VF and the prevalence of ED utilizing a representative cohort of US adults. Our findings underscore a nonlinear positive linkage between METS-VF and ED prevalence, extending this correlation to participants with more pronounced ED severity. Furthermore, we exhibit the heightened predictive utility of METS-VF relative to conventional obesity benchmarks such as BMI and WC.

Earlier research has ascertained that age, smoking, sedentary behavior, and obesity exhibit robust correlations with ED development, with a subset of cases (20%) attributed to psychological factors (23). The global economic shift has facilitated the widespread emergence of obesity as a significant public health concern due to a Westernized dietary pattern adopted by populations worldwide (24). Despite this, the precise mechanistic underpinnings of obesity-related ED remain elusive (11), motivating researchers to focus on effective preventive strategies for ED within the obese demographic. Consequently, accurate assessment of an individual's genuine obesity status has become paramount. In this context, researchers have increasingly cast doubt on the adequacy of BMI as a precise marker, instead viewing it as a rudimentary indicator for identifying obesity or overweight status (25). This arises from BMI's inability to differentiate between fat mass and lean (muscle) mass, while also failing to elucidate localized fat distribution patterns (26). While WC exhibits better sensitivity to obesity, particularly abdominal obesity (15), its use as a solitary measure remains inadequate for distinguishing between subcutaneous and visceral fat deposits (27, 28).

Prior research has indicated that variables such as age, sex, waist-to-height ratio (WhtR), METS-IR, and fasting glucose (FPG) and triglycerides (TG) within the METS-VF hold promise as indicators of intra-abdominal fat content response (18). The accumulation of intra-abdominal fat is notably associated with more perilous health implications than fat accumulation in other regions. In our study, we confirm a positive correlation between METS-VF and ED prevalence, suggesting potential advantages for METS-VF in assessing ED prevalence. First, it is widely established that the prevalence of ED escalates with advancing age, notably being considerably higher in men above 40 years of age compared to their younger counterparts (4). Our investigation similarly highlights the elevated ED risk in men over 40 years. Secondly, earlier cohort-based studies have demonstrated diabetes to be the leading risk factor for ED, associated with a 1.3- to 3-fold amplified risk of ED onset, even after accounting for diabetes type and age (29–31). Although METS-VF isn't a diagnostic tool for diabetes or

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TABLE 3 Two-piecewise linear regression and logarithmic likelihood ratio test explained the threshold effect analysis of METS-VF index with ED prevalence.

METS-VF Index	ULR Test	PLR Test	LRT test
	OR (95% CI)	OR (95% CI)	P value
< 6.63	3.47 (2.83, 4.24)	1.82 (1.42, 2.33)	< 0.0001
≥ 6.63		20.28 (11.63, 35.38)	

ULR, univariate linear regression; PLR, piecewise linear regression; LRT, logarithmic likelihood ratio test, statistically significant: p<0.05.

its type, the inclusion of fasting glucose contributes significantly to diabetes presence assessment. Notably, the METS-IR effectively reflects insulin resistance degree and possesses advantages in assessing adverse outcomes in type 2 diabetes (32). This aligns with prior observations that insulin resistance status might contribute to ED development through impaired vascular nitric oxide (NO) production and vasodilation, underscoring the need to incorporate insulin resistance diagnosis and management into ED

TABLE 4 Subgroup regression analysis between METS-VF index with ED prevalence.

Characteristic	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 3 OR (95% CI)
Stratified by age (years)			
20-49	1.44 (1.07, 1.94)	1.47 (1.07, 2.02)	1.33 (0.95, 1.88)
50-85	2.74 (2.10, 3.57)	2.76 (2.11, 3.62)	2.05 (1.54, 2.73)
Stratified by race			
Mexican American	4.94 (3.25, 7.49)	4.90 (3.21, 7.49)	2.71 (1.70, 4.33)
White	8.88 (6.77, 11.66)	8.53 (6.49, 11.21)	5.01 (3.76, 6.67)
Black	3.55 (2.49, 5.06)	3.60 (2.48, 5.21)	2.19 (1.46, 3.29)
Other Race	2.24 (0.90, 5.55)	1.94 (0.75, 5.04)	1.76 (0.53, 5.84)
Stratified by hypertension			
Yes	4.78 (3.44, 6.65)	4.65 (3.32, 6.49)	3.64 (2.56, 5.18)
No	4.58 (3.62, 5.80)	4.40 (3.45, 5.62)	3.38 (2.63, 4.35)
Stratified by diabetes			
Yes	4.09 (2.26, 7.40)	3.77 (2.06, 6.90)	3.79 (1.86, 7.71)
No	5.21 (4.26, 6.37)	5.09 (4.14, 6.25)	3.49 (2.82, 4.32)
Stratified by CVD			
Yes	3.43 (1.55, 7.55)	3.44 (1.50, 7.87)	3.82 (1.41, 10.36)
No	5.64 (4.64, 6.86)	5.58 (4.57, 6.82)	3.47 (2.82, 4.27)

Model 1 was adjusted for no covariates;
Model 2 was adjusted for race,marital status and education;
Model 3 adjusted for all covariates except effect modifier.

TABLE 5 Logistic regression analysis between METS-VF index with serious ED prevalence.

Characteristic	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 3 OR (95% CI)	Model 4 OR (95% CI)
METS-VF	7.98 (5.86, 10.87)	6.97 (5.11, 9.49)	3.86 (2.80, 5.33)	2.26 (1.12, 4.53)★
Categories				
Lower (3.40-6.47)	1	1	1	1
Higher (6.47-7.38)	4.83 (3.68, 6.34)	4.35 (3.29, 5.74)	2.66 (1.96, 3.59)	1.65 (1.10, 2.48)★

Model 1 was adjusted for no covariates;
Model 2 was adjusted for race,marital status and education;
Model 3 was adjusted for covariates in Model 2+diabetes,blood pressure, PIR, total water,total kcal, total sugar, total fat, smoked, physical activity, alcohol use, serum cholesterol, coronary artery disease, asthma, testosterone and estradiol were adjusted.
Model 4:The covariates that need to be adjusted were consistent with those in Model 3.
★ = IPTW analysis only in model 4.

care preceding diabetes onset (33). Third, though direct comparisons of WhtR and WC in predicting ED remain lacking, WhtR has demonstrated superior predictive power for diabetes mellitus, hypertension, and cardiovascular disease relative to WC (34). Furthermore, our study substantiates that METS-VF outperforms WC in diagnosing ED, accentuating the robustness of our findings.

Lastly, the generation of active adipokines stemming from abnormal visceral adiposity emerges as a pivotal driver of chronic inflammation within the body (35). A potent connection exists between inflammation and ED development, particularly pronounced in obese individuals (36). Nonetheless, preceding investigations have underscored that abnormal lipid profiles wield a stronger correlation with ED severity compared to inflammatory markers (37). Moreover, while the precise mechanisms linking obesity and ED development remain elusive, it is well-accepted that obesity can trigger diminished androgen production (e.g., total testosterone), heightened conversion of androgens to estrogens, and hypogonadotropic hypogonadism (11, 38, 39). These adverse effects associated with obesity are similarly evident in individuals with diabetes mellitus, insulin resistance, and dyslipidemia (40, 41).

The present study holds several strengths that contribute to its significance. Foremost, it stands as the inaugural cross-sectional examination delving into the interplay between visceral fat distribution and the prevalence of ED through the application of a simplified scoring system. Moreover, our study is underscored by the selection of a robust and representative sample, further bolstering its merit. Nevertheless, the study also carries certain limitations that warrant acknowledgment. First and foremost, the inherent nature of cross-sectional studies restricts our ability to deduce causality. Establishing whether a causal link exists between METS-VF and ED, and deciphering the unidirectional or bidirectional nature of this association, demands further

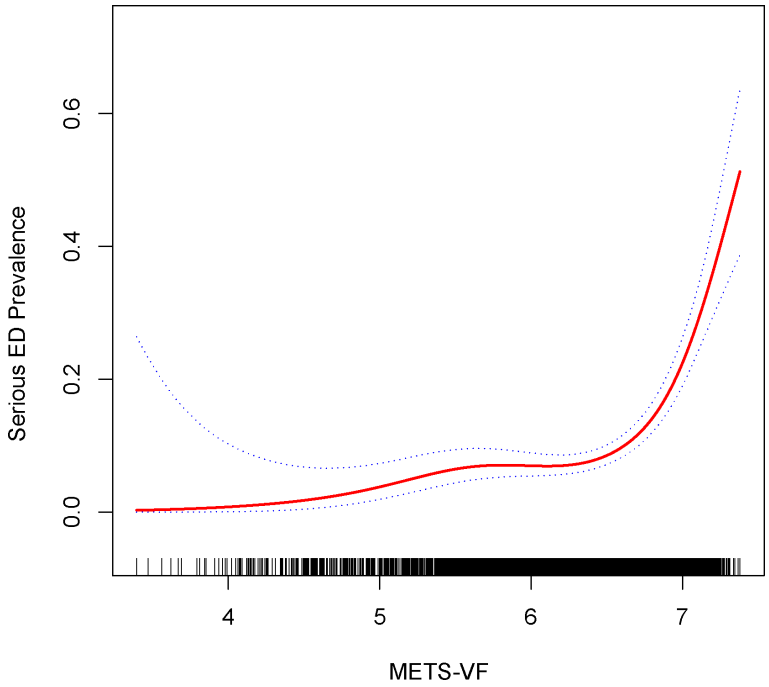

FIGURE 3
Density dose-response relationship between METS-VF index with serious ED prevalence. The area between the upper and lower dashed lines is represented as 95% CI. Each point shows the magnitude of the index and is connected to form a continuous line. Adjusted for all covariates except effect modifier.

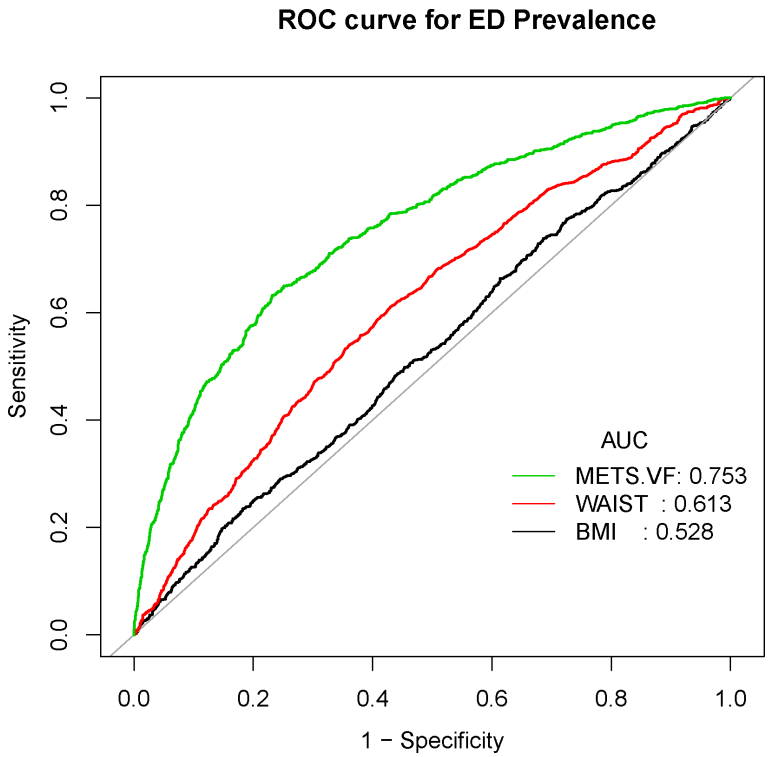

FIGURE 4
Receiver operating characteristic (ROC) curve analysis for predicting ED. Comparison of area under curve (AUC) value between WWI and BMI, WC.

TABLE 6 Two-piecewise linear regression and logarithmic likelihood ratio test explained the threshold effect analysis of METS-VF index with serious ED prevalence.

METS-VF Index	ULR Test	PLR Test	LRT test
	OR (95% CI)	OR (95% CI)	P value
< 6.68	3.86 (2.80, 5.33)	1.63 (1.10, 2.41)	<0.0001
≥ 6.68		23.75 (11.31, 49.85)	

substantiation in subsequent investigations. Second, the assessment of ED in this study was reliant upon self-reported participant surveys, inherently susceptible to recall bias. Consequently, prospective follow-up studies are imperative to provide more robust insights. Third, the potential influences stemming from ED and METS-VF are multifaceted. While extensive endeavors were undertaken to encompass relevant covariates within our model for adjustments, it remains an ongoing challenge to entirely mitigate the potential impact of other covariates that may be at play.

5 Conclusions

Our study harnessed data derived from a representative U.S. population sample, effectively unveiling a robust and affirmative linkage between METS-VF and the prevalence of ED. Notably, our findings indicate that METS-VF levels surpassing 6.63 and 6.68 correspondingly usher in a notable surge in the risk of ED and heightened ED severity. Additionally, this observed positive correlation highlights the need for heightened vigilance among participants aged 50-85 years, those of Caucasian ethnicity, individuals with hypertension, diabetes, and coronary heart disease.

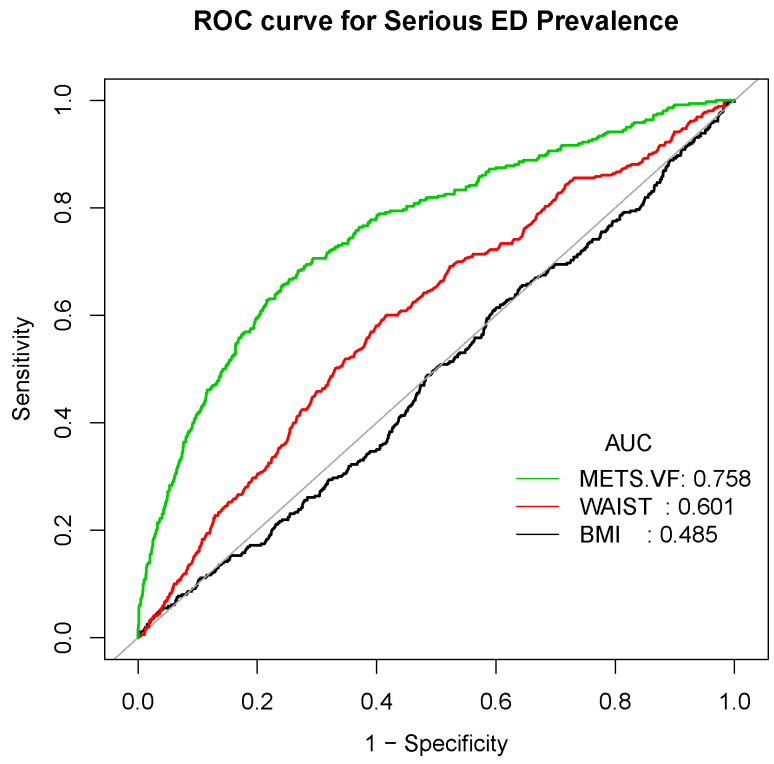

FIGURE 5
Receiver operating characteristic (ROC) curve analysis for predicting serious ED. Comparison of area under curve (AUC) value between WWI and BMI, WC.

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 NCHS Research Ethics Review Committee approved the NHANES survey protocol, and all participants of the study provided informed written consent. The NHANES database is open to the public and therefore the ethical review of this study was exempt.

Author contributions

LH: Conceptualization, Investigation, Writing – original draft. HL: Data curation, Investigation, Methodology, Writing – original draft. LL: Formal analysis, Project administration, Writing – review & editing. SW: Data curation, Software, Validation, Writing – original draft. GS: Conceptualization, Investigation, Methodology, Software, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1283545/full#supplementary-material

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EDITED BY
Baohua Ma,
Northwest A&F University, China

REVIEWED BY
Cristina Corina Clement,
Cornell University, United States
Sergio Minucci,
University of Campania Luigi Vanvitelli, Italy
James Nyirenda,
University of Zambia, Zambia

*CORRESPONDENCE
Xiang Xiao
xxiao@zjams.cn
Ya Ni
niya99@126.com

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Exploring the evolving function of soluble intercellular adhesion molecule-1 in junction dynamics during spermatogenesis

Xiang Xiao[1,2]*, Yating Han[1,3], Qin Li[4], Dongwang Zheng[1], C. Yan Cheng[5] and Ya Ni[1]*

[1]Center for Reproductive Health, School of Pharmaceutical Sciences, Hangzhou Medical College (Zhejiang Academy of Medical Sciences), Hangzhou, China, [2]Engineering Research Center of Novel Vaccine of Zhejiang Province, Hangzhou Medical College, Hangzhou, China, [3]School of Basic Medical Sciences and Forensic Medicine, Hangzhou Medical College, Hangzhou, China, [4]School of Pharmaceutical Sciences, Hangzhou Medical College, Hangzhou, China, [5]Department of Urology and Andrology, Sir Run-Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China

Intercellular adhesion molecule-1 (ICAM-1) is a transmembrane glycoprotein expressed on immune, endothelial, and epithelial cells. Its ectodomain can be proteolytically cleaved to release a circulating soluble form called sICAM-1. Clinical studies demonstrate sICAM-1 is upregulated in various diseases and associated with disease severity. Research has identified sICAM-1 as a regulator of the blood-testis barrier (BTB) and spermatogenesis. Overexpression of sICAM-1 weakened the BTB *in vitro* and *in vivo*, downregulated junction proteins including N-cadherin, γ-catenin, and connexin 43, and caused germ cell loss. This contrasts with barrier-strengthening effects of membrane-bound ICAM-1. sICAM-1 may act as a molecular switch enabling germ cells to open BTB and Sertoli-germ cell adhesion for transport across the seminiferous epithelium. While the mechanism remains unclear, reduced SRC family kinase (SFK) signaling was observed following sICAM-1 overexpression. SRC promotes BTB protein endocytosis and degradation, influences cytoskeletal dynamics, and affects cell polarity. As sICAM-1 overexpression phenocopies SRC inhibition, SRC may operate downstream of sICAM-1 in regulating BTB dynamics and spermatogenesis. Investigating sICAM-1's structure-function regions and downstream targets will elucidate the molecular mechanisms of junction disruption. This knowledge could enable strategies targeting sICAM-1/SRC to modulate BTB permeability and treat male infertility or diseases involving endothelial/epithelial barrier dysfunction.

KEYWORDS

soluble intercellular adhesion molecule-1 (sICAM-1), Sertoli cells, blood-testis barrier (BTB), testis, spermatogenesis, cytoskeleton, male fertility

1 Introduction

Male infertility is a major global health challenge according to a recent World Health Organization (WHO) report analyzing infertility prevalence worldwide from 1990 to 2021. The report found that around 17.5% of adults, approximately 1 in 6 people, experience infertility, with comparable rates across high-, middle-, and low-income countries. Male infertility negatively impacts the physical, psychological, and social well-being of men of reproductive age worldwide. A separate study estimated that over 56 million men suffered from infertility up to 2019, representing a 76.9% increase from 1990 (1). In addition to impacts on reproductive capacity, male infertility also causes substantial psychosocial distress and introduces treatment costs (2, 3).

Multiple factors can contribute to male infertility, including abnormalities in sperm function and quality, as well as failure to produce sperm. However, the precise molecular mechanisms remain poorly understood, making diagnosis and treatment challenging. Improved semen parameters after treatment do not guarantee identifying the underlying causes (4–8). Studies show close links between male fertility and overall health. Infertility increases risks of illnesses unrelated to reproduction, such as cancer, diabetes, and cardiovascular disease. Infertile men also face higher hospitalization and mortality rates when seriously ill (9–11). This correlation suggests a coordinated regulatory system involving male reproduction and other body systems. Shared effector molecules and regulatory proteins may underlie different disease processes.

One such molecule is intercellular adhesion molecule-1 (ICAM-1), which exists in both membrane-bound and circulating soluble forms (sICAM-1). In rodent testes, germ cells appear to secrete sICAM-1 to alter Sertoli cell adhesion as they move across the seminiferous epithelium during spermatogenesis. Overexpression of sICAM-1 in rat testes severely impairs the blood-testis barrier (BTB) function and germ cell adhesion (12–14). Clinical evidence has associated elevated sICAM-1 levels with diverse pathological states, including male infertility (Table 1). This positions sICAM-1 as a putative shared effector that links reproductive and systemic damage. However, the mechanism behind sICAM-1's action remains unclear. A comprehensive investigation into sICAM-1's impacts on testis function and on Sertoli and germ cell adhesion is needed. Deeper insights into sICAM-1's role and mechanisms could provide therapeutic targets for infertility while elucidating systemic disease mechanisms. This could significantly advance infertility

TABLE 1 sICAM-1/ICAM-1 expression in body fluids across different pathological conditions[a].

Diseases		Body fluid(s)	sICAM-1	ICAM-1	Pathway(s)	Inhibitor	Ref (s)
Autoimmune diseases	Celiac disease	Serum	↑				(15)
	Graves' ophthalmopathy	Serum	↑			Carbimazole	(16–18)
	Idiopathic pulmonary fibrosis	Serum	↑	↑			(19)
	Psoriasis	Serum, plasma	↑		IL-18↑		(20–23)
	Rheumatoid arthritis	Serum, plasma, synovial fluid	↑		TNF-α↑	TNFi	(24–26)
	Scleroderma	Serum	↑				(27)
	Spontaneous urticaria	Serum	↑				(28)
	Systemic lupus erythematosus	Plasma	↑	↑			(29, 30)
	Ulcerative colitis	Serum	↑			Prednisolone	(31)
	Vitiligo	Skin tissue fluid	↑	↓	IL-6↑, IL-17↑, TNF-α↑		(32, 33)
Cancers	Colorectal cancer	Serum	↑		TNF-α↑		(34–37)
	Gastric cancer	Serum	↑	↑			(38–40)
	Invasive bladder cancer	Urine, serum	↑	↑			(41)
	Lung cancer	Serum	↑	↑	TNF-α↑		(42–45)
	Mammary cancer	Serum	↑				(46, 47)

(Continued)

TABLE 1 Continued

Diseases		Body fluid(s)	sICAM-1	ICAM-1	Pathway(s)	Inhibitor	Ref (s)
Cardiovascular diseases	Coronary heart disease	Blood	↑		IL-6↑, sTNFR1↑, sTNFR2↑		(48)
	Dilated cardiomyopathy	Serum	↑				(49)
	Hypertension	Plasma	↑				(50)
Liver Diseases	Alcoholic liver cirrhosis	Serum	↑	↑	TNF-α↑		(51)
	Cholestasis	Serum	↑				(52)
	Chronic hepatitis	Serum	↑			IFN-α	(53)
	Primary biliary cirrhosis	Serum	↑	↑			(54)
	Primary sclerosing cholangitis	Serum	↑	↑			(54)
	Schistosomiasis japonica	Serum	↑				(55)
Nervous system diseases	Alzheimer's disease	Cerebrospinal fluid	↑		IL-8 ↑		(56, 57)
	Aseptic meningitis	Cerebrospinal fluid	↓		IL-8 ↑		(58)
	Bipolar disorder	Blood	↑	↑			(59)
	Schizophrenia	Plasma	↑			chlorpromazine	(59)
	Multiple sclerosis	Serum, cerebrospinal fluid	↑	↑			(60–63)
Other diseases	Endometriosis	Peritoneal fluid	↑				(64)
	Thoracic inflammation	Pleural fluid	↑				(65)
	Male infertility	Seminal plasma	↑		IL-6↑		(66)

[a]This table provides an illustrative, non-exhaustive summary of sICAM-1/ICAM-1 levels in various pathological states. Carbimazole, TNFi, prednisolone, IFN-α and chlorpromazine have been shown to decrease sICAM-1 levels and are therefore considered inhibitors. Abbreviations used in this table include: IL (Interleukin), TNF (Tumor Necrosis Factor), sTNFR1 (Soluble Tumor Necrosis Factor Receptor-1), sTNFR2 (Soluble Tumor Necrosis Factor Receptor-2), IFN-α (Interferon-α), and TNFi (Tumor Necrosis Factor Inhibitor). Up-regulation is indicated by an upward arrow (↑) and downregulation is indicated by a downward arrow (↓).

prevention and treatment, as well as therapies targeting common regulatory pathways for systemic diseases.

2 Background of sICAM-1

2.1 Origins and variants

ICAM-1, also known as Cluster of Differentiation 54 (CD54), is a single-chain transmembrane glycoprotein that consists of an extracellular region with five immunoglobulin (Ig)-like domains, a transmembrane segment, and a short cytoplasmic tail. ICAM-1 is expressed at relatively low levels by immune cells, endothelial cells, epithelial cells, and other normal tissues. However, multiple inflammatory stimuli, including cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and interferon (IFN)-γ, as well as lipopolysaccharide (LPS), can increase ICAM-1 expression through transcription (67–70).

Under inflammatory or cellular stress conditions, the ectodomain of ICAM-1 can be proteolytically cleaved and shed from the cell surface. This releases the soluble form, sICAM-1, into extracellular fluids. Pro-inflammatory cytokines enhance this shedding, leading to increased sICAM-1 levels. Circulating in body fluids like blood, sICAM-1 is a truncated form of ICAM-1 that consists solely of the five Ig-like extracellular domains D1-D5, without the transmembrane and cytoplasmic regions (Figure 1A). Being heavily glycosylated, the molecular weights of ICAM-1 and sICAM-1 can vary between 75-115 kDa and 50-90 kDa respectively (13, 70–72).

The origin and generation of sICAM-1 is not fully understood. It is thought to primarily occur through the proteolytic cleavage of ICAM-1's ectodomain by proteases. Proteases like serine proteases, matrix metalloproteinases (MMPs), and members of the "a disintegrin and metalloproteinase" (ADAM) family may cleave at different sites on ICAM-1. Both the ICAM-1 cleavage by different proteases and the differences in glycosylation could produce variants of sICAM-1 with subtle structural differences. Alternative mRNA splicing can also generate other variants of sICAM-1. Six other splice variants of ICAM-1 have been reported, varying in their combination and number of Ig domains (Figure 1B) (73). These variants are more susceptible to proteolytic cleavage and may contribute additional sICAM-1. Some studies suggest mRNAs that encode sICAM-1 directly may exist (13, 74). Each of these mechanisms could give rise to sICAM-1 variants that differ in

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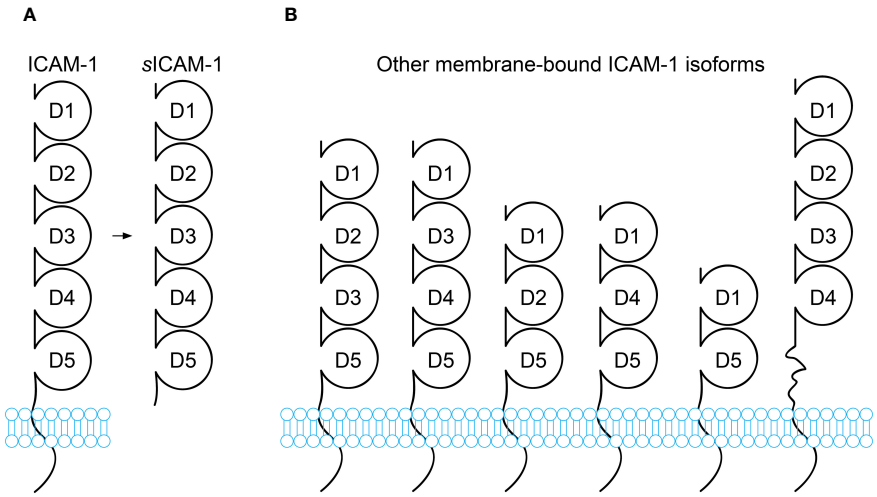

FIGURE 1
Schematic diagram of ICAM-1 isoforms. **(A)** Full-length ICAM-1 is a transmembrane protein containing 5 immunoglobulin(Ig)-like extracellular domains (D1-D5), a transmembrane domain, and a short cytoplasmic domain. Soluble ICAM-1 (sICAM-1) lacks the transmembrane and cytoplasmic domains. **(B)** In addition to the full-length isoform, alternative splicing generates transmembrane ICAM-1 isoforms with truncated extracellular regions containing 2, 3, or 4 Ig-like domains. While these alternatively spliced isoforms have been reported, most functional research pertains to full-length ICAM-1. The isoforms likely differ in tissue distribution, expression levels, and functions. Some may only appear under certain conditions, such as pathological conditions.

structure and function. Studies using isoform-deficient mice found they have sharply contrasting disease phenotypes. The ability of ICAM-1 variants to bind ligands like lymphocyte function-associated antigen-1 (LFA-1) *in vitro* also varies depending on present Ig domains. Expression of isoforms differs between cell types and may change with inflammation (73). However, it remains unclear if these isoforms could differentially regulate ligand interactions, dimerization, intracellular signaling and disease outcomes through their structural variations expressed on different cell types. It is still unknown if any hypothetical fragments have unique roles. Nevertheless, the only soluble form found in body fluids contains domains D1-D5, appearing most physiologically relevant (13, 70, 74). Further work is needed to characterize and compare sICAM-1 variants from splicing versus protease cleavage to understand their properties and potential functions. This may provide insight into the complex origins of sICAM-1.

2.2 General functions and disease associations

sICAM-1 plays complex roles in regulating inflammation and immunity. It is proposed that sICAM-1 retains the characteristics of membrane-bound ICAM-1 and can compete with ICAM-1 for binding to the integrin receptor LFA-1. As a competitive inhibitor, sICAM-1 can influence leukocyte adhesion and migration by inhibiting ICAM-1-mediated interactions between leukocytes and endothelial cells. This potentially weakens the body's immunity (70, 74, 75). The concentration of sICAM-1 affects cytokine release, immune cell adhesion, angiogenesis and tissue repair in different ways. At low concentrations, sICAM-1 can promote cytokine release and immune cell activation. In contrast, high levels of sICAM-1 may limit leukocyte adhesion while promoting angiogenesis and tissue repair (70). Specific genetic variations in the ICAM-1 gene can also affect sICAM-1 levels (69). Additionally, sICAM-1 acts as a signaling molecule. The intensity of signaling induced by sICAM-1 is regulated by the completeness of N-glycosylation and sialylation. Sialylation does not affect ICAM-1 binding to LFA-1 in astrocytes. However, it is critical for the signaling function of sICAM-1 in inducing production of the inflammatory chemokine MIP-2/CXCL2 (macrophage inflammatory protein 2/C-X-C motif chemokine ligand 2). Fully sialylated sICAM-1 induces a more rapid, robust, and prolonged MIP-2 response compared to nonsialylated or high mannose glycoforms. Sialylation may regulate receptor interactions and signaling kinetics of sICAM-1 (76).

sICAM-1 has been detected in various human body fluids including serum, cerebrospinal fluid, bile, amniotic fluid, and urine (71, 72, 74, 77–80). Elevated sICAM-1 levels correlate with disease severity and prognosis in numerous diseases like cancer, cardiovascular disease, autoimmune disorders, nervous system disease, inflammation, and viral infections (70, 72, 80–86). For instance, recent studies have found that higher levels of sICAM-1 in the serum of COVID-19 patients are positively associated with disease severity and can even predict the risk of mortality (81, 82). In chronic pain patients, serum sICAM-1 levels have been found to significantly correlate with pain intensity. However, in acute experimental pain models with healthy volunteers, sICAM-1 levels did not directly correlate with perceived pain levels. Instead, sICAM-1 underwent short-term changes after acute nociceptive stimuli (87). Additionally, an increase in sICAM-1 levels in seminal plasma may be associated with immune infertility in men (66).

Unlike ICAM-1 whose cellular expression is difficult to clinically assess, sICAM-1 in body fluid is easily measurable and is often used as a common marker for inflammatory diseases (71, 74, 77–79). However, the mechanism behind its action is still unclear. Efforts to therapeutically modulate sICAM-1 levels have shown limited efficacy in treating diseases so far. Currently, most research focuses on exploring relationships between sICAM-1 levels in fluids and disease onset, progression, and prognosis (75, 82, 84, 88) (Table 1). Alternatively, studies have generally viewed ICAM-1 and sICAM-1 as ubiquitous adhesion molecules expressed on epithelial or endothelial cell surfaces in response to environmental stimuli. It is believed that high levels of sICAM-1 may stem from corresponding vascular endothelial dysfunction (70, 72). For example, research has demonstrated that high sICAM-1 levels in cerebrospinal fluid are associated with increased phosphorylation of the microtubule-associated protein tau, which is implicated in blood-brain barrier (BBB) dysfunction, and with elevated levels of total tau protein. However, few studies have examined the links between sICAM-1 and endothelial barrier permeability. Limited existing findings suggest that sICAM-1 can influence the transport of immune cells across the BBB and their communication with surrounding cells (72, 89–92).

The precise impact of sICAM-1 on germ cell transport across the BTB and throughout the seminiferous epithelium remains unclear. It also remains unknown whether sICAM-1 acts as a key signaling molecule for communication between germ cells and surrounding Sertoli cells. At present, these mechanisms remain mysterious, as only one study to date has linked sICAM-1 to spermatogenesis and BTB permeability (12). In the upcoming section of this review, cell adhesion and junction dynamics in the testis will first be introduced. Hypothetical molecular mechanisms for the role of sICAM-1 during spermatogenesis will then be proposed. Exploring potential explanations could help further the understanding of how sICAM-1 may regulate both the BTB and germ cell adhesion. Ultimately, this may provide useful insights to advance future research on sICAM-1's function. While questions remain, continued investigation of sICAM-1's involvement holds promise to elucidate the intricate process of spermatogenesis.

3 Cell adhesion and junction dynamics in the testis

3.1 Cell junctions facilitate cell adhesion

Cell migration and morphogenesis, which are central to developmental processes, require dynamic changes in cell adhesion properties. Fundamentally, cell adhesion refers to interactions between cells (cell-cell adhesion) and between cells and the extracellular matrix (ECM, cell-ECM adhesion). These interactions play a pivotal role in maintaining tissue integrity, homeostasis, and function. Cell adhesion is primarily facilitated through specialized structures known as cell junctions, which include tight junctions (TJ), adherens junctions (AJ), desmosomes, and gap junctions (GJ). These junctions work in concert to provide structural stability, coordinate cellular behavior, and maintain tissue architecture. Nonetheless, each junction type serves unique and indispensable roles in connecting cells, facilitating intercellular signaling, and providing cell adhesion. TJ, composed of integral membrane proteins such as occludin and claudins, interact with the actin cytoskeleton through adapter proteins like ZO-1, thereby forming a permeability barrier through tight sealing between cells. This restricts the passage of molecules and plays an integral part during the development and remodeling of epithelial tissue. AJ, with core components like cadherins/catenins and nectins/afadin complexes, typically form first between cells during epithelial development, providing mechanical attachment. This is followed by formation of TJ at the apical region to AJ, sealing the paracellular space. At desmosomes, the adhesion proteins desmoglein and desmocollin provide linkage to intermediate filaments within cells. GJ, formed by clustering of connexins, create intercellular channels that directly transfer molecules between cells (93–97).

3.2 Specialized junctions in the testis

In the adult mammalian testis, spermatogenesis exemplifies the vital role of cell adhesion and junction dynamics. This highly coordinated and cyclical process involves the continual division and differentiation of germ cells, which are tightly bound to the surrounding Sertoli cells within seminiferous tubules. Cell-cell adhesion is mediated by TJ, ectoplasmic specializations (ES), GJ, and desmosomes, all relying on Sertoli cells (98). ES are unique testis-specific actin-based AJ structure located at basal sites between adjacent Sertoli cells. Characterized by their hexagonal actin arrays sandwiched between the Sertoli cell plasma membrane and endoplasmic reticulum, basal ES comprise part of the BTB. They coexist and intermix with the other three junction types (TJ, GJ, and desmosomes) to form the intricate, multifaceted BTB structure (99–102), as depicted in Figure 2 showing the different junction types labeled with colors.

ES are also found at apical sites between Sertoli cells and spermatids, termed apical ES. Once formed, apical ES serve as the sole anchoring junction at these sites until spermiation. Different Sertoli-germ cell junction types predominate during specific maturation stages, enabling diverse germ cell activities (98–105). In rodent models, early germ cells including spermatogonial stem cells, spermatogonia, spermatocytes, and pre-step 8 spermatids, primarily connect to Sertoli cells through GJ and desmosomes. However, as germ cells mature into step 8 and beyond spermatids, apical ES takes precedence (99–101, 103, 104). This junctional shift underscores the complexity and specificity of cell-cell interactions within the dynamic environment of the testis. At apical ES sites, hexagonal actin filament bundles are restricted to the Sertoli cell side. Apical ES anchor spermatids and guide their orientation and bidirectional movement in the epithelium, potentially via linkage to microtubule motors like kinesins and dyneins (106–110).

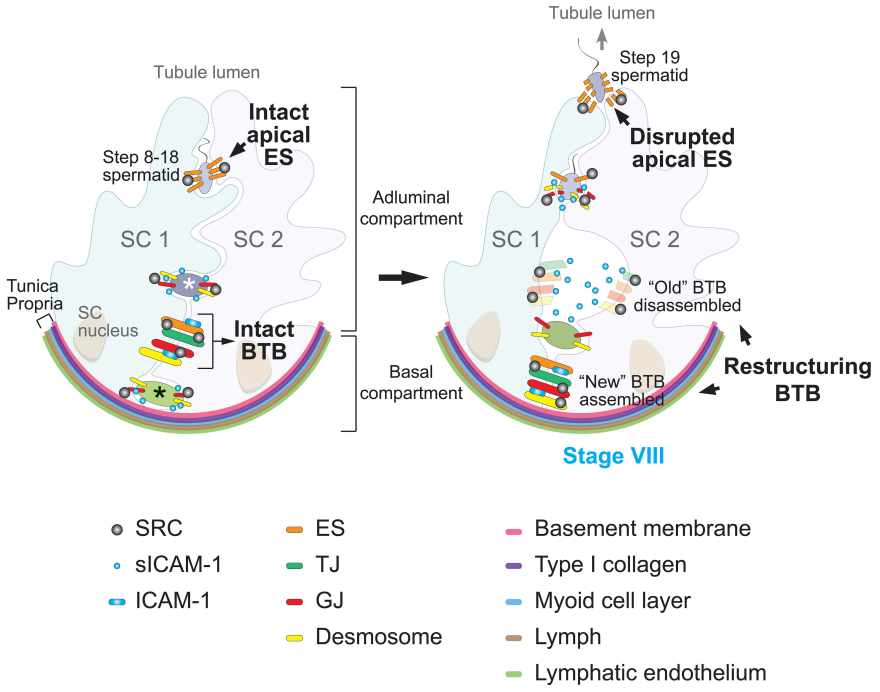

FIGURE 2
Proposed model of sICAM-1 and SRC signaling in regulating blood-testis barrier (BTB) dynamics and spermatogenesis. In the rat seminiferous tubule (left), the seminiferous epithelium is composed of Sertoli cells (SC) and germ cells at different developmental stages. The BTB, formed between adjacent Sertoli cells (SC 1 and SC 2), divides the epithelium into basal and adluminal compartments. Less mature germ cells (such as spermatogonia and preleptotene spermatocytes, marked with a black asterisk) reside in the basal compartment, while more advanced germ cells (such as primary and secondary spermatocytes, as well as round spermatids, marked with a white asterisk) reside in the adluminal compartment. The BTB comprises four distinct cell junction types, color-coded in the diagram: basal ectoplasmic specializations (ES), tight junctions (TJ), gap junctions (GJ), and desmosomes. Sertoli cells also form apical ES with step 8 and beyond spermatids, acting as the primary anchoring junction until spermiation. Pre-step 8 germ cells are linked to Sertoli cells through GJ and desmosomes. Src functions as a pivotal regulator of both the BTB and Sertoli-germ cell adhesion. At stage VIII of the seminiferous epithelial cycle (right), the BTB restructures to enable transit of preleptotene spermatocytes. This includes the formation of a "new" BTB beneath spermatocytes and the disintegration of the "old" BTB above the spermatocytes, enabling spermatocyte passage into the adluminal compartment without compromising the BTB. Concurrently, the apical ES is broken down to release mature sperm, specifically the step 19 spermatid. During these events, SRC facilitates BTB remodeling and regulates the dynamics of Sertoli-germ cell junctions, particularly apical ES disintegration. Through protein endocytosis and degradation pathways, SRC promotes transport of spermatocytes across the BTB and release of mature spermatozoa from the seminiferous epithelium. Previous studies show membrane-bound ICAM-1 promotes BTB assembly, whereas its soluble form sICAM-1 expressed by germ cells impairs the BTB to facilitate spermatocyte transit. Overexpression of sICAM-1 also disrupts GJ and desmosomes between Sertoli and germ cells, causing loss of immature germ cells. SRC may act downstream of sICAM-1 to regulate BTB restructuring and Sertoli-germ cell adhesion during spermatogenesis.

3.3 BTB dynamics

The BTB is a defining feature of the testis, fundamentally consisting of specialized cell junction structures that create a selectively permeable barrier within the seminiferous epithelium. It separates the epithelium into adluminal (apical) and basal compartments (Figure 2) and constitutes one of the tightest tissue barriers in the body. The BTB prevents substances in the blood like drugs and antibodies from accessing the adluminal compartment, providing an immunological and physical shield to developing germ cells. However, the BTB must be dynamic—continuous reorganization of intricate junctional complexes enables spermatocyte transit from the basal to adluminal compartment, a critical step in sperm development. This meticulous BTB remodeling facilitates extensive germ cell transport while maintaining barrier integrity (102).

The BTB's constant restructuring without compromising its immunological barrier function is made possible by coordinated interplay between its cell junction components. As shown in Figure 2, movement of preleptotene spermatocytes across the BTB involves localized assembly of "new" junctions below transiting spermatocytes, followed by disassembly of "old" junctions above them. Accordingly, junctional proteins undergo endocytosis, then intracellular transport including protein degradation, recycling back to the cell surface, and transcytosis across cells (102, 105, 111–113). This intricately orchestrated BTB dynamics enables the extensive germ cell development needed for the remarkable sperm production capacity of the mammalian testes.

4 Detection and roles of sICAM-1 in the rat testis

4.1 Expression of sICAM-1

A previous study has shown that both Sertoli cells and germ cells express membrane-bound ICAM-1 in the rat testis, as examined

using commercial antibodies against the cytoplasmic region of ICAM-1 (12). During stage VIII of the seminiferous epithelial cycle, ICAM-1 expression at the BTB significantly increases when examined by immunofluorescence microscopy, coinciding with BTB restructuring and the transit of preleptotene spermatocytes across it as they differentiate into leptotene and zygotene spermatocytes. This suggests ICAM-1 involvement in spermatocyte transport across the BTB. However, these antibodies cannot recognize sICAM-1. Using a custom polyclonal antibody targeting the extracellular D2-D3 domain of ICAM-1, the authors detected sICAM-1 in rat testes. The identified sICAM-1 comprised all five Ig-like domains and had a molecular weight of around 70 kDa. This is lower than the full-length ICAM-1 in rat testes with a molecular weight of approximately 97 kDa. The antibody also detected additional protein fragments with lower molecular weights, indicating potential alternative forms of sICAM-1 may be present (12). By immunoblotting analysis, sICAM-1 was found to be highly expressed in germ cells (12). This suggests germ cells may secrete sICAM-1 to regulate Sertoli cell adhesion and facilitate their own crossing of the BTB and transport in the epithelium (12–14).

4.2 Contrasting roles of sICAM-1 and ICAM-1

The authors further discovered that overexpression of sICAM-1, via a plasmid containing only the extracellular domains D1-D5, exerted an opposing effect on BTB permeability compared to overexpression of the full-length membrane-bound ICAM-1 (12). While overexpression of full-length ICAM-1 strengthens the BTB, overexpression of sICAM-1 severely impairs BTB function and causes loss of adhesion between spermatocytes and round spermatids with supportive Sertoli cells within the seminiferous epithelium (12–14).

Specifically, overexpression studies in a Sertoli cell culture model, which mimics the BTB *in vitro*, found ICAM-1 and sICAM-1 have antagonistic effects on Sertoli cell barrier function (12). ICAM-1 strengthened the barrier, mimicking "new" BTB assembly, whereas sICAM-1 weakened it, corresponding to "old" BTB disassembly during restructuring (Figure 2). Moreover, overexpressed sICAM-1 downregulated expression of BTB proteins including N-cadherin (an ES protein), γ-catenin (the only known protein present at both ES and desmosomes, also called plakoglobin (114–116)), and connexin 43 (a GJ protein). These differential effects were verified *in vivo*, where sICAM-1 overexpression in rat testes disrupted the BTB, downregulated N-cadherin and connexin 43, and caused loss of spermatocytes and round spermatids (step 1-7 spermatids). Compared to membrane-bound ICAM-1, sICAM-1 may act as a molecular switch and promoter of germ cell transit across the BTB. By interfering with Sertoli-germ cell junctions and BTB protein expression, sICAM-1 may facilitate transport of germ cells during differentiation.

In summary, sICAM-1 dually assists germ cell movement by disrupting adhesion at ES, desmosomes and GJ, and downregulating proteins like N-cadherin, γ-catenin and connexin 43 (Figure 2). This contrasts with membrane ICAM-1 which reinforces adhesion. sICAM-1 thus fine-tunes BTB dynamics to support spermatogenesis.

5 The mechanisms of sICAM-1 regulation of cell junctions

5.1 sICAM-1 downregulates SRC signaling pathways

The mechanisms by which sICAM-1 downregulates the expression of BTB and cell adhesion proteins, or "opens" cell junctions in the testis remain unclear. Overexpression studies in cultured primary Sertoli cells provide insights. sICAM-1 overexpression also reduced levels of signaling molecules important in SRC signaling, including SRC, PYK2, p-SRC-Y530 and p-PYK2-Y402 (12). This implies SRC pathways likely mediate intracellular sICAM-1 effects. Phosphorylation at tyrosine 530 (Y530) of SRC renders the kinase in an inactive confirmation (117). Thus, reduced p-SRC-Y530, in the context of decreased total SRC, indicates lower inactive and overall SRC levels, suggesting either a general decline in SRC activity, or a shift in the normal balance between active and inactive SRC conformations. As a known SRC substrate, PYK2 autophosphorylation at tyrosine 402 (Y402) recruits SRC to further regulate PYK2 activity (118). Diminished SRC and p-PYK2-Y402 levels indicate potential downregulation of PYK2 signaling involved in cell adhesion, migration, and possibly calcium-induced signaling events (119, 120). However, more research on PYK2 in the testis is needed. Collectively, this data shows sICAM-1 overexpression decreases SRC-related signaling pathways. This supports SRC pathways as probable intracellular mediators of sICAM-1.

5.2 Role of SRC in regulating BTB dynamics

SRC family kinases (SFKs), a family of non-receptor tyrosine kinases, play crucial roles in various cellular processes through signal transduction. Key members involved in the testis include SRC and YES. SFKs are known to regulate cell adhesion by modulating adhesion complexes and cytoskeletal rearrangement. They also participate in junction remodeling by phosphorylating junction proteins. Additionally, SFKs regulate endocytosis through phosphorylating endocytic vesicle proteins (121–124).

Previous studies have shown SFKs, particularly SRC, are critical modulators of BTB dynamics and spermatogenesis. SRC alters the phosphorylation state of BTB and apical ES proteins. This triggers endocytosis and intracellular transport of the junctional components, controlling opening/closing of the BTB and dissociation of spermatozoa from Sertoli cells via disruption of the apical ES (98, 102, 105). Specifically, during stage VIII of the seminiferous epithelial cycle in rodent testes, BTB restructuring coincides with sperm release through apical ES disruption (Figure 2). SRC promotes BTB disintegration and apical ES disruption by inducing protein endocytosis and degradation, mediating both "old" BTB disassembly during spermatocyte transit and loss of mature sperm association with Sertoli cells during spermiation. SRC also facilitates adhesion between immature germ cells and Sertoli cells. In summary, SRC signaling plays multifaceted regulatory roles on BTB and Sertoli-germ cell

interactions throughout spermatogenesis (124–129). While its precise interaction with sICAM-1 requires further clarification, they may coordinately influence critical junction events.

5.3 Similar phenotypes suggest SRC mediates sICAM-1 effects

Previous research shows that SRC contributes to the organization and remodeling of F-actin structures in Sertoli cells (124, 128). Excess sICAM-1 undermines SRC signaling in rat testes, severely impacting the structure and alignment of actin filaments (F-actin) (12), corresponding to SRC knockdown phenotypes (128). In other words, overexpressing sICAM-1 or inhibiting SRC produce similar results on Sertoli cell F-actin cytoskeleton.

Additionally, SRC is known to regulate intracellular protein transport, facilitating junction dynamics and aiding "old" BTB disassembly during spermatocyte transit (124–128). It binds and interacts with specific BTB protein complexes, such as N-cadherin/β-/γ-catenin, desmoglein-2/γ-catenin, and connexin 43/plakophilin-2 (102, 124, 130). SRC phosphorylation of N-cadherin, β-catenin, or γ-catenin leads to catenin dissociation from N-cadherin at the BTB sites, resulting in cadherin/catenin complex degradation, breakdown of basal ES at the BTB, and consequently increasing BTB permeability (13, 102, 130–132). These SRC-induced effects closely resemble effects of overexpressing sICAM-1, which downregulates BTB proteins like the N-cadherin/γ-catenin complex, impairing overall BTB integrity.

Excess sICAM-1 in rat testes also led to a disordered arrangement of post-step 8 spermatids within the seminiferous tubules. The sperm heads, rather than maintaining a neat alignment towards the basement membrane, exhibited a loss in polarity. The SRC-PYK2 pathway is associated with cell polarity and is likely involved in germ cell transport in the seminiferous epithelium (126, 133, 134). As sICAM-1 overexpression reduced the protein levels of SRC and PYK2, it may impede the SRC-PYK2 signaling in Sertoli cells, potentially impairing germ cell polarity and their movement within the seminiferous epithelium. Evidence also shows that intraperitoneal injection of the SRC inhibitor PP1 into rats induces sloughing of spermatocytes and round spermatids from the seminiferous epithelium (130). This phenotype of germ cell loss mimics the effects of excessive sICAM-1 in the testis. Together, sICAM-1 overexpression reduces SRC-related signaling while phenocopying global SRC inhibition outcomes, such as disoriented spermatids and their premature leaving. Thus, when secreted by germ cells, sICAM-1 appears to target SRC in Sertoli cells, affecting Sertoli-germ cell adhesion.

5.4 Summary and future directions

In conclusion, the evidence presented here demonstrates that sICAM-1 and SRC both contribute to junction disassembly and F-actin cytoskeleton remodeling. sICAM-1 overexpression diminishes activity of the tyrosine phosphorylation pathways driven by SRC and its related kinases. This impairs GJ and/or desmosomes, leading to BTB dysfunction and premature germ cell release. This pattern mirrors the effects seen when overall SRC activity is suppressed. Regarding F-actin cytoskeleton, the consequences of sICAM-1 overexpression parallel those of an SRC deficiency. Evidence also indicates sICAM-1 overexpression reduces levels of SRC and PYK2, possibly inhibiting downstream signaling in Sertoli cells, which could impair germ cell polarity and transport by disrupting SRC-PYK2. SRC thus mediates sICAM-1 impacts on BTB integrity, Sertoli-germ cell adhesion, and underlying cytoskeleton. When germ cells secrete sICAM-1 at the Sertoli-Sertoli or Sertoli-germ cell interface, SRC in Sertoli cells responds, so as to facilitate germ cell transport via cytoskeletal modulation and/or junction restructuring (Figure 3).

Future directions could explore these proposed mechanisms further. Research has indicated that the four predominant cytoskeletal structures in the testis—namely, microfilaments (F-actin), microtubules (MT, composed of tubulin polymers), intermediate filaments (e.g., vimentin filaments), and septins—interact with and influence each other (135–137). Additional investigations may delve into the impacts of sICAM-1 overexpression on these various cytoskeletal structures, as well as the subsequent changes in the Sertoli cell cytoskeleton after modulating SRC expression or activity. Approaches that alter SRC might offer a way to counteract the adverse outcomes instigated by sICAM-1 overproduction, such as BTB compromise, premature germ cell loss, and defects in spermatid polarity.

Further studies could also investigate transcription factors and broader gene expression changes resulting from sICAM-1-mediated effects on SRC signaling. The testis has specialized transcription complexes that coordinate the spermatogenic differentiation program (138). SRC can phosphorylate and/or activate various transcription factors like forkhead box class O proteins (FOXO), signal transducer and activator of transcription (STAT) proteins, and nuclear factor-κB (NF-κB) (122, 139–141). Low sICAM-1 levels may also trigger NF-κB and ERK activation, releasing inflammatory cytokines (70). However, limited information exists on specific testicular transcription factors regulated by SRC or the sICAM-1/SRC pathway. It would be interesting to decipher how main transcriptional factors are regulated in response to changes in phosphorylation status of the SRC kinase signaling pathway, and also understand the potential effect of signaling mediated by sICAM-1 on the transcriptional networks that are activated or downregulated in response to changes in sICAM-1/ICAM-1 ratios during spermatogenesis. Profiling genome-wide expression changes at defined spermatogenic stages after sICAM-1 exposure could reveal roles of particular transcription factors and gene networks altered by SRC-mediated signals. Integrating transcriptomic and proteomic data will enable constructing detailed signaling cascades from surface sICAM-1/ICAM-1 ratios and adhesive interactions to nuclear transcriptional responses governing spermatogenesis and cytoskeletal remodeling.

In summary, sICAM-1 and SRC are pivotal regulators of BTB function and spermatogenesis. Overproduction of sICAM-1 mirrors the effects of suppressing SRC, implying that SRC may be positioned downstream of sICAM-1, governing the Sertoli cell

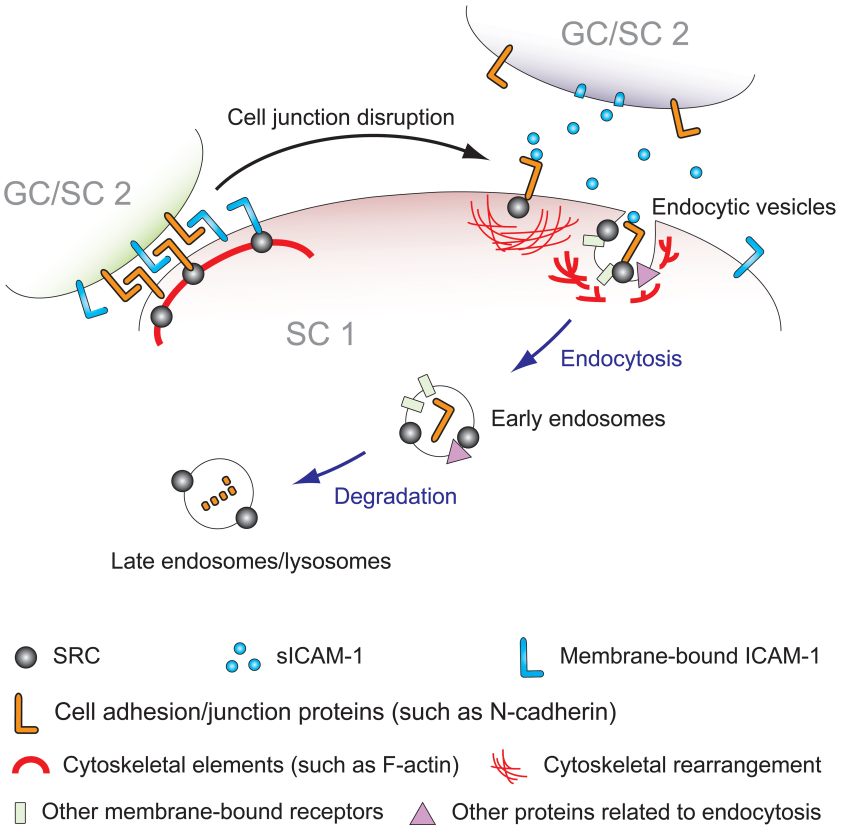

FIGURE 3
Schematic model depicting the mechanism of sICAM-1 and SRC interplay during Sertoli-Sertoli (SC 1 and SC 2) and Sertoli-germ (SC 1 and GC) cell adhesion. In mammalian testes, germ cell adhesion and the dynamic reorganization of the blood-testis barrier (BTB) primarily rely on protein endocytosis and intracellular transport of internalized proteins, including protein degradation, recycling, and transcytosis. SRC is known to regulate adhesion between immature germ cells and Sertoli cells, as well as the release of mature sperm from the seminiferous epithelium, playing a role in cell junction dissociation and restructuring. Previous studies have shown that SRC promotes endocytosis and degradation of BTB proteins in Sertoli cells, disassembling the "old" BTB. Overexpression of sICAM-1 in primary Sertoli cells and rat testes damages the BTB, GJ, and desmosomes, leading to increased BTB permeability, loss of immature germ cells, and decreased expression levels of SRC, p-SRC-Y530, and SRC substrate PYK2/p-PYK2-Y402. This affects the F-actin cytoskeleton, consistent with the phenotype of SRC deficiency or inhibition. As sICAM-1 is a germ cell-secreted extracellular signal, SRC likely acts downstream as the intracellular mediator of sICAM-1 effects on Sertoli cells, propagating sICAM-1 disruption of the BTB and Sertoli-germ cell adhesion. This model illustrates that at Sertoli-Sertoli (BTB) and Sertoli-germ cell junctions, sICAM-1 signals through cell adhesion proteins to recruit SRC, triggering cytoskeletal reorganization and adhesion protein endocytosis and degradation to regulate the BTB and spermatogenesis.

cytoskeleton, BTB stability, and the adhesion of germ cells. A more in-depth understanding of the sICAM-1/SRC signaling mechanisms promises to yield valuable insights for manipulating BTB permeability and addressing male infertility.

6 Understanding sICAM-1 interactions and knowledge gaps

6.1 sICAM-1 interactions and binding partners

ICAM-1 and its soluble form sICAM-1 must interact with various partners to mediate their functions. One of the best characterized binding partners is LFA-1, a member of the β2-integrin family. As the classical interacting protein of ICAM-1, studies show that sICAM-1 can competitively bind to LFA-1 similarly to ICAM-1 (142, 143). However, the precise role of this interaction in the testis remains unclear. While LFA-1 has been detected in mouse testicular germ cells, it does not appear to directly bind ICAM-1 in this tissue (144). This suggests there may be alternative, testis-specific binding partners for both ICAM-1 and sICAM-1 that influence their function locally. Co-immunoprecipitation experiments shows ICAM-1 physically associates with actin cytoskeletal filaments as well as several important tight junction proteins in the rat testis, including occludin and N-cadherin (12). These proteins represent plausible interacting partners for ICAM-1 and sICAM-1 at testicular junctional sites. However, it remains unknown whether the associations are direct or indirect. Further studies are still needed to conclusively identify the ligand(s) that ICAM-1 and sICAM-1 bind to on adjacent Sertoli and germ cells.

Questions also remain regarding the precise mechanisms and functional outcomes of these putative interactions in the testicular environment. Do ICAM-1 and sICAM-1 directly engage occludin, N-cadherin or other proteins at junctions? Elucidating their binding

modes of action could provide crucial insights into how these molecules regulate testicular permeability and germ cell transport.

6.2 Functional regions of sICAM-1

Beyond its binding partners, understanding the specific domains that facilitate interactions is important for elucidating ICAM-1 and sICAM-1 function. Examining their signaling mechanisms further illuminates the roles of these molecules in the testis.

Structural analyses indicate the D1 domain of ICAM-1 predominantly mediates its binding to transmembrane partners like LFA-1. Additionally, the D3 or D3-D4 regions interact with other β2-integrin family members such as Mac-1 and p150,95 (74). As sICAM-1 likely binds extracellular targets on adjacent Sertoli cells, one hypothesis is that sICAM-1 engages N-cadherin extracellularly and recruits SRC signaling through N-cadherin's cytoplasmic tail, thereby initiating SRC signaling intracellularly. Analyzing individual or combined sICAM-1 fragments could help identify key motifs enabling these interactions. Understanding the specific domains involved in partner binding and signaling initiation provides insights into the mechanisms by which ICAM-1 and sICAM-1 exert their functions in the testis.

6.3 Differences between membrane-bound ICAM-1 and tailless sICAM-1 signaling

The cytoplasmic tail of ICAM-1 plays a pivotal regulatory role in intracellular signaling, influencing various cellular processes (70). Deletion of this tail impairs ICAM-1 function, reducing adhesion and stress fiber formation. The RKIKK motif within the tail plays a critical part in regulating ICAM-1 dynamics on the cell surface, inducing actin cytoskeleton rearrangement and stress fiber formation through interactions with actin binding proteins (145). Additionally, the tail establishes important interactions with the actin cytoskeleton. It is essential for efficient RhoA activation and also interacts with myosin-II and Rac1, contributing to downstream effects on actin cytoskeleton remodeling and cell adhesion (70, 146, 147). Moreover, it is crucial for the ICAM-1 cleavage process, particularly through tyrosine residues Y474 and Y485 within its cytoplasmic region (148). The tail's association with proteins like α-actinin, ezrin, and moesin is also pivotal for ICAM-1 localization and functions related to adhesion and migration (149, 150).

In contrast to membrane-bound ICAM-1, as the tailless form, sICAM-1 lacks the ability to directly impact key intracellular signaling entities. For example, sICAM-1 cannot influence SRC or components of the actin cytoskeleton in the same way as ICAM-1. The precise mechanisms of sICAM-1 signaling remain uncertain. It is unclear whether signaling solely involves sICAM-1 functioning as a cleaved and released molecule, or if signaling may still occur through residual interactions between the tail and intracellular proteins after cleavage. Alternatively, sICAM-1 could signal independently upon binding extracellular receptors. Elucidating these uncertainties surrounding sICAM-1 signaling pathways requires further investigation.

6.4 Target molecules of sICAM-1

While sICAM-1 shares similarities with ICAM-1, emerging evidence indicates it functions as a distinct signaling molecule in the testis (12–14). Both Sertoli and germ cells express ICAM-1, but germ cells predominantly express sICAM-1 and not ICAM-1. Since sICAM-1 overexpression alone triggers downstream signaling, it likely transmits signals generated by germ cells to Sertoli cells. One hypothesis is that sICAM-1 functions to open the BTB, enabling spermatocyte transit across. It may also help interrupt and reassemble Sertoli-germ cell adhesion to facilitate transport of developing germ cells through the epithelium. Elevated sICAM-1 associates with decreased SRC signaling and lower levels of specific adhesion proteins such as N-cadherin, connexin 43, and γ-catenin. Thus, sICAM-1 could prompt degradation of BTB/adhesion proteins like N-cadherin via SRC pathways, compromising junction integrity. N-cadherin and other membrane proteins may be direct targets on Sertoli cells that enter SRC-mediated degradation pathways upon receiving extracellular sICAM-1 signals from germ cells (Figure 3). Identifying sICAM-1's exact molecular targets and deciphering its role in regulating barrier permeability and germ cell movement are fundamental goals for advancing our understanding.

6.5 Linking sICAM-1 to testicular pathology and dysfunction

While studies establish associations between sICAM-1 and barrier integrity proteins, several questions remain regarding its links to pathological conditions in the testis. The inverse relationship between sICAM-1 levels and proteins involved in cell contacts points to its potential involvement in adhesion dysregulation. However, causality has yet to be proven. Exposure to toxicants is known to disrupt the BTB and cause germ cell loss. Given sICAM-1's role in barrier function, it may mediate some aspects of toxicant susceptibility in the testis. Some studies have shown that air pollutants such as diesel exhaust particles and particulate matter up-regulate sICAM-1 and/or ICAM-1 expression in both humans and animals (151–153). Additionally, drug treatments like statins mostly decreased sICAM-1 and/or ICAM-1 levels in patients (154, 155). However, direct evidence in the testis is still lacking. The precise circumstances influencing sICAM-1 level changes in the testis remain unknown, as does the identification of treatments that could reduce its levels.

To move from correlation to elucidating disease mechanisms, more in-depth investigation of sICAM-1 signaling pathways is needed. Answering questions about how it specifically modifies junction formation and barrier properties, as well as its effects in toxicant exposure models, could help link sICAM-1 functions to pathological barrier breakdown in the testis. Addressing key gaps through techniques like overexpression, knockdown and toxicological models will help determine sICAM-1's precise role in testis dysfunction. Its intricate regulation of cell adhesions indicates its potential as a target for diagnosing and treating male reproductive conditions.

7 Future avenues and opportunities for sICAM-1 study

The study of sICAM-1, an endogenous regulatory molecule with unique biological activity, presents promising opportunities to advance our understanding of processes beyond its established role in BTB restructuring and spermatogenesis. One exciting area of future research is the BBB. Like the BTB, the BBB's vital protective function also prevents drug delivery to the brain. sICAM-1's ability to modulate barrier function makes it a candidate for strategies to enable efficient drug delivery across the BBB, potentially enabling new treatments for neurological disorders. Pairing sICAM-1 fragments with specific drugs could be explored, developing targeted brain delivery approaches. Additionally, sICAM-1's association with inflammatory diseases like rheumatoid arthritis suggests the possibility of elucidating molecular mechanisms to inform targeted therapies. Its influence on tumor progression and metastasis also indicates significance for cancer research, perhaps leading to innovative therapeutic approaches for patients. Deeper investigation into sICAM-1's role across tissue systems may provide pivotal insights. For cases of unexplained male infertility or testicular inflammation, exploring BTB damage and the expression of sICAM-1, SRC, and related signals could illuminate underlying causes. In summary, sICAM-1's diverse implications across biology and medicine offer promising opportunities to advance both scientific understanding and therapeutic innovation.

Author contributions

XX: Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing. YH: Investigation, Methodology, Writing – review & editing. QL: Investigation, Methodology, Writing – review & editing. DZ: Investigation, Methodology, Writing – review & editing. CC: Methodology, Resources, Validation, Writing – review & editing. YN: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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EDITED BY
C Yan Cheng,
St. Francis College, United States

REVIEWED BY
Wei Zhang,
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Massimo Venditti,
University of Campania Luigi Vanvitelli, Italy

*CORRESPONDENCE
Layla Simón
lsimon@uft.cl
María Salomé Mariotti-Celis
mmariotti@uft.cl

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Bioactive compounds as potential alternative treatments to prevent cancer therapy-induced male infertility

Layla Simón* and María Salomé Mariotti-Celis*

Nutrition and Dietetic School, Facultad de Medicina, Universidad Finis Terrae, Santiago, Chile

About 8-12% of couples experience infertility, with male infertility being the cause in 50% of cases. Several congenital and acquired conditions, including chronic diseases and their treatments, can contribute to male infertility. Prostate cancer incidence increases annually by roughly 3%, leading to an increment in cancer treatments that have adverse effects on male fertility. To preserve male fertility post-cancer survival, conventional cancer treatments use sperm cryopreservation and hormone stimulation. However, these techniques are invasive, expensive, and unsuitable in prepubertal patients lacking mature sperm cells. Alternatively, nutritional therapies enriched with bioactive compounds are highlighted as non-invasive approaches to prevent male infertility that are easily implementable and cost-effective. In fact, curcumin and resveratrol are two examples of bioactive compounds with chemo-preventive effects at the testicular level. In this article, we summarize and discuss the literature regarding bioactive compounds and their mechanisms in preventing cancer treatment-induced male infertility. This information may lead to novel opportunities for future interventions.

KEYWORDS

fertility, testicular, chemotherapy, radiation, tumor, resveratrol, curcumin

1 Introduction

Infertility is a major health problem that affects approximately 8% to 12% of couples worldwide. Male infertility stands as the primary cause in 20% to 30% of cases and contributes to infertility in an additional 20% of couples. Collectively, male factors account for 50% of infertility cases. Various congenital, acquired and idiopathic factors contribute to male infertility. In terms of acquired factors, chronic diseases and their treatments, such as cancer, chemotherapy and radiation, respectively, play a role in male infertility (1).

Cancer is a major health problem worldwide, with incidence and death rates historically higher in men than women. In fact, the probability of developing invasive cancer within lifetime was 1 in 2 men compared to 1 in 3 women in the United States

between 2017-2019. Indeed, men are more exposed to carcinogenic factors, such as endogenous hormones, smoking, height, and immune response. In this sense, it is expected that around one-half of cancers in men will be concentrated in prostate (29%), lung (12%) and colorectal (8%) cancers in 2023. In the United States, prostate cancer incidence has increased by roughly 3% annually, similar to lung cancer (2%) (2). Therefore, the sustaining increase in cancer incidence leads to an increment in cancer treatments that can have adverse effects on male fertility.

Hopefully, the 5-year cancer survival rate has increased from 49% in the mid-1970s to 68% in the last decade. Moreover, prostate cancer (97%) has the highest survival rate after thyroid cancer (98%) (2), thereby increasing the number of patients surviving after cancer treatment.

Male infertility after cancer treatment is caused by: (a) decreased gonadotropin secretion from the pituitary gland caused by immune inhibitors, cranial irradiation and central nervous system tumors surgeries; (b) spermatogenic dysfunction due to chemotherapy or irradiation; (c) obstruction of seminal tracts caused by intrapelvic surgeries; (d) sexual dysfunction due to intrapelvic or retroperitoneal surgeries or irradiation. In fact, almost 46% of young cancer survivors overcome male infertility, and 30% of patients have testicular dysfunction due to chemo- or radio-therapy (3). Unfortunately, male survivors of childhood cancer have a higher desire for children compared with their siblings (25% *vs.* 7%) (4), thereby developing interventions that preserve male fertility is a necessity for cancer patients.

Adult cancer patients use sperm cryopreservation and hormone stimulation to preserve male fertility post-cancer treatments. However, these techniques are invasive, expensive, and unsuitable in prepubertal patients lacking mature sperm cells. Using less gonadotoxic chemo- and radio-therapies, organ-sparing surgeries and cryopreserving testicular tissue are some methods to preserve or restore fertility in prepuberal males undergoing cancer therapies. Unfortunately, these approaches are even more expensive, invasive, possible only for some patients and available in only few medical centers (5).

On the other hand, nutritional therapies enriched with bioactive compounds seem to be cost-effective, easily-implementable, and non-invasive approaches to prevent male infertility. In a metanalysis review, L-Carnitine administrated with micronutrients, antioxidants and herbal supplements increases pregnancy rates (6). In fact, antioxidants such as L-Carnitine, Coenzyme-Q10, ω3 fatty acid and selenium improves sperm quality parameters (7).

In this article, we summarize and discuss the literature concerning bioactive compounds and their mechanisms of action within preventing cancer treatment-induced male infertility. This information may contribute to develop novel opportunities for future interventions.

2 Etiology of male infertility

Male infertility is classified into four categories: (a) hypothalamic-pituitary axis disturbances, (b) spermatogenic qualitative and (c) quantitative defects, and (d) ductal obstruction or dysfunction (8). Independently of the category, about 30% of infertility cases are due to idiopathic conditions, and 70% are caused by genetic mutations or acquired conditions (9).

Genetic mutations affect almost 15% of males with infertility and 25% of men with azoospermia (no spermatozoa in the ejaculate). Some genetic alterations are chromosomal numerical or structural abnormalities, Y chromosomal deletions, azoospermia factor (AZF) deletions, androgen receptor (AR) gene mutations, cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations (8).

Some acquired conditions that affect male fertility are obesity, pesticide exposure, smoking and medications (9). For instance, obesity is a health problem that course with concomitant diseases such as cardiovascular disease, type 2 diabetes and cancer. Moreover, obesity affects the hypothalamic-pituitary-gonadal axis, disrupts testicular steroidogenesis, and induces erectile dysfunction, poor semen quality and prostatitis. Some mediators of infertility-induced obesity are hyperinsulinemia, hyperleptinemia, chronic inflammation and oxidative stress (10). In the case of pesticide exposure, workers and exposed populations have deleterious semen quality (volume and sperm count, motility and morphology), DNA fragmentation and chromosome aneuploidy (11). Males with smoking habits have a high probability of being infertile, which increases with long smoking duration (>10 years) (12). Also, medications have adverse effects on reproductive achievement. For example, males receiving major antidepressant drugs have reduced conceptional rates (13).

Similarly, other environmental pollutants that have shown to affect male fertility are heavy metals, microplastics and endocrine-disrupting chemicals such as bisphenols, phthalates, and parabens. Heavy metals such as Zn, Se, Pb, and Cd are known to increase lipid peroxidation, reduce antioxidant capacity, and thereby impair sperm function (14). Also, high blood and seminal levels of Pb, Cd, Ba and U are associated with low sperm viability (15). In the case of microplastics, it has been proved in animals that microplastics impair semen quality in an equivalent human dose of 0.016 mg/kg/d, which is nearly achieved in Japan and South Korea (16). In addition, bisphenols, phthalates, and parabens increase ROS production, DNA damage and apoptosis, leading to abnormal sperm count and semen quality (17).

Testicular tumors represent almost 2% of all cancers in men. Testicular tumors are classified into two main groups: germ cell and sex cord tumors. Germ cell tumors include germ cell neoplasia *in situ*, seminoma, teratoma, non-seminomatous germ cell tumors, embryonal carcinoma, Yolk sac tumors, trophoblastic and regressed germ cell tumors. Sex cord stromal tumors cover Sertoli and Leydig cells tumors, and myoid gonadal stromal tumors (18). Although testicular cancer is a disease that reduces male fertility by disturbing spermatogenesis, vasculogenesis and the secretion of paracrine factors, the reduction in fertility is more a consequence of cancer treatment than the primary testicular tumor effects. In the case of surgery, 50% of patients after orchiectomy have a decrease in sperm number. Radiotherapy effects on spermatogenesis depend on doses. In fact, doses less than 1 Gy allow spermatogenesis recovery after 18 months, doses up to 3 Gy after 30 months, and up to 4 Gy after 5

years. Moreover, the chemo- and radiotherapy combination increases their gonadotoxic effects that when are administrated alone. The main adverse effect of chemotherapy on fertility is a consequence of targeting proliferating cells as germ cells (19). Spermatogonia are sensitive to the cytotoxic effects of cancer treatments, with increased susceptibility during the spermatogenesis undergoing differentiation (3). In addition, chemotherapy induces mutations in stem cell spermatogonia, thereby causing permanent damage to spermatogenesis. Some alkylating agents such as vincristine, prednisolone and cyclophosphamide induce permanent germ cell depletion and azoospermia in 80% of patients. Etoposide and doxorubicin promote azoospermia in 90% of patients (19).

In addition, other types of tumors dismiss male fertility by endocrine, nutritional, metabolic and immune alterations (19). In fact, germ cells are sensitive to the toxic effects of cancer therapy. Due to spermatogonia are more sensitive than highly differentiated germ cells, initially cancer treatment reduces the number of spermatogonia but does not affect the number of spermatocytes, spermatids and sperm cells. So, the sperm count is maintained at the beginning of the treatment but is reduced dramatically after 1 or 2 months. The azoospermia occurs after 12 weeks of cancer treatment, and in the case of low cytotoxic drugs (vinblastine, bleomycin, methotrexate, 5-fluorouracil), sperm count is recovered after 12 weeks of discontinuation chemotherapy. But, the highly cytotoxic drugs (cyclophosphamide, cisplatin, busulfan) induce more prolonged and even permanent azoospermia. Furthermore, cranial surgery or irradiation affect the hypothalamic-pituitary-gonadal axis, reduce gonadotrophin secretion and thereby spermatogenesis (3).

3 Treatment for male infertility

3.1 Conventional treatments preventing male infertility

Treatments for male fertility are selected in function of the etiology. For instance, male hypogonadotropic hypogonadism is secondary to gonadotropin deficiency and is treated with hormone stimulation to replace the missing hormones. Therapeutic GnRH stimulates pituitary gonadotropin secretion. Gonadotropins imitate LH and FSH. Selective Oestrogen Receptor Modulators (SERMs) inhibit Oestrogen Receptors in the hypothalamus and pituitary and suppress the oestrogen-mediated negative feedback on the hypothalamic-pituitary-gonadal axis. Aromatase inhibitors inhibit the conversion of testosterone to oestradiol, thereby decreasing the oestradiol-mediated negative feedback (20). Although hormone stimulation has promising effects on animals, it is ineffective in human cancer patients (5).

In the case of male infertility due to cancer treatment in reproductive age, an alternative to preserve fertility is sperm cryopreservation followed by *in vitro* fertilization or intracytoplasmic sperm injection. These approaches are effective when sperm is collected for 2-3 ejaculates and before the cancer treatment. But, in some cases starting cancer treatment is an urgency that no contemplate time for sperm cryopreservation. Moreover, this option is expensive and difficult to develop in some centers (19). Furthermore, sperm cryopreservation and assisted-reproductive technology have 30% pregnancy and 25% live birth rates, respectively. Although being compared to general fertility treatment, is not effective in all the patients (3).

Cryopreserving sperm is impossible in prepuberal patients. The cryopreservation of testicular tissue obtained through a biopsy is possible in these patients (19). Moreover, cryopreserving testicular tissue is the only option in men unable to ejaculate and those with azoospermia (3). However, testicular biopsy has potential risks in leukemia patients. Furthermore, fertility restoration is still a problem in young cancer survivors for clinical reasons (19). In fact, the alternatives for using cryopreserving spermatogonia stem cells are under experimentation. For instance, testicular tissue auto- or xeno-grafting, spermatogonial stem cells transplantation and *in vitro* spermatogenesis are being studied in animals (5). However, culturing sperm and germ cells has been unsuccessful in humans. In addition, these approaches have some disadvantages: the small number of spermatogonia stem cells, contamination with cancer cells, surgical procedures, intracytoplasmic sperm injection and virus infection (3).

3.2 Bioactive compounds as potential alternative treatments preventing male infertility

Regarding nutritional aspects, abnormal sperm parameters and hormone levels are associated with a high intake of alcohol, processed starchy and meat foods and foods rich in trans and saturated fatty acids (21–27). On the other hand, nutritional healthy habits such as high consumption of vegetables, fruits and seafood products have shown a positive association with the prevention of male infertility (28).

The Western diet is rich in saturated fatty acids, carbohydrates and proteins found in processed foods. It is also reduced in polyunsaturated fatty acids, dietary fibers and antioxidants, consequently having a negative effect on sperm quality. In this sense, the Western diet is associated with obesity, dyslipidemia, insulin resistance, oxidative stress, and aromatase activity. As a result, the Western diet reduces testosterone levels leading to a decrease in sperm count, motility and morphology. Moreover, the Western diet modifies the metabolism of sperm cells by decreasing glycolysis and mitochondrial respiration, reducing ATP content and sperm motility. Contrary, vegetarian diets are rich in plant-based antioxidants such as polyphenols with beneficial effects on sperm quality. For instance, quercetin is a flavonoid that interacts with mitochondrial membranes at the coenzyme Q-binding site, suppresses superoxide generation and promotes ATP synthesis (29). Similarly, the Mediterranean diet is characterized by a high intake of polyphenols, monounsaturated fatty acids, fiber, ω3 fatty acids, and vitamins, while being reduced in saturated and trans-fatty acids. Therefore, the Mediterranean diet is associated with a positive impact on male fertility, high sperm count and motility (28). Furthermore, the Mediterranean diet reduces oxidative stress

and increases ATP generated through energetic metabolism, thus improving sperm quality (29).

As we previously described, there exists an intricate connection between nutritional habits and fertility. *In vivo* models have confirmed that administrating several bioactive compounds found in plant sources can help to mitigate the detrimental effects caused by unhealthy habits and environmental pollutants, thereby aiding in the recovery of fertility. Table 1 summarizes the nutritional habits known to prevent male infertility, classifying the sources and bioactive compounds that control the detrimental effects.

Mice fed with a high-fat diet develop a metabolic syndrome-like condition (increased body weight, hypercholesterolemia, hyperglycemia and glucose intolerance) associated with deleterious reproductive status. For instance, high-fat diet-fed

TABLE 1 Bioactive compounds preventing male infertility.

Source or Bioactive Compound	Model	Effect	Reference
Olive oil	Rabbit model of high-fat diet-induced hypercholesterolemia	Recover the semen quality and sperm function	(30–32)
Lepidium meyenii (Maca)	Healthy men	Increase sperm count and motility	(33)
Acanthopanacis senticosi	Men with asthenospermia	Activate sperm motility	(34)
Sesamum indicum	Infertile men	Improve sperm count, motility and normal morphology	(35)
Sesamum indicum	Caffeine-induced sperm toxicity in male albino rats	Increase the weight of epididymis and sperm count, and reduce sperm head abnormalities	(36)
Withania somnifera	Infertile men	Improve energy metabolism and quality of semen and reproductive hormone levels	(37)
Morinda officinalis-Lycium barbarum with ohioensin-A, quercetin and sitosterol	Men with oligoasthenozoospermia	Reduce oxidative stress and apoptosis	(38)
Camellia sinensis leaves contain flavonol and epigallocatechin gallate	Wistar rats	Increase sperm concentration and viability	(39)
Chlorella vulgaris	Deltamethrin-intoxicated rats	Increase total sperm number and testicular antioxidant enzymes	(40)
Chlorella vulgaris	Rats with sodium nitrite-induced reproductive toxicity	Prevent sodium nitrite-induced alterations of sperm parameters, hormonal concentrations and testicular oxidative–antioxidant status	(41)
Spirulina platensis	Cadmium-intoxicated rats	Improve spermatogenesis and steroidogenesis after Cadmium exposure	(42)
Spirulina platensis	Furan-intoxicated rats	Improve semen quality, reproductive hormone levels and redox status in furan-intoxicated rats	(43)
Spirulina platensis	Mercuric chloride-intoxicated rats	Improve mercuric chloride-induced testis injuries and sperm quality alterations	(44)
Chlorella vulgaris and *Spirulina platensis*	Rats treated with lead acetate	Mitigate lead acetate-induced testicular oxidative stress and apoptosis	(45)
Halopteris scoparia	Mice with cadmium-induced reproductive toxicity	Recover sperm count, viability and motility, and reduce apoptosis	(46)
Laminaria japonica	Streptozotocin-nicotinamide-induced diabetic rats	Restore sperm motility and testosterone level, decrease abnormal sperm number, and inhibit lipid peroxidation	(47)
Resveratrol	Mice intoxicated with Cadmium and Lead	Improve sperm parameters, redox balance, testicular histology, and reduce signaling pathways such as Akt	(48)
Resveratrol- loaded nanostructured lipid carriers	Cryopreserved rooster sperms	Increase motility, viability, membrane function, mitochondrial activity, antioxidant capacity and reduce apoptosis	(49)
Resveratrol	Cryopreserved human sperms	Decreases DNA fragmentation. Increase markers of male fertility (protamine 1 and 2) and pregnancy success (adducin 1 alpha) by activating 5' AMP-activated protein kinase	(50)
Curcumin	Cadmium-intoxicated mice	Increase antioxidant enzymes. Recover REDOX status	(51)
Curcumin	Artesunate-intoxicated Swiss Albino mice	Increase antioxidant enzymes. Recover REDOX status	(52)
Curcumin	Cadmium-intoxicated mice	Reduce oxidative stress via nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway	(53)

REDOX, Oxidation-Reduction.

mice have an increment in gonadal fat, associated with a reduction in epididymis weight and sperm count (54). However, switching from a high-fat diet to a normal diet recovers the fertility potential in obese male mice. In this sense, obese mice changing to a normal diet have a reduction in gonadal fat content, and an increment in FSH serum levels and fertility potential (55). In addition, the impact of a high-fat diet on semen parameters has been studied in a rabbit model of diet-induced hypercholesterolemia. In fact, a high-fat diet is associated with a reduction of semen volume, sperm count and motility, but an increment in sperm cholesterol content, lipid droplets, functional (acrosomal reaction) and morphological abnormalities. The testicular inefficiency is associated with reduced testosterone levels, decreased differentiation from spermatogonia to sperm cells, and increased apoptosis of germ cells. On the other hand, the addition of olive oil to the diet recovered the semen quality and sperm function dismissed by hypercholesterolemic diet in rabbits Table 1) (30–32).

Plants-based diets are alternative and sustainable approaches managing male infertility. Active principles and crude extracts of medicinal plants are used because of their antioxidant, anti-inflammatory, and positive effects on the testis. They have bioactive compounds such as polyphenols (anthocyanins, proanthocyanidins), phyto-oestrogens, diosgenin and thymoquinone (56). For instance, *Lepidium meyenii* (Maca), administrated to healthy men at 1.75 g/day for 3 months, increases sperm count and motility (33). Furthermore, *Acanthopanacis senticosi* activates sperm motility when administrated in humans (34). Moreover, *Sesamum indicum*, administrated at 0.5 mg/kg during 3 months, improves sperm count, motility and normal morphology (35, 36). In addition, roots of *Withania somnifera* administrated 5 g/day during 3 months improve energy metabolism and quality of semen and reproductive hormone levels in infertile men (37). *Zingiber officinale* (Ginger) powder or root are used because of their antioxidant, anti-inflammatory, anti-tumorigenic and androgenic activity. In fact, ginger contains gingerdiol, gingerol, shogaols, zingerone, zingibrene, folic acid, sesquiterpenes and vitamin C (57).

Morinda officinalis-Lycium barbarum coupled herbs are traditional Chinese medicines that reduce oxidative stress and apoptosis, thereby improving male fertility. These herbs contain ohioensin-A, quercetin and sitosterol that target androgen and estrogen receptors, MAPK, PI3K/Akt and glyceraldehyde-3-phosphate dehydrogenase (38). *Camellia sinensis* (green tea) leaves contain flavonol and epigallocatechin gallate and increase sperm concentration and viability when administrated for 52 days in rats (39, 57). Microalgae such as *Chlorella vulgaris* and *Spirulina platensis* improve spermatogenesis and steroidogenesis and protect against oxidative stress in rats (40–45). Algae such as *Halopteris scorapia* and *Laminaria japonica* increase sperm count, motility and testosterone levels, meanwhile decreasing sperm abnormalities, inflammation and oxidative stress, in mice and rats, respectively (46, 47, 57).

Vitis vinifera (grape) contains resveratrol and flavonoids (catechin, quercetin, anthocyanin and pro-anthocyanidins) with protective effects on testicles. In this sense, grape seed extracts reduce oxidative stress and apoptosis, meanwhile improve testicular histology, hormone levels, and sperm count and morphology (57). In mice, resveratrol reduces the toxic effects of cadmium and lead at the testicular level. Moreover, resveratrol prevents the development of testicular germ cell neoplasia *in situ* promoted by heavy metals. In this sense, resveratrol improves sperm parameters, redox balance, testicular histology, and reduces signaling pathways such as Akt (48). Moreover, resveratrol supplementation in a cryopreservation medium improves the post-thawed sperm quality and fertility of roosters. Indeed, rooster semen cryopreserved with 40 μM resveratrol-loaded nanostructured lipid carriers has higher motility, viability, membrane function, mitochondrial activity, antioxidant capacity and lower apoptosis than non-treated frozen sperm (49). In human cryopreserved semen samples, 15 μM resveratrol decreases DNA fragmentation mean increases markers of male fertility (protamine 1 and 2) and pregnancy success (adducin 1 alpha) by activating 5' AMP-activated protein kinase (50).

Antioxidants lead the list of natural products that are protective agents for male infertility. In this regard, oxidative stress damages sperm membranes and DNA, thereby promoting infertility. Curcumin is a bioactive compound present in the turmeric plant *Curcuma longa* that reduce oxidative stress, lipid peroxidation and oxidative DNA damage. Curcumin increases the levels of GSH, glutathione peroxidase, superoxide dismutase and catalase. In addition, curcumin increases testosterone, FSH and LH levels in mice (51–53). Ellagic acid is a polyphenol with similar effects to curcumin. Vitamin C protects spermatogenesis, prevents sperm agglutination, and increases testosterone, FSH and LH levels. Moreover, vitamin C induces antioxidant enzymes, and reduces LDL cholesterol and lipid peroxidation. Vitamin E is an antioxidant that protects cell membrane components from oxidative damage. At reproductive level, vitamin E also protects spermatogenesis and testosterone levels (57).

3.3 Bioactive compounds as potential alternative treatments preventing cancer therapy-induced male infertility

Because of their availability, safety and low cost, bioactive compounds contained in fruits, vegetables and spices are potential agents for the prevention and treatment of cancer. Even though they have limitations (low bioavailability, high metabolism, poor water solubility), these molecules have antitumorigenic effects against a wide range of cancers: colon, lung, prostate, breast, gastric, liver, and brain cancer (58). Moreover, contrary to current cancer treatments, bioactive compounds are potential chemo-preventive and -therapeutic agents with low side effects on the health of patients (59, 60).

Bioactive compounds target cancer cells, macrophages and adipocytes in the tumor microenvironment, thereby preventing cancer development and progression (61). In this way, curcumin, myricetin, geraniin, genistein, tocotrienol, fucoxanthin, anthocyanin, epigallocatechin gallate, gallic and ellagic acids have anti-proliferative, pro-apoptotic and anti-metastatic effects. For instance, curcumin, in nanoparticles, piperine, phospholipid

complexes and liposomes, inhibits PI3K/Akt and NF-κB pathways, but upregulates p53 and Bax expression, thereby promoting apoptosis of cancer cells. In addition, curcumin downregulates MMP-9 expression and reduce the metastatic potential of cancer cells (59). Furthermore, *Tripterygium wilfordii* used in the Chinese medicine contains bioactive compounds such as triptolide, celastrol and tripchlorolide that also inhibit PI3K/Akt and NF-κB pathways thereby exerting anticancer and anti-inflammatory effects (62).

As previously mentioned, several plant-derived bioactive compounds are effective in improving male infertility. In addition, these compounds have been shown to reverse cancer-induced infertility. Table 2 summarizes the sources and bioactive compounds known to prevent cancer-associated male infertility by controlling the detrimental effects of cancer therapy.

For instance, ginger and algae extracts have beneficial reproductive effects post cancer-therapy (57). Rats treated with a single intraperitoneal (IP) injection of 5mg/kg busulfan (chronic myelogenous leukemia treatment) and with 100-150 mg/kg ginger extract for 48 days have increased volume of seminiferous tubules, sperm count and testosterone levels, previously impair by busulfan (Table 2) (63). Another group demonstrated that oral administration of 300-600 mg/kg ginger extract recovers the epithelium thickness and germ cell count of rat testis affected after a single IP dose of 100 mg/kg cyclophosphamide treatment by increasing antioxidant and testosterone serum levels (64). In addition, oral administration of fucoxanthin-rich brown algae *Sargassum glaucescens* extract ameliorates cisplatin-induced testicular damage in hamsters. Hamsters were intraperitoneal injected with 7 mg/kg cisplatin and treated with 100, 200 and 500 mg/kg fucoxanthin extract orally administrated. In fact, fucoxanthin-enriched extract recovers the testosterone level, seminiferous tubules morphology and sperm number, motility and morphology affected by cisplatin treatment (65).

Additionally, several bioactive compounds such as curcumin, ellagic acid and vitamin E are involved in reverting cancer-induced male infertility (57). For instance, rats treated with a single IP dose of 5 mg/kg cisplatin and 100 mg/kg/day curcumin during 7 days reverse testicular damage by reducing NF-κB and caspase-3 activated by cisplatin (66). Rats treated with a single IP dose of 7 mg/kg cisplatin and orally administrated 200 mg/kg/day curcumin for 10 days recover oxidative stress and testicular damage induced by cisplatin via mitogen-activated protein kinase and nuclear factor-kappa B signaling pathways (67). Mice treated with 4 mg/kg curcumin nanocrystals recover the negative effects of cyclophosphamide in sperm activity, sperm chromatin condensation, and seminiferous tubule architecture, meanwhile have a reduction in germ cells apoptosis induced by the treatment with 200 mg/kg cyclophosphamide (IP) (68). Mice with colon cancer treated with 5 mg/kg cisplatin (IP) have a reduction in sperm count which is recovered after 5 weeks of treatment with 10 mg/kg ellagic acid (oral) (69). Rats receiving 250 mg/kg vitamin E with 500 mg/kg L-carnitine control the oxidative stress induced by the treatment with 20 mg/kg methotrexate (IP) via reducing malondialdehyde and

TABLE 2 Bioactive compounds preventing cancer therapy-induced male infertility.

Source or Bioactive compound	Model	Chemotherapy	Dose	Effect	Reference
Ginger extract	Rat	5 mg/kg busulfan, IP	100-150 mg/kg for 48 days	↑ volume of seminiferous tubules ↑ sperm count ↑ testosterone levels	(63)
Ginger extract	Rat	100 mg/kg cyclophosphamide, IP	300-600 mg/kg, oral	↑ antioxidant and testosterone serum levels	(64)
Fucoxanthin-rich extract obtained from *Sargassum glaucescens*	Hamster	7 mg/kg cisplatin, IP	100-500 mg/kg, oral	↑ antioxidant enzymes ↑ testosterone serum levels ↑ sperm count and motility ↓ sperm abnormality	(65)
Curcumin	Rat	5 mg/kg cisplatin, IP	100 mg/kg/day for 7 days	↓ NF-κB and caspase-3 activation	(66)
Curcumin	Rat	7 mg/kg cisplatin, IP	200 mg/kg/day for 7 days	↓ MAPK and NF-κB	(67)
Curcumin nanocrystals	Mouse	200 mg/kg cyclophosphamide, IP	4 mg/kg	↑ sperm activity ↑ sperm chromatin condensation ↑ seminiferous tubule architecture ↓ germ cells apoptosis	(68)
Ellagic acid	Mouse	5 mg/kg cisplatin, IP	10 mg/kg for 5 weeks, oral	↑ sperm count	(69)
Vitamin E and L-carnitine	Rat	20 mg/kg methotrexate, IP	250 mg/kg vitamin E with 500 mg/kg L-carnitine	↓ malondialdehyde ↑ superoxide dismutase	(70)
Resveratrol	Rabbit	5 mg/kg paclitaxel, IV	4 mg/kg for 8 weeks, IV	↓ DNA fragmentation and abnormal DNA integrity in epididymal sperms	(71)
Resveratrol	Mouse	30 mg/kg busulfan, gavage	100 mg/kg/day for 2 weeks	↑ proliferation of germ cells	(72)

IP, Intraperitoneal; IV, Intravenous.

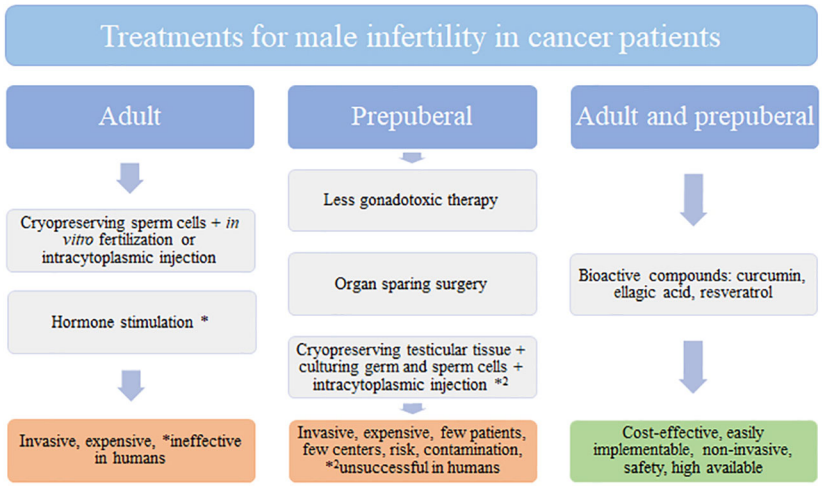

FIGURE 1
Treatments for male infertility in cancer patients.

increasing superoxide dismutase (70). In rabbits, the treatment with 4 mg/kg resveratrol (intravenous) for 8 weeks ameliorates the DNA fragmentation and abnormal DNA integrity in epididymal sperms induced by 5 mg/kg paclitaxel (71). In addition, 20 μM resveratrol induces the proliferation of spermatogonia stem cells *in vitro*. In mice, 100 mg/kg/day resveratrol for 2 weeks promotes the proliferation of germ cells thereby reversing the loss of spermatogenic cells in the testis and sperm cells in the epididymis induced by 30 mg/kg busulfan treatment (gavage) (72).

4 Conclusion

Cancer incidence is increasing worldwide and men are more affected than women. Current cancer treatments involve surgery, chemotherapy and radiation, which have side-effects such as infertility. Moreover, male infertility is responsible for 50% of couples with fertility problems. Adult cancer patients are subjected to invasive and expensive techniques to recover fertility after cancer treatment such as cryopreservation of sperm cells and *in vitro* fertilization (Figure 1). However, prepuberal patients without sperm cells are unable to access to these techniques. In this age group, cryopreserving testicular tissue to culture germ and sperm cells and do intracytoplasmic injection seems to be another invasive, expensive technique that is unsuccessful in humans. In this way, looking for alternative treatments for cancer and male infertility is a need to alleviate patient suffering post-cancer survival. Natural products rich in bioactive compounds are increasing interest in this scenario as alternative, novel, non-invasive agents to prevent and treat cancer preserving male fertility. Curcumin, ellagic acid, and resveratrol seem to be potential compounds that recover testicular function by increasing proliferation, reducing oxidative stress, inflammation, and apoptosis of germ cells. However, further research regarding bioavailability, solubility and metabolism of these natural compounds must be taken in consideration to improve the current therapy approaches.

Author contributions

LS: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. MM-C: Funding acquisition, Writing – review & editing.

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Conflict of interest

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

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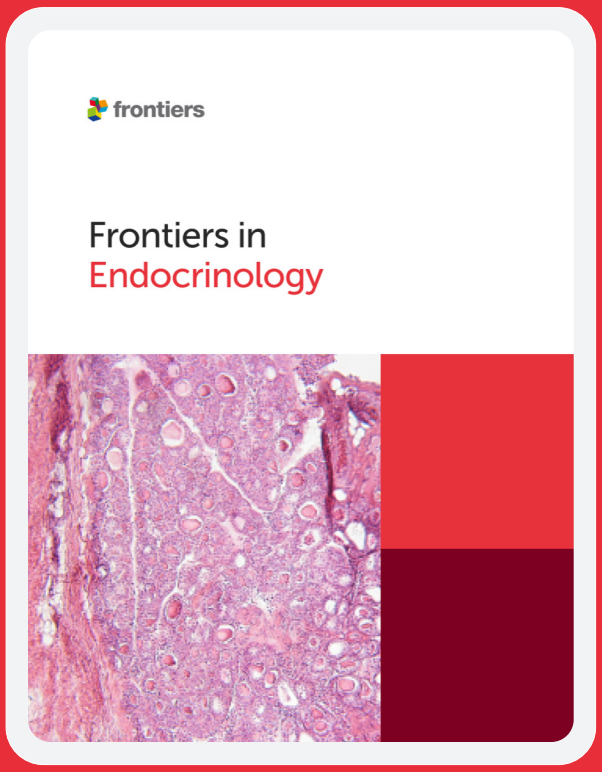

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