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The moderation effect of *GSTM1*/*GSTT1* gene polymorphisms on the association of sperm mitochondrial DNA copy number and sperm mobility

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Oxidative stress (OS) is believed to be a significant factor in the decline of semen quality, with mitochondrial DNA copy number (mtDNAcn) serving as a sensitive biomarker for both semen quality and mitochondrial dysfunction resulting from oxidative stress. While glutathione S-transferases (GSTs) are commonly known as 'antioxidant' enzymes, there is ongoing debate regarding the relationship between *GST* genotypes and semen quality. In a study involving 568 male volunteers from the outpatient department of Puyang Reproductive Medicine Center, sperm mtDNAcn, semen quality, and *GSTM1*/*GSTT1* genotypes were analyzed to investigate the potential link between *GSTM1*/*GSTT1* gene variations and semen quality, as well as the impact of *GSTs* gene variations on the connection between sperm mtDNAcn and semen quality. Adjusting for variables such as age, BMI, smoking, and alcohol consumption, it was found that mtDNAcn was significantly correlated with decreased sperm concentration and total sperm count ($b = -0.109, -0.128$, respectively; $P = 0.002, 0.001$, respectively). *GSTM1* was associated with progressive motility (OR 0.390, 95% CI 0.218, 0.697), Straight line velocity (VSL) (OR = 0.606, 95% CI 0.385, 0.953), and Straightness (STR) (OR 0.604, 95% CI 0.367, 0.994), while *GSTT1* was linked to progressive motility (OR 0.554, 95% CI 0.324, 0.944) and Beat crossover frequency (OR 0.624, 95% CI 0.397, 0.982). The *GSTT1* was found to moderate the relationship between mtDNAcn and sperm motility parameters linearity (LIN), STR, and Wobble (WOB), with additive interaction effects observed between *GSTT1* and mtDNAcn on LIN, STR, and WOB (P for interaction = 0.008, 0.034, 0.010, respectively). Overall, this study suggests that *GSTT1* and *GSTM1* gene variations may play a role in sperm motility, with *GSTT1* potentially influencing the impact of oxidative stress on sperm motility.

Keywords Semen quality, Sperm mitochondrial DNA copy number, *GSTM1*, *GSTT1*, Moderation, Interaction

The World Health Organization (WHO) recognizes that infertility is a disease that is increasingly becoming a global public health issue. Infertility affects about 8–12% of reproductive-aged couples worldwide[1–3]. Evidence suggests a decline in semen quality among men globally in recent decades[4,5], with the male component contributing 50% to infertility cases[6]. While the precise reasons for this decline are not fully understood, various environmental pollutants have been implicated. Exposure to pollutants like petroleum products, agrochemicals,

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industrial chemicals, and heavy metals, particularly lead (Pb) and cadmium (Cd), may negatively impact the male reproductive system.

Oxidative stress (OS) refers to the imbalance between pro-oxidation and antioxidant states, which is linked to apoptosis and germ cell apoptosis[7]. It is known to play a role in the negative impact of various environmental pollutants on semen quality. Mitochondrial DNA, lacking histones and DNA repair capabilities found in nuclear DNA, is particularly vulnerable to oxidative stress[8]. Mitochondrial DNA copy number (mtDNAcn) serves as a valuable indicator of mitochondrial genome abundance[9] and has been recognized as a sensitive biomarker for semen quality and mitochondrial dysfunction caused by oxidative stress[10–12]. Following acute chemical exposure and oxidative stress, mtDNAcn tends to increase as a compensatory mechanism due to reduced energy production. However, prolonged oxidative stress can eventually overwhelm mitochondria, leading to mitochondrial dysfunction and a decline in mtDNAcn levels[13]. During epididymal transport, sperm undergo a series of biochemical and morphological changes, gradually maturing in the process. As sperm mature, they lose most of their cytoplasmic components and become more vulnerable to oxidative stress, which can damage sperm structure and function. An optimal level of reactive oxygen species (ROS) not only protects against oxidative damage but also aids in sperm capacitation[14,15].

Glutathione S-transferases (GSTs) are a group of enzymes that are widely present and have multiple functions. They are crucial for cellular detoxification in phase II reactions and for defending against oxidative stress, often referred to as 'antioxidant' enzymes[16,17]. Some GST enzymes are produced by genes with known functional variations. *GSTM1* (encoding GST mu1) and *GSTT1* (encoding GST theta1) are important in detoxifying both internal and external toxins. Deletions in *GSTM1* and *GSTT1* are quite common in human populations[18]. Variations in genes that encode enzymes involved in drug metabolism and detoxification in phase II can impact their activity, potentially affecting how the male reproductive system processes harmful substances and influencing susceptibility to conditions like infertility[19–21]. In a previous study, we observed that *GSTM1/GSTT1* gene variations moderated the relationship between metals and semen quality in male volunteers at the Puyang Reproductive Medicine Center outpatient department. Individuals with *GSTT1+* and *GSTM1+* genotypes were found to have a protective effect against the harmful impacts of heavy metal exposure[18].

While some studies have linked mtDNAcn and *GSTs* gene polymorphisms to decreased semen quality, there are inconsistencies in the results. Previous research has often focused on the impacts of mtDNAcn or *GSTs* genotypes individually, without considering potential interactions. Given that *GSTs* possess antioxidant properties that protect against oxidative stress, it is hypothesized that *GSTs* gene polymorphisms may influence the association between sperm mtDNAcn and impaired semen quality. This study aims to investigate the relationship between sperm mtDNAcn and gene polymorphisms of *GSTT1* and *GSTM1* in adult male volunteers recruited from the outpatient department of Puyang Reproductive Medicine Center. Additionally, the study will explore the role of gene polymorphisms in the relationship between mtDNAcn and semen quality.

Materials and methods
Study population and design
In our cross-sectional study, we recruited male partners in couples who attended the outpatient department of Puyang Reproductive Medicine Center in Henan, China for semen examination, which has been reported in our previous report[18]. The sample size calculation formula used is as follows:

$$n = \frac{(Z_{\alpha/2} + Z_{\beta})^2 \times 2\sigma^2}{\delta^2} \tag{1}$$

In Eq. (1), the variables have the following meanings.

$Z_{\alpha/2} = 1.96$ (significance level with α = 0.05, Two-tailed test); $Z_{\beta} = 1.28$ (power of test 1 − β = 0.9, One-tailed test); σ = The estimate of the standard deviation of the two populations is based on findings from published literature[22] and the sample collection experience, which is 15 in this case. δ = The mean difference of 4.5 between the two groups is based on findings from published literature and sample collection experience. $n_1 = n_2 = 234$. In order to ensure sufficient sample size, the sample size was expanded by 20% on this basis, and N was chosen as 568.

Following the exclusion criteria, 568 participants were ultimately included in the final analytic sample. Upon signing the informed consent form, volunteers completed a comprehensive social-body-behavioral questionnaire covering basic demographics, disease history, lifestyle, and other relevant factors. Height, weight, and various indicators were measured, and body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Semen samples were collected for semen parameter and genetic polymorphism detection, with storage at − 80 °C designated for research purposes only, not for conception.

Including and excluding criteria
The same inclusion and exclusion criteria were applied as described in our previous study. The inclusion criteria required participants to abstain from sexual activity for 2–7 days and be at least 18 years old, as well as accept the questionnaire and provide informed consent. Additionally, subjects were excluded if they had a history of testicular injury, epididymitis, or genitourinary inflammation diagnosed by a urologist, as well as if they had received treatment for varicocele, had a history of incomplete testicular dislocation, or exhibited certain physical abnormalities identified by the urologist during the physical examination stage, such as absence of pubic hair, breast abnormalities, or penile abnormalities. Individuals with azoospermia, mitochondrial loss, or mtDNAcn deletion were also excluded.

Semen collection and semen parameters measurement

Prior to semen collection, volunteers were instructed to abstain from sexual activity for a period of two to seven days. Each subject received individual guidance before entering the designated semen collection room. Semen samples were obtained through masturbation without the use of a condom in a secluded room adjacent to the semen analysis area. All procedures adhered to the guidelines outlined in the WHO laboratory manual for the examination and processing of human semen (Fifth Edition)[23]. The collected semen samples were placed in containers and incubated at 37 °C for liquefaction. Computer-assisted sperm analysis (CFT-9201, Jiangsu Ruiqi Life Science Co., LTD, Jiangsu, China) was utilized for the evaluation of semen parameters, including volume, sperm concentration, total sperm count, progressive motility, as well as various sperm motility parameters such as curve line velocity (VCL, μm/s), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), wobble (WOB), the head of lateral movement (ALH, μm), linearity (LIN, %), the average degree of movement (MAD, °), straightness (STR, %), beat crossover frequency (BCF, Hz). Sperm morphology assessment was conducted using a microscope, which included evaluation of normal morphology rate, teratozoospermia index, sperm deformity index, and normal acrosome.

mtDNAcn analysis

Genomic DNA was isolated from the semen sample for the mtDNAcn assay. For each semen sample, centrifuged at 3500 r/min for 5 min, and the upper spermatoplasm was discarded. DNA was isolated from the remaining sperm cells using a DNA isolation kit (Life Feng Biotechnology Co, Shanghai) following the manufacturer's instructions. The method for measuring the relative mtDNAcn was consistent with that described by Gong et al.[24]. Ct values of the mitochondrial gene mtND1 and the reference gene human β-globulin (HBG) were measured using the QuantiNAVA STBR Green PCR Kit. The relative mtDNAcn was calculated through quantitative real-time polymerase chain reaction (PCR) based on the copy number of mitochondrial gene mtND1 and the copy number of the nuclear gene HBG. The primer sequences used were as follows:

mtND1:forward5′-CACCCAAGAACAGGGTTTGT-3′, reverse5′-TGGCCATGGGTATGTTGTTA-3′;
HBG:forward5′-GCTTCTGACACAGTGTTCACTAGC-3′,
reverse5′-CACCAACTTCATCCACGTTCACC-3′.

The reaction procedure consisted of an initial activation of DNA polymerase at 95 °C for 3 min, followed by 35 cycles. Each cycle included denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 60 s. A standard curve with 5 data points (1.25, 2.50, 5.00, 10.00, and 20.00 ng/μL) was generated for each plate, and the amplification efficiency was calculated, which ranged from 90 to 110% for each plate. Three replicates were measured in each sample. The calculation of relative mtDNAcn was as follows:

$$\text{The relative mtDNAcn} = 2^{Ct_{HBG} - Ct_{mtND1}}$$

GSTT1/GSTM1 polymorphism detection

Genomic DNA was utilized for analyzing GSTT1/GSTM1 polymorphisms following a previously reported method[25]. DNA was extracted from peripheral blood using a DNA isolation kit (Life Feng Biotechnology Co, Shanghai) as per the manufacturer's instructions, and stored at − 80℃. The polymorphisms of GSTM1 and GSTT1 were examined through restriction fragment length polymorphism (RFLP) analysis. For each genotype RFLP method, several positive and negative samples were selected randomly to do DNA sequencing to verify the accuracy of the method, and about 20% of samples were repeated secondly to prove the repeatability. Multiplex polymerase chain reaction was employed to determine GSTT1, GSTM1, and ALB (albumin) levels, with the following primer sequences:

Gene	Primer sequence (5′–3′)
GSTT1	Forward: TTCCTTACTGGTCCTCACATCTC
	Reverse: TCACCGGATCATGGCCAGCA
GSTM1	Forward: GAACTCCCTGAAAAGCTAAAGC
	Reverse: GTTGGGCTCAAATATACGGTGG
ALB	Forward: GCCCTCTGCTAACAAGTCCTAC
	Reverse: GCCCTAAAAAGAAAATCGCCAATC

Statistical analysis

All analyses were conducted using SPSS 22.0 and R 4.3.0 (R Development Core Team, Vienna, Austria). The total sperm count, sperm concentration, normal morphology rate, and mitochondrial DNA copy number were transformed using the natural-log(measure) (ln) method to achieve a normal distribution approximation. Semen quality parameters and mtDNAcn for the study population were described using data prior to transformation and were presented as mean ± SD or M (P_{25}, P_{75}). Descriptive analysis was utilized to summarize characteristics with counts or proportions for variables such as smoking status (smokers and ever smokers, non-smokers), alcohol usage (users, non-users), and GST genotypes (−, +).

In the association analysis, the multivariate linear regression was utilized initially to investigate the association of semen parameters as continuous variables with mtDNAcn, GSTM1/GSTT1 genotypes. Additionally, Independent sample t-tests and ANOVA tests were employed to compare semen quality parameter levels among

different genotype populations. To ensure the reliability of our results, sensitivity analyses were conducted. Firstly, mtDNAcn levels were categorized into quartiles based on the overall study population's distribution to examine the relationships. Secondly, associations were explored when each semen quality parameter was dichotomously classified as 'normal' or 'abnormal' according to WHO guidelines, which define normal as semen volume ≥ 1.5 ml, sperm concentration $\geq 15 \times 10^6$/ml, total sperm number $\geq 39 \times 10^6$, normal morphology rate $\geq 4\%$, and progressive motility $\geq 32\%$, respectively. For semen parameters without established standards, the P_{25} was used as a classification criterion in this study. A multivariate logistic model was employed to assess the association between semen parameters as dichotomous variables and mtDNAcn, *GST* genotypes adjusting for age (continuous), body mass index (BMI; continuous), smoking (categorical), and drinking status (categorical). The regression results were presented as effect estimates and *95%* confidence intervals (*CI*).

SPSS PROCESS[26] was utilized to investigate the moderation effect of *GSTs* gene polymorphisms on the relationships between mtDNAcn and semen quality. The variables were centered to ensure that the coefficients for the product of the two variables were interpretable within the data range. To further explore the impact of gene polymorphisms on the connection between sperm mtDNAcn and semen quality, we conducted a stratified analysis based on *GSTM1* and *GSTT1* genotypes. The correlation between semen parameters and mtDNAcn in each stratum was examined. When assessing biological interaction, emphasis was placed on additive interaction rather than multiplicative interaction. In linear regression analysis, the regression coefficient of the product term indicated whether there was additive interaction or departure from additivity. Therefore, interaction terms of the stratified variables and the independent variable were included in a linear regression model, and the coefficient of the interaction term was directly assessed to determine if there was an interaction effect between *GSTM1*/*GSTT1* gene polymorphisms and mtDNAcn on semen parameters using R. The statistical significance level was set at $P < 0.05$ (two-tailed).

Ethical approval
This study was approved by the Ethics Committee of Xinxiang Medical University (December 15, 2017) (Ethical review approval document No: XYLL-20170311). The research content and process of this project adhere to the ethical requirements for biomedical research set forth by international and national governments. All methods were conducted in alignment with the applicable guidelines and regulations, and informed consent was obtained from all participants and/or their legal guardians.

Consent for publication
Informed consent was obtained from all the participants who answered the questionnaire and provided samples.

Results
Participant characteristics and semen parameters
A total of 568 male volunteers were recruited for the study. Table 1 displays the characteristics, semen parameters, *GSTT1* and *GSTM1* genotype distribution, as well as other basic information of the participants. The average age of the participants was 29.61 years, with an average BMI of 24.74 kg/m². Among the participants, 50.30% were *GSTT1+*, 45.70% were *GSTM1+*, 49.90% were *GSTT1+/GSTM1+*, and 23.00% were both *GSTT1+* and *GSTM1+*. Furthermore, 49.80% of the participants were smokers and 39.50% were drinkers. The median values for sperm concentration, total sperm number, and mtDNAcn were 58.20 million/mL, 202.90 million, and 91.77 copies, respectively. The mean levels for various semen parameters were as follows: VCL 57.78 μm/s, LIN 65.71%, VSL 37.48 μm/s, STR 68.56%, VAP 49.34 μm/s, WOB 80.04%, ALH 2.67 μm, BCF 5.65 Hz, and MAD 38.68°.

The association of semen quality with mtDNAcn, *GSTM1* and *GSTT1* gene polymorphisms
Relationship between mtDNAcn and semen parameters
Sperm mitochondrial DNA copy number (mtDNAcn) was found to be inversely correlated with semen quality even after adjusting for age, BMI, smoking, and alcohol consumption. Specifically, the study revealed a significant negative association between mtDNAcn and sperm concentration ($b = -0.109$, $P = 0.002$) as well as total sperm count ($b = -0.128$, $P = 0.001$) (Fig. 1). Supplementary Table S1 further supported these findings by showing a negative correlation between mtDNAcn levels and sperm concentration ($P = 0.004$) as well as total sperm count ($P = 0.004$). Additionally, an increase in mtDNAcn was associated with a 0.076 (95% CI 0.000, 0.153) reduction in BCF.

Relationship between GSTM1 and GSTT1 gene polymorphisms and semen parameters
The study found that the levels of STR ($P = 0.034$) and ALH ($P = 0.042$) were significantly higher in the *GSTM1+* genotype group compared to the *GSTM1−* genotype group. Additionally, sperm concentration ($P = 0.047$), total sperm count ($P = 0.025$), progressive motility ($P = 0.020$), VCL ($P = 0.055$), VSL ($P = 0.049$), and BCF ($P = 0.008$) levels were all notably higher in the *GSTT1+* genotype group than in the *GSTT1−* genotype group, as illustrated in Fig. 2 and Supplementary Table S2.

The results presented in Fig. 3 indicate that *GSTM1* was significantly associated with progressive motility (OR 0.390, $P = 0.001$), sperm motility parameters VSL (OR 0.606, $P = 0.030$) and STR (OR 0.604, $P = 0.047$), while *GSTT1* showed a correlation with progressive motility (OR 0.554, $P = 0.030$) and BCF (OR 0.624, $P = 0.042$). Individuals with '*GSTM1+/GSTT1+*' had a reduced risk of abnormal progressive motility and VSL ($P = 0.022$, 0.005, respectively), as did those with '*GSTM1+* and *GSTT1+*' ($P = 0.001$, 0.009, respectively) compared to individuals with '*GSTM1−* and *GSTT1−*' (Fig. 3 and Supplementary Fig. S1).

Characteristics	Data
Age (years)[a]	29.61 ± 5.72
BMI (kg/m²)[a]	24.74 ± 3.91
Smoking status[b]	
Smokers and ever smokers	283 (49.80)
Non-smokers	285 (50.20)
Alcohol usage[b]	
Users	221 (39.50)
Non-users	339 (60.50)
GSTT1[b]	
+	249 (50.30)
−	246 (49.70)
GSTM1[b]	
+	226 (45.70)
−	269 (54.30)
GSTT1TM1[b]	
−	134 (27.10)
+−/−+	247 (49.90)
++	114 (23.00)
Semen parameters[a]	
Semen volume (mL)	3.63 ± 1.40
Sperm concentration (millions/mL)[c]	58.20 (34.00, 91.30)
Total sperm number (millions)[c]	202.90 (111.40, 312.90)
Progressive motility (%)	48.79 ± 17.77
Normal morphological (%)[c]	2 (1, 4)
TZI	1.84 ± 1.05
SDI	1.83 ± 1.42
Normal acrosome	32.92 ± 19.26
VCL (µm/s)	57.78 ± 10.71
LIN (%)	65.71 ± 7.57
VSL (µm/s)	37.48 ± 7.32
STR (%)	68.56 ± 9.91
VAP (µm/s)	49.34 ± 9.26
WOB (%)	80.04 ± 7.60
ALH (µm)	2.67 ± 0.60
BCF (Hz)	5.65 ± 0.98
MAD (°)	38.68 ± 5.84
mtDNAcn[c]	91.77 (22.05, 493.71)

Table 1. Characteristics, semen parameters, *GSTM1, GSTT1* and mtDNAcn in the study population (n = 568). BMI: body mass index, TZI: Teratozoospermia index, SDI: Sperm deformity index, VCL: curve line velocity, VSL: straight line velocity, VAP: velocity of average path, ALH: lateral head movement, MAD: average motion degree, LIN: linearity, STR: straightness, WOB: wobble and BCF: beat cross frequency. [a]The results are presented as the mean ± SD. [b]The results are presented as the No. (%). [c]The results are presented as the M (P$_{25}$, P$_{75}$).

Moderation effect of the *GSTM1* and *GSTT1* gene polymorphisms on association between mtDNAcn and semen quality

Supplementary Table S3 illustrates the moderating impact of *GSTT1* gene polymorphisms on the correlation between mtDNAcn and semen quality. Specifically, for the sperm motility parameter LIN, the regression coefficient of the product term of mtDNAcn and *GSTT1* (mtDNAcn*GSTT1) was 1.7738 (*P* = 0.0077), while the regression coefficient of mtDNAcn was −0.5755, showing opposite signs. *GSTT1* appears to have a negative regulatory effect. The change in R-square was 0.0167, indicating that the moderating effect contributes approximately 1.67%. Furthermore, *GSTT1* may also modulate the negative relationships between mtDNAcn and STR (*b* = 4.0424, *t* = 2.0450, *P* = 0.0415) and WOB (*b* = 4.0494, *t* = 2.6683, *P* = 0.0079). Additionally, individuals with *GSTM1*+/*GSTT1*+ genotypes may mitigate the adverse impact of mtDNAcn on LIN, STR, and WOB (*P* = 0.016, 0.026, 0.003, respectively) (Supplementary Table S4).
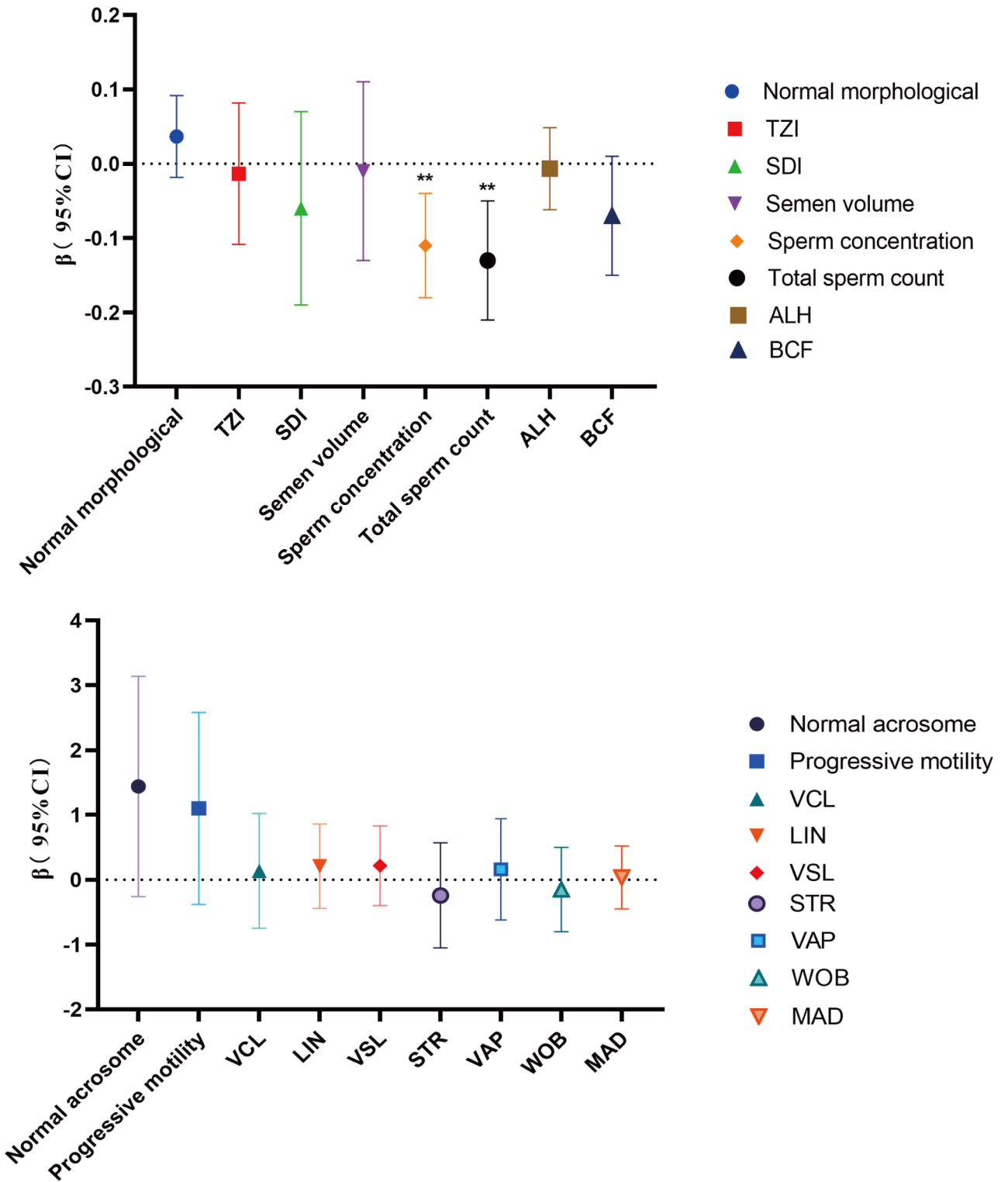
Fig. 1. The association between mtDNAcn and semen parameters in multiple linear model while adjusting for age (continuous), BMI (continuous), smoking (categorical) and drinking status (categorical). Sperm concentration, total sperm count, and normal morphological rate and mtDNAcn were ln-transformed. *$P < 0.05$ and **$P < 0.01$. TZI teratozoospermia index, SDI sperm deformity index, VCL curve line velocity, VSL straight line velocity, VAP velocity of average path, ALH lateral head movement, MAD average motion degree, LIN linearity, STR straightness, WOB wobble, BCF beat cross frequency.
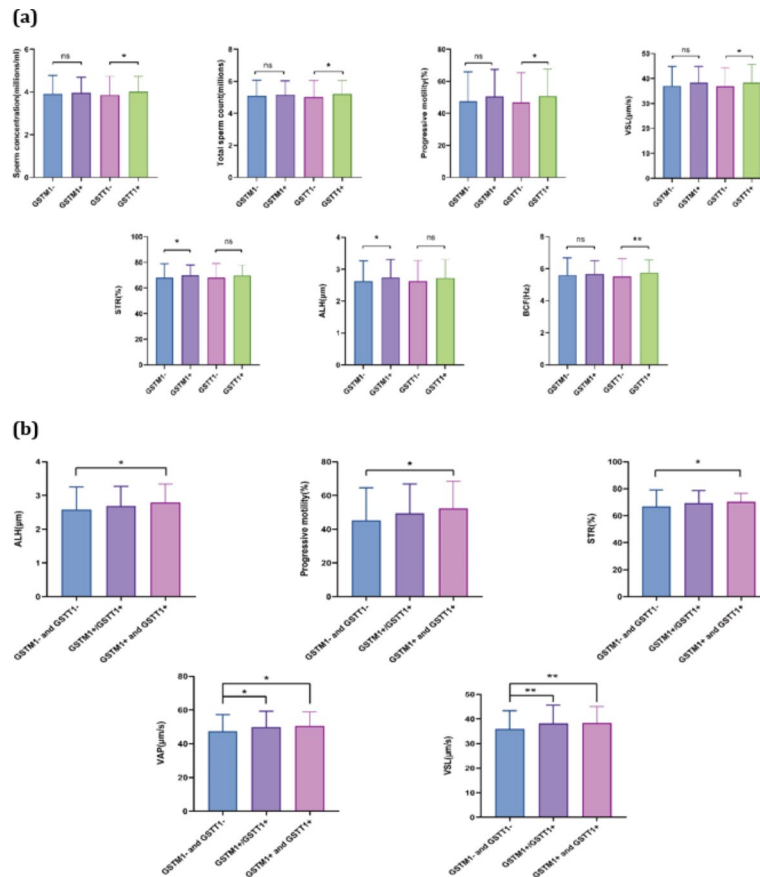
Fig. 2. (**a**) Semen quality parameters levels of the population with different genotypes (*GSTM1, GSTT1*) Independent sample t test was applied. *P < 0.05 and **P < 0.01. (**b**) Semen quality parameters levels of the population with different genotypes (*GSTT1TM1*). ANOVA test was applied. "*"indicates *P* < 0.05 and "**"indicates *P* < 0.01 gotten from LSD or Dunnett's T3 method.

Interaction effect of mtDNAcn and *GSTM1* and *GSTT1* gene polymorphisms on sperm mobility parameters

The results presented in Table 2 indicate a positive correlation between mtDNAcn and sperm mobility parameters LIN (β = 1.186, 95% CI 0.265, 2.107) in individuals with the *GSTT1*+ genotype. Additionally, an association was observed between mtDNAcn and an increase in LIN (β = 1.266, 95% CI 0.240, 2.293) and WOB (β = 1.013, 95% CI 0.001, 2.025) in subjects with the *GSTM1*+/*GSTT1*+ genotype. And a negative correlation between mtDNAcn and ALH (β = − 0.111, *P* = 0.042) and MAD (β = − 1.068, *P* = 0.036) in individuals with the *GSTT1*− *and GSTM1*− genotype was found. Further analysis showed that the association between mtDNAcn and LIN, when examined by quartiles of mtDNAcn level, was evident in men with *GSTT1*+ genotype (β = 1.230, 95% CI 0.384, 2.076), as well as with *GSTM1*+ and *GSTT1*+ (β = 1.146, 95% CI 0.269, 2.023) (Supplementary Table S5).

Moreover, significant interaction effects were observed between *GSTT1* gene polymorphisms and mtDNAcn on sperm motility parameters LIN (*P* for interaction = 0.008), STR (*P* for interaction = 0.034), and WOB (*P* for interaction = 0.010). However, there was limited evidence for interaction effects on other semen quality parameters as detailed in Table 3. The interaction effect results by mtDNAcn quartile grouping, as presented in Supplementary Table S6, further support the significant interaction between *GSTT1* and mtDNAcn on LIN (*P* for interaction = 0.014) and WOB (*P* for interaction = 0.040), consistent with the aforementioned findings.

Discussion

In this study, it was discovered that mtDNAcn was significantly inversely correlated with sperm concentration and total sperm count, even after adjusting for age, BMI, smoking, and alcohol consumption. Additionally, a positive correlation was observed between *GSTM1* and *GSTT1* gene polymorphisms and sperm mobility. Further analysis suggested that *GSTT1* gene polymorphisms may moderate the relationship between mtDNAcn and sperm LIN, STR, and WOB.

During sperm differentiation and maturation in the epididymis, plasma droplets gradually fall off, cytoplasmic content decreases, and the sperm head becomes predominantly occupied by DNA. This leads to an increase in polyunsaturated fatty acids (PUFA) in the sperm plasma membrane, making mature sperm more sensitive to ROS[27]. Exposure to oxidative stress impairs the functionality and vitality of human sperm, affecting the fertilization process. The mtDNAcn serves as an indicator of mitochondrial DNA quantity. In semen of
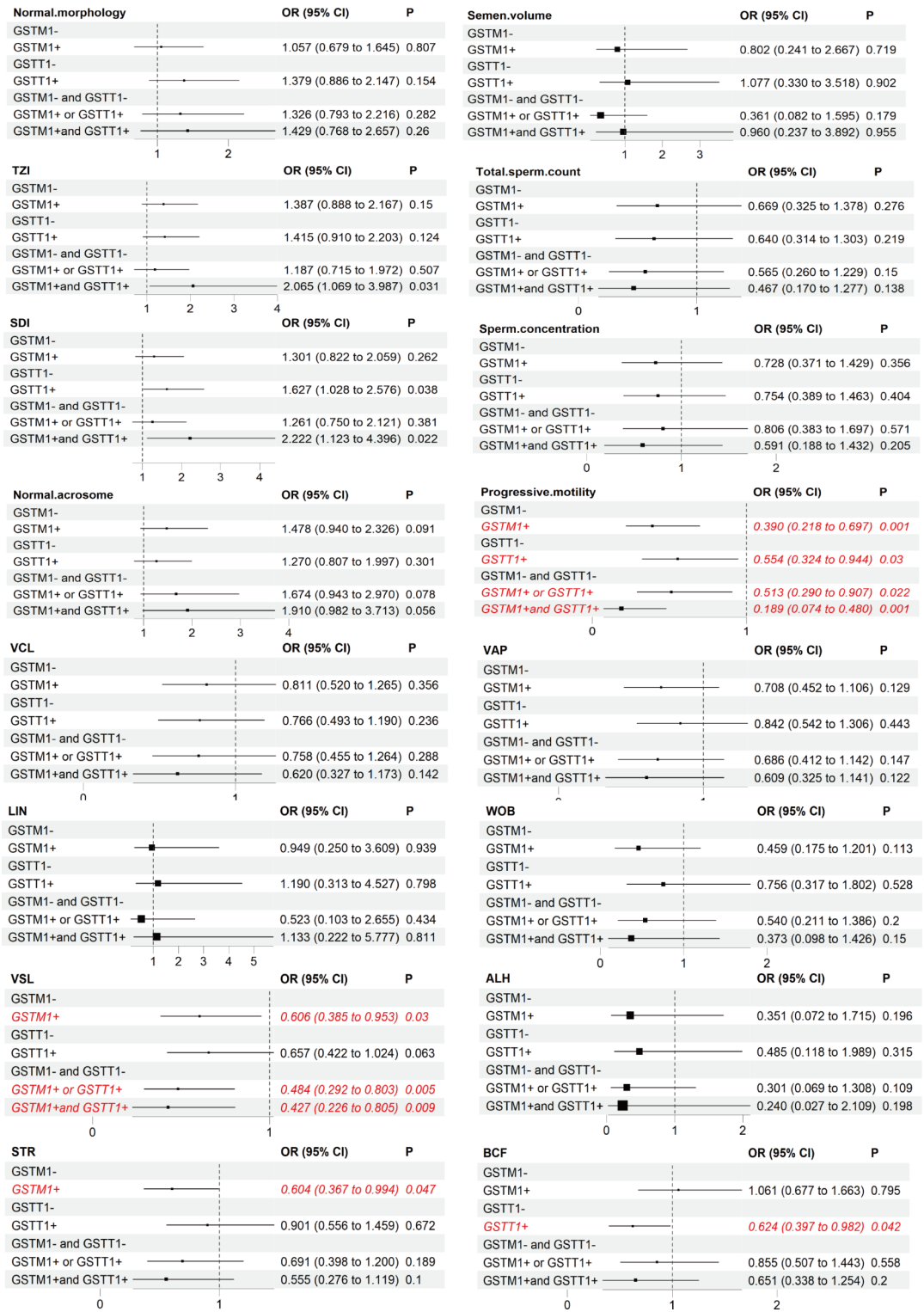
Fig. 3. The associations of *GSTT1*, *GSTM1* and *GSTT1TM1* gene polymorphisms with sperm parameters (binary categorical variable). The logistic regression model was applied adjusting for age (continuous), BMI (continuous), smoking (categorical) and drinking status (categorical). *GSTT1TM1* was a multicategory variable and the reference group was *GSTM1−* and *GSTT1−*.

poor quality, the increase in sperm mtDNAcn can be attributed to two main factors. Mild oxidative stress promotes mitochondrial biosynthesis and mtDNAcn[28]. However, prolonged exposure to excessive ROS impairs mitochondrial respiratory chain function, hindering ATP synthesis. As a compensatory mechanism, mtDNA copy number increases to address functional defects caused by damage, although this compensation may not

Variable	LIN	STR	WOB	ALH	MAD
mtDNAcn	0.210 (−0.437, 0.857)	−0.239 (−1.046, 0.568)	−0.152 (−0.804, 0.501)	−0.006 (−0.056, 0.045)	0.038 (−0.445, 0.521)
GSTM+ (N = 226)					
mtDNAcn[a]	0.241 (−0.545, 1.026)	0.804 (−0.198, 1.807)	0.284 (−0.254, 0.822)	0.042 (−0.035, 0.120)	0.652 (−0.058, 1.362)
GSTM− (N = 269)					
mtDNAcn[b]	0.394 (−0.640, 1.428)	−0.928 (−2.241, 0.385)	−0.255 (−1.369, 0.860)	−0.028 (−0.105, 0.049)	−0.572 (−1.292, 0.148)
GSTT+ (N = 249)					
mtDNAcn[c]	**1.186 (0.265, 2.107)***	0.696 (−0.363, 1.755)	0.842 (−0.037, 1.722)	0.053 (−0.022, 0.127)	−0.097 (−0.712, 0.518)
GSTT− (N = 246)					
mtDNAcn[d]	−0.591 (−1.547, 0.364)	−1.156 (−2.499, 0.188)	−0.918 (−1.892, 0.055)	−0.067 (−0.147, 0.014)	−0.079 (−0.894, 0.737)
GSTM− and *GSTT−* (N = 134)					
mtDNAcn[e]	−0.778 (−2.182, 0.627)	−1.773 (−3.693, 0.147)	−1.421 (−2.967, 0.125)	**−0.111 (−0.218, −0.004)***	**−1.068 (−2.064, −0.073)***
GSTM+/GSTT+ (N = 247)					
mtDNAcn[f]	**1.266 (0.240, 2.293)***	0.515 (−0.792, 1.821)	**1.013 (0.001, 2.025)***	0.066 (−0.018, 0.150)	0.731 (−0.121, 1.583)
GSTM+ and *GSTT+* (N = 114)					
mtDNAcn[g]	0.266 (−0.774, 1.305)	0.689 (−0.352, 1.730)	0.188 (−0.450, 0.826)	0.028 (−0.071, 0.126)	−0.079 (−0.725, 0.567)

Table 2. The association between mtDNAcn and sperm mobility parameters in different genotypes population. The association between mtDNAcn and sperm mobility parameters in different genotypes population. The multiple linear model was applied adjusting for age (continuous), BMI (continuous), smoking (categorical) and drinking status (categorical). Significant values are in [bold]. LIN: linearity, STR: straightness, WOB: wobble, ALH: lateral head movement and MAD: average motion degree. "*"indicates $P < 0.05$, "**"indicates $P < 0.01$. [a]When *GSTM+*. [b]When *GSTM−*. [c]When *GSTT+*. [d]When *GSTT−*. [e]When *GSTM−* and *GSTT−*. [f]When GSTM+/GSTT+. [g]When GSTM+ and GSTT+.

	GSTM1	GSTT1	GSTT1/TM1
Semen parameters	*P* for interaction	*P* for interaction	*P* for interaction
Normal morphology	0.750	0.438	0.508
TZI	0.835	0.840	0.686
SDI	0.444	0.504	0.261
Normal acrosome	0.158	0.275	0.855
Semen volume	0.121	0.592	0.478
Sperm concentration	0.792	0.565	0.601
Total sperm count	0.661	0.896	0.781
Progressive motility	0.681	0.333	0.564
VCL	0.722	0.138	0.335
LIN	0.661	**0.008****	0.205
VSL	0.999	0.106	0.433
STR	0.059	**0.034***	**0.018***
VAP	0.822	0.126	0.371
WOB	0.447	**0.010***	**0.045***
ALH	0.217	**0.039***	0.059
BCF	0.887	0.449	0.840
MAD	0.057	0.691	0.230

Table 3. Interaction effect between mtDNAcn and *GSTs* gene polymorphisms on semen parameters (*P* for interaction). The interaction terms between *GSTs* gene polymorphisms and mtDNAcn were constructed and incorporated into the regression model to directly obtain the effect value of the interaction terms $P < 0.05$ was considered statistically significant. Significant values are in [bold]. "*"indicates $P < 0.05$, "**"indicates $P < 0.01$.

fully restore mitochondrial function and normal spermatogenesis. Additionally, during normal spermatogenesis, sperm mtDNAcn decreases significantly to ensure low mtDNAcn levels at fertilization[29,30], as mitochondrial DNA is maternally inherited. Abnormal spermatogenesis or disrupted apoptosis processes may result in an elevated mtDNA copy number in human sperm[31]. Our findings support the notion that mtDNAcn negatively impacts sperm concentration and total sperm count.

GSTs, a supergene family of related isozymes, are known for detoxifying electrophiles by facilitating the binding of electrophilic groups from harmful substances to the sulfhydryl groups of reduced glutathione. This

process results in the formation of a more soluble, non-toxic derivative that is easily excreted from the body[32]. *GSTs* are typically composed of two subunits of 25–27 kDa that polymerize in a homologous or heterologous manner. Each subunit contains two ligand binding sites, including a glutathione (GSH)-specific binding site (G-site) located at the N-terminal end. A conserved serine/tyrosine (Ser/Tyr) residue at this site forms a hydrogen bond with the sulfhydryl group of GSH, leading to the ionization of GSH and the formation of a stable, highly reactive thiolate anion. Genetic variations in human *GST* can influence gene activity, with *GSTM1* and *GSTT1* potentially impacting an individual's susceptibility to OS damage[33]. Studies have shown that the *GSTT1-* genotype is associated with lower levels of *GSTT1* expression, which may increase vulnerability to oxidative DNA damage and excessive ROS[34]. Our results suggest a link between the *GSTT1* gene polymorphisms and improved sperm motility parameters, indicating that individuals with the null genotype may have reduced detoxification capacity and a higher risk of poorer semen quality.

The impact of *GSTM1* and *GSTT1* gene polymorphisms on the association between mtDNAcn and semen quality was investigated. It was observed that *GSTT1* acts as a moderator in the relationship between mtDNAcn and semen parameters LIN, STR, and WOB, mitigating the inverse correlation. Previous research indicated that individuals with the *GSTT1-* genotype exhibited higher levels of oxidative stress markers such as malondialdehyde (MDA) and nitric oxide (NO), suggesting a potential mechanism[35]. Males with the *GSTT1-* genotype may have increased mtDNAcn, with the *GSTT1* gene helping to maintain a balance between pro-oxidation and anti-oxidation states. Certain oxidative stress biomarkers could be influenced by a *GST* null genotype, leading to elevated ROS levels and reduced antioxidant capacity[36]. The absence of histones and DNA repair mechanisms in mitochondrial DNA makes it more vulnerable to oxidative stress, potentially resulting in mitochondrial dysfunction and decreased mtDNAcn. Significant DNA damage in sperm cells could accelerate germ cell death, lower sperm count, and impact semen quality. Our study highlighted the significant moderating effect of *GSTT1*, rather than *GSTM1*, on the relationship between mtDNAcn and sperm motility parameters, suggesting that *GSTT1* may play a more substantial role in mitigating the adverse impact of mtDNAcn on semen quality.

Sperm motility parameters were a key focus of this research, with nine parameters considered. This is in contrast to most studies which typically only report conventional semen parameters that indicate semen quality. In our analysis of moderating effects, we utilized the PROCESS method and ensured that variables were centered. Centering is essential in estimating a moderation model as it helps reduce multicollinearity between the product and its constituent terms, ensuring that coefficients remain interpretable within the range of the data. Furthermore, this study incorporated interaction terms of *GSTM1*/*GSTT1* gene polymorphism and mtDNAcn in a linear regression model to assess their combined effect on semen quality, providing valuable insights into biological interaction. However, it is important to acknowledge several limitations of this study. Firstly, the male volunteers were recruited from the Center for Reproductive Medicine, potentially introducing admission bias. Secondly, despite adjusting for some potential confounders, residual confounding from unmeasured covariates or unknown factors cannot be entirely ruled out. Lastly, although the primary focus was on oxidative stress, direct observation of parameters related to oxidative stress was not conducted.

Conclusions

Our results indicated a significant negative correlation between sperm mtDNAcn and semen parameters, suggesting that oxidative stress may have a detrimental impact on semen quality. Furthermore, *GST* gene polymorphisms were found to play a protective role against oxidative stress, potentially reducing the decline in semen quality. Specifically, *GSTT1* gene polymorphisms may moderate the relationship between oxidative stress and sperm motility parameters. Furthermore, studies on glutathione S-transferases gene polymorphisms and levels of oxidative stress should be done in a multicenter, multi-ethnic population in the future. And its biological significance still needs to be verified in future longitudinal studies.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

T.Z. and S.Z.: Writing—original draft, data curation, methodology, resources. C.Z., H.L., M.L., and G.Z.: Investigation, methodology, project administration, resources. G.D.: Supervision, validation, writing−review and editing. S.C.: Writing—review and editing. J.R.: Conceptualization, formal analysis, resources, supervision, writing—review and editing.

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