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Shenqi Qiangjing Granules Ameliorate Asthenozoospermia in Mice by Regulating Ferroptosis through the METTL3/GPX4 Signaling Axis

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Asthenozoospermia is a leading cause of male infertility, yet current pharmacotherapies yield suboptimal outcomes, underscoring the urgent need for novel treatment modalities. Herein, we induced asthenozoospermic mouse models using busulfan and investigated the therapeutic effects of Shenqi Qiangjing Granules (SQ) on testicular pathology, serum sex hormone and steroidogenic enzyme levels, and ferroptosis. Furthermore, utilizing GC-1 spg cell lines, we elucidated the role of the METTL3-mediated m^6A modification in GPX4 mRNA stability. Treatment with SQ or Fer-1 (an inhibitor of ferroptosis) significantly ameliorated testicular pathological injury, restored abnormal serum sex hormone levels, and enhanced testicular steroidogenic enzyme expression, highlighting the therapeutic potential of targeting ferroptosis in asthenozoospermia. In elucidating the molecular mechanism of METTL3 in ferroptosis, we found that METTL3 regulates GPX4 mRNA stability, subsequently impacting ferroptosis and sperm quality. Knockdown of METTL3 mimicked the effects of SQ treatment, while overexpression of METTL3 partially reversed SQ-mediated effects on ferroptosis and asthenozoospermia, underscoring the pivotal role of METTL3 in SQ therapy. In conclusion, the METTL3-GPX4-ferroptosis axis emerges as a novel regulatory pathway in the pathogenesis of asthenozoospermia. Targeting this axis, particularly through interventions such as SQ treatment, holds promise for the management of male infertility.

Keywords: male infertility; METTL3-GPX4-ferroptosis axis; traditional Chinese medicine
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Introduction

The incidence of infertility has risen significantly in recent years, with surveys showing that about 15% of couples are afflicted with infertility (Urdinguio et al. 2015). According to the guidelines established by the European Association of Urology, 20-30% of all infertile couples are exclusively caused by male infertility, while 50% of these patients have poor semen quality (Minhas et al. 2021). With further research, asthenozoospermia has gained increasing attention as a cause of male infertility and is defined as reduced or absent sperm motility (Tu et al. 2020). For asthenozoospermia, a series of hormone-based empirical drug treatments or direct assisted reproductive technol-

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ogy treatments are usually used in clinical practice, but most drugs for asthenozoospermia lack ideal therapeutic effects, so it is very important to develop new drug treatments (Shahrokhi et al. 2020).

According to traditional Chinese medicine (TCM), asthenozoospermia is mainly characterized by sperm deficiency, and is located in the liver and kidney. The treatment should be to tonify the kidney and benefit the essence, promote blood circulation and remove blood stasis, and remove dampness and heat (Zheng et al. 2020). TCM studies have found that Codonopsis pilosula, Astragalus membranaceus, and Epimedium have the function of enhancing renal function and improving sperm motility (Hu et al. 2013). We prepared 13 herbs, including Codonopsis pilosula, Lycium barbarum, Astragalus membranaceus, Angelica sinensis, Dipsacus, and Epimedium, into Shenqi Qiangjing Granules (SQ), which have the effects of tonifying the kidney and invigorating the spleen, activating blood circulation and nourishing blood, clearing away heat and dampness, and generating sperm and strengthening essence, and it may be useful in the treatment of asthenozoospermia. In the study of male infertility, observations of low levels of peroxidase suggest a potential link between iron and sperm health (Bell et al. 2013; Mirshahvaladi et al. 2023). Iron, as an essential element within cells, may induce oxidative stress reactions when present in excess, particularly by catalyzing the Fenton reaction to produce harmful hydroxyl radicals, thereby causing cellular and tissue damage (Almashhedy et al. 2023). As previous research has shown, peroxiredoxin 2 (PRDX2) plays a crucial role in this process. PRDX2, as an antioxidant protein, participates in regulating the redox balance within cells, thereby inhibiting the occurrence of oxidative stress. However, when sperm are subjected to oxidative stress, their vitality and health may be compromised (Xu et al. 2023). The study identifies ferroptosis as a specific cell death mechanism closely linked to sperm health and fertility (Zhao et al. 2020b). Moreover, recent research, as self-referenced, underscores the critical role of methyltransferase-like 3 (METTL3) in regulating glutathione peroxidase 4 (GPX4) expression and function within sperm, thus suggesting the involvement of the METTL3-GPX4-ferroptosis axis in sperm vitality decline (Song et al. 2023). Additionally, it is crucial to note that alongside ferroptosis, other cell death mechanisms like apoptosis and necrosis may also contribute to conditions such as oligoasthenoteratozoospermia (OAT), highlighting the need for further exploration beyond ferroptosis to comprehend the comprehensive regulatory mechanisms of sperm health (Su et al. 2022). The pathogenesis of asthenozoospermia is complex and has not yet been fully elucidated, but some related studies suggest that it may be the result of a combination of factors, such as low sperm motility, insufficient sperm energy metabolism, and abnormal signal transduction pathways (Shahrokhi et al. 2020; Jia et al. 2021). Ferroptosis is a newly defined programmed cell death pathway characterized by iron overload and lipid peroxidation. It has been shown that ferroptosis is closely associated with male infertility (Zeng et al. 2021). A recent study found that GPX4 and cystine/glutamate antiporter solute carrier family 7 member 11 (SLC7A11) expressions were downregulated and iron level was upregulated in patients with asthenozoospermia (Zhai et al. 2023), suggesting that sperm function impairment is associated with ferroptosis. This leads us to hypothesize that the mechanism of SQ in treating asthenozoospermia may be associated with regulating ferroptosis.

N^6-methyladenosine (m^6A) is the most ubiquitous internal RNA modification and regulates mRNA degradation, splicing, translation, and stability. Evidences suggest that m^6A regulatory molecules are expressed in virtually all types of testicular cells, including spermatogenic and somatic cells (Cai et al. 2021; Liu et al. 2021). Upregulation of m^6A methylation levels as well as METTL3 expression have been found in semen samples from asthenozoospermic patients (Yang et al. 2016). An additional study has shown that METTL3 regulates ferroptosis through induced GPX4 m^6A modification (Zhang et al. 2022), but the role of METTL3 in asthenozoospermia remains unclear.

Based on these findings, we proposed the hypothesis that SQ may improve asthenozoospermia by regulating METTL3/GPX4-mediated ferroptosis and performed the following experiments.

Materials and Methods

Animals and treatments

Male ICR mice (nine-week-old) were purchased from Beijing Vita River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mice were housed in separate cages for one week to adapt to the new environment, and were fed ad libitum with a national standard rodent diet with temperature regulated to 23-25°C and relative humidity to 40%-60%. Experiments (Supplementary Fig. S1) were performed in strict accordance with the animal care and use guidelines, and were approved by Guangxi University of Chinese Medicine Institutional Welfare and Ethical Committee (Approval No. DW20230615-035). Mice were first subjected to direct testicular injections of Busulfan, a chemotherapeutic agent, to induce asthenozoospermia, a condition characterized by reduced sperm motility (Qin et al. 2016b). Busulfan (4 mg/kg per side) was directly injected from the scrotum into testicular transverse diameter on both sides to induce asthenozoospermia in the model group (Qin et al. 2016a), and then they were further divided into asthenozoospermia group (n = 6), SQ group (n = 6) and ferrostatin-1 (Fer-1; ferroptosis inhibitor) group (n = 6). One week after busulfan injection, SQ group was given 800 mg/kg/d of SQ for intragastric administration, and Fer-1 group mice were given 1 mg/kg of Fer-1 intraperitoneally three times per week for 21 days. In addition, to investigate the role of METTL3, researchers prepared shRNA lentiviral plasmids targeting METTL3 (sh-METTL3; Shanghai Gene

Pharmaceutical Co., Ltd., China) and overexpression vectors for METTL3 (oe-METTL3). These lentiviral vectors (1×10^7 titers/0.05 mL) were separately injected into the tail vein of mice using microneedles before establishing the asthenozoospermia models. The control group mice were given an equal amount of saline. At the end of the experiments, serum and testicles were collected for further analysis. The body weights and testicular weights of the mice were also recorded. SQ was provided by The First Affiliated Hospital of Guangxi Traditional Chinese Medical University (China).

Histomorphological observation of testis

Histological sections of testis were taken and dewaxed routinely for hematoxylin and eosin (H&E) staining. Morphological and structural changes of testis were observed using Olympus light microscope (including quantization of seminiferous tubule diameter, lumen diameter, seminiferous epithelium height).

Epididymal sperm count and motility analysis

The epididymis was taken, the adherent blood vessels and other tissues were stripped off. When the sperm suspension was obtained from cauda epididymis, a haemocytometer was used for sperm counting. Two hundred sperm motility grades and counts were rapidly completed at room temperature (26℃) under a microscope to calculate sperm motility. Sperm were divided into grade A (rapid progressive motility), grade B (slow progressive motility); grade C (non-progressive motility), grade D (immobility), and sperm motility = (A + B + C)/200 × 100%.

Enzyme linked immunosorbent assay (ELISA)

For serum hormone of ICR mice measurements, follicle-stimulating hormone (FSH; E-EL-M0511c, Elabscience, Wuhan, China), luteinizing hormone (LH; E-EL-M3053, Elabscience), estradiol (E_2; E-OSEL-M0002, Elabscience) and testosterone (E-OSEL-M0003, Elabscience) levels were detected by ELISA kits according to the manufacturer's instructions.

Cell culture and transfection

The mouse spermatogonial cell line GC-1 spg cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified eagle's medium (DMEM; Hyclone, South Logan, UT, USA) with 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY, USA) and 1% penicillin-streptomycin (Hyclone). For cell transfection, plasmids were transfected into cells with Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) at a final concentration of 200 ng/mL following the manufacturer's protocols.

Quantitative real-time PCR (qPCR) assay

Total RNA was isolated from testicular tissues of ICR mice or GC-1 spg cells using Trizol reagent (Invitrogen), followed by reverse transcription into cDNA with a with PrimeScript RT Master Mix (TaKaRa, Tokyo, Japan). Then, gene expressions were measured by qPCR using SYBR Green PCR Master Mix (Beyotime, Shanghai, China). β-actin was used as an internal reference. The relative quantitative expression was determined using the $2^{-\Delta\Delta CT}$ method. Primer sequences were listed as follows: METTL3: 3′-CTG GGC ACT TGG ATT TAA GGA A-5′ (forward), 3′-GTA TCC CAT CCA GTT GGT TTC-5′ (reverse); GPX4: 3′-CCT CTG CTG CAA GAG CCT CCC-5′ (forward), 3′-CTT ATC CAG GCA GAC CAT GTG C-5′ (reverse); steroidogenic factor 1 (SF-1): 3′-GTG CAT GGT CTT TAA GGA GCT GG-5′ (forward), 3′-GGA TGC TGT CTT CCT TGC CGT A-5′ (reverse); steroidogenic acute regulatory protein (StAR): 3′- AAA GCC AGC AGG AGA ACG GGG A-5′ (forward), 3′-GCC TCC ATG CGG TCC ACA AGT T-5′ (reverse); P450 cholesterol side-chain cleavage enzyme (P450scc): 3′-TGC TCA ACC TGC CTC CAG ACT T-5′ (forward), 3′-ACT GGC TGA AGT CTC GCT TCT G-5′ (reverse); cytochrome P450 family 17 subfamily a member 1 (P450c17): 3′-AGC TCT GTG CTG AAC TGG ATC C-5′ (forward), 3′-AGA CGG TGT TCG ACT GAA GCC T-5′ (reverse); β-actin: 3′-CAT TGC TGA CAG GAT GCA GAA GG-5′ (forward), 3′-TGC TGG AAG GTG GAC AGT GAG G-5′ (reverse); METTL14: 3′-AGA GTG CGG ATA GCA TTG GTG C-5′ (forward), 3′-CTC CTT CAT CCA GAC ACT TCC G-5′ (reverse); fat mass and obesity-associated protein (FTO): 3′-GCC TCG GTT TAG TTC CAC TCA C-5′ (forward), 3′-GTC GCC ATC GTC TGA GTC ATT G-5′ (reverse); Wilms' tumor 1-associated protein (WTAP): 3′-AGT GCC TGG AAG TTT ACG CCT G-5′ (forward), 3′-GCT TCA AGC TGT GCA ATA CGG C-5′ (reverse); AlkB homolog 5 (ALKBH5): 3′-TCG GAA CCT GTG CTT TCT CTG C-5′ (forward), 3′-CTT CCT GAG AAT GAT GAC CGC C-5′ (reverse).

Western blot

Total proteins from ICR mouse testicular tissues or GC-1 spg cells were extracted using radio immunoprecipitation assay (RIPA) buffer (Beyotime) supplemented with protease and phosphatase inhibitors. Protein concentrations were determined using a bicinchoninic acid (BCA) kit (Beyotime) and were uniformly 2 μg/μL. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk for 1 h, membranes were incubated with the following antibodies: GPX4 (ab125066, 1:5,000, Abcam, Cambridge, MA, USA), METTL3 (ab195352, 1:1,000, Abcam), SF-1 (ab168380, 1:2,000, Abcam), StAR (ab233427, 1:500, Abcam), P450scc (ab272494, 1:1,000, Abcam), P450c17 (ab134910, 1:10,000, Abcam) and GAPDH (ab181602, 1:10,000, Abcam) overnight at 4℃. Then, membranes were conjugated with a secondary anti-rabbit antibody (ab6721, 1:10,000, Abcam) at room temperature for 1 h.

Chemical enhancing reagents were added and incubated for 1-5 min to visualize the bands. Protein bands were imaged using an X-ray imaging system (Bio-Rad, Hercules, CA, USA). Image J software was used to analyze the gray values of the bands and calculate the relative expression of the proteins to be tested.

Malondialdehyde (MDA), glutathione (GSH), iron and adenosine-triphosphate (ATP) level detections

Testicular MDA (E-EL-0060c, Elabscience), GSH (E-EL-0026c, Elabscience), ferrous iron (E-BC-K773-M, Elabscience) levels and ATP content (E-BC-K157-M, Elabscience) were measured according to the instructions of corresponding kits.

m^6A level test

Total RNA was isolated from testis tissues by the Trizol method, followed by RNA quality validation. Then, levels of m^6A in testis tissues were assessed with a m^6A RNA methylation quantification kit (ab185912, Abcam) according to the operating instructions.

RNA immunoprecipitation (RIP)

GC-1 spg cells (1×10^6) were seeded into 6-well plates and transfected with indicated plasmids. After cell transfection, total RNA extraction, mRNA separation and purification were carried out in order. IgG (ab97046, 1:500, Abcam) or m^6A (ab151230, 1:500, Abcam) antibody and IP buffer (2 mM EDTA, 1% NP-40, 140 mM NaCl and 20 mM Tris, pH 7.5) was used for 1-hour incubation with protein A/G beads. Afterwards, IP buffer was incubated with bead-antibody complex and mRNA overnight at 4°C. The beads with RNA binding were washed, purified, and quantified the GPX4 mRNA by qPCR assay.

mRNA stability assay

For GPX4 mRNA stability, transfected GC-1 spg cells were treated with Actinomycin D (5 μg/mL, ab141058, Abcam) for 0, 3, 9, 12 h. Then, RNA was isolated for reverse transcription, followed by the detection of GPX4 mRNA remaining using qPCR method.

Statistical analysis

Data were presented as the mean ± standard deviation (SD). All statistical analyses were conducted using SPSS18.0 software, and pictures required were drawn with GraphPad 8 software. Student's t test for comparison between two groups, one-way ANOVA for comparison between multiple groups, LSD-t test for post-hoc comparison, repeated measures analysis of variance for multi-time point expression, and Bonferroni for post-hoc test. It was considered statistically significant when $p < 0.05$.

Results

SQ improve the pathological injury of testis

To evaluate if SQ and Fer-1 can repair testicular damage induced by busulfan, focusing on structural changes and weight ratios as indicators of testicular health. Asthenozoospermic mice were treated with SQ or ferroptosis inhibitor (Fer-1), and H&E staining results showed that model group mice had testicular atrophy and seminiferous tubule vacuolation, while SQ and Fer-1 could improve the abnormal testicular structure (Fig. 1A). The diameter of seminiferous tubules and the height of seminiferous epithelium were significantly decreased and the lumen diameter was significantly increased in the model group, but SQ and Fer-1 intervention could alleviate these changes (Fig. 1B-D). Measuring and calculating testicular weight/body weight results showed that the testis to body weight ratio was significantly decreased in the model group, but SQ and Fer-1 intervention could increase such ratio (Fig. 1E). In addition, sperm count and motility test results showed that SQ and Fer-1 could improve the decreased sperm count and motility in the model group (Fig. 1F, G). The above results indicated that SQ was able to ameliorate testicular pathological damage in asthenozoospermic mice, which may be associated with inhibition of ferroptosis.

Effects of SQ on sex hormone and steroidogenic enzyme levels

To determine how SQ and Fer-1 affect hormonal profiles and enzyme levels involved in steroidogenesis, which is crucial for understanding their impact on male fertility. ELISA detected the decrease of serum FSH, LH and testosterone levels and an increase of E_2 level in the model group, while SQ and Fer-1 treatment could significantly improve the changes of serum sex hormone levels (Fig. 2A). qPCR and western blot results showed that SF-1, StAR, P450scc and P450c17 mRNA expressions and protein levels in the testis of model group were significantly decreased, but SQ and Fer-1 treatment could partially reverse these changes (Fig. 2B, C). Taken together, SQ could improve serum sex hormone levels and testicular steroidogenic enzyme levels in asthenozoospermic mice, which may be related to the inhibition of ferroptosis.

Ferroptosis is involved in the anti-testicular injury process of SQ

To establish if SQ and Fer-1 mitigate oxidative stress and ferroptosis by assessing biomarkers like MDA, GSH, and iron levels, clarifying the role of ferroptosis in testicular protection. The results showed that busulfan increased MDA and decreased GSH levels, while SQ and Fer-1 significantly reversed their effects on MDA and GSH levels (Fig. 3A, B). Moreover, busulfan induced the increase of divalent iron level, while SQ and Fer-1 remarkably decreased iron level (Fig. 3C). ATP level in testis was decreased in the model group, which were partly reversed SQ or Fer-1 (Fig. 3D). qPCR and western blot results showed that busulfan decreased GPX4 mRNA and protein levels, while SQ and Fer-1 were able to reverse the changes in GPX4 expression (Fig. 3E, F). Collectively, ferroptosis

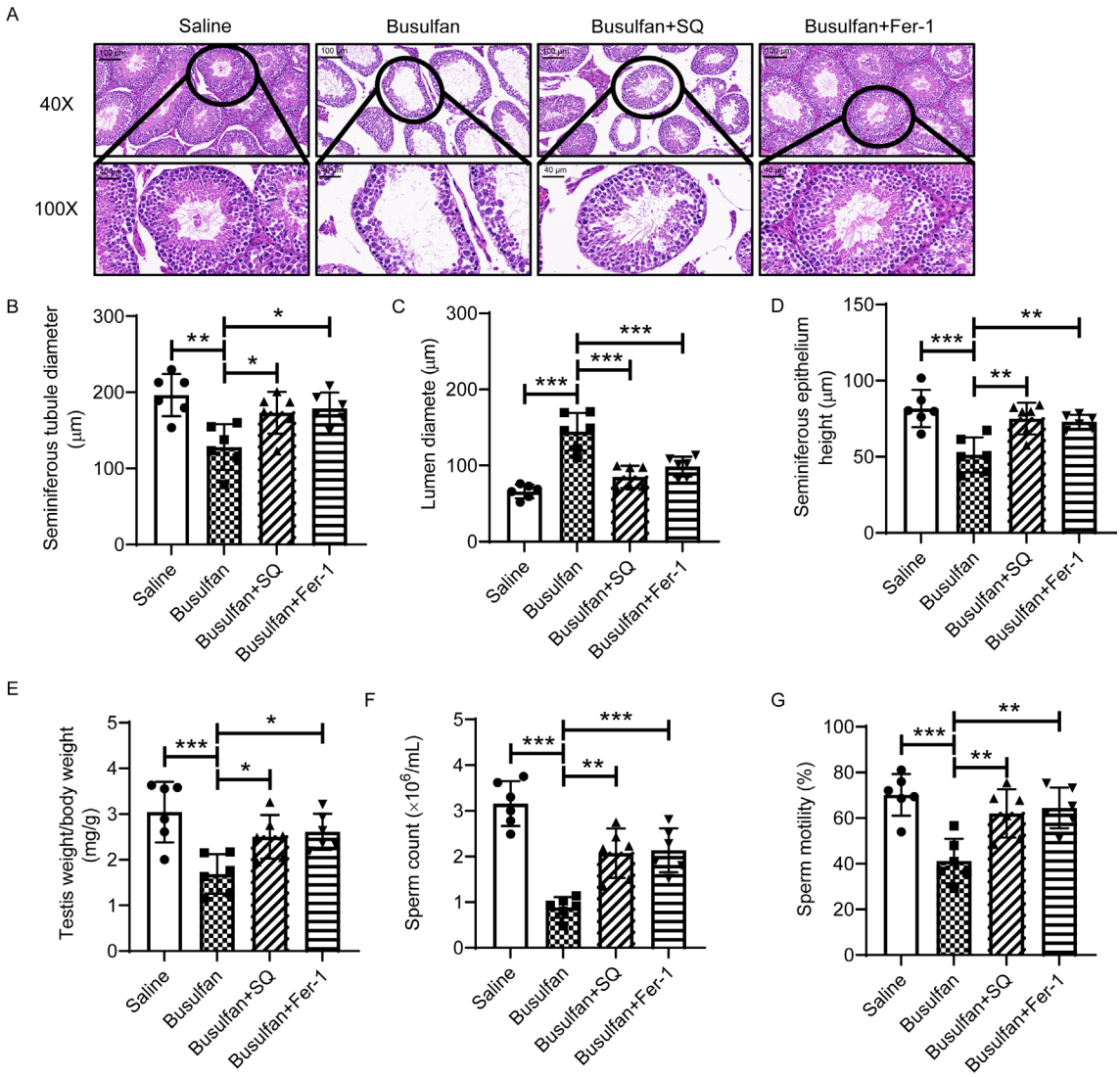

Fig. 1. SQ improve the pathological injury of testis.
A: H&E staining was used to observe testicular injury of mice of Saline, Busulfan, Busulfan + SQ, and Busulfan + Fer-1 groups. B-D: quantitative statistics of seminiferous tubule diameter, lumen diameter, seminiferous epithelium height. E: measurement and calculation of testicular weight/body weight. F: statistics of sperm count; G: statistics of sperm motility. *p < 0.05, **p < 0.01, ***p < 0.001.

may be an important mechanism of SQ against testicular injury in asthenozoospermia.

SQ regulate METTL3-mediated GPX4 m^{6}A modification

To investigate how SQ affects GPX4 expression via m^{6}A methylation, controlled by METTL3, linking epigenetic regulation to ferroptosis and cell survival. Using the m^{6}A RNA methylation quantification kit, we found that m^{6}A level was evidently increased in the model group mice, but SQ treatment could reduce m^{6}A level (Fig. 4A). Moreover, METTL3 mRNA and protein levels were significantly upregulated in mouse models, which could be downregulated by SQ treatment (Fig. 4B, C). RIP detection showed that GPX4 m^{6}A level was increased in the model group, while SQ treatment decreased GPX4 m^{6}A level (Fig. 4D). In addition, in vitro qPCR/western blot assays indicated that overexpression of METTL3 obviously reduced GPX4 mRNA and protein levels, while knockdown of METTL3 had the opposite effects (Fig. 4E, F). Overexpression of METTL3 elevated GPX4 m^{6}A level and knockdown of METTL3 decreased GPX4 m^{6}A level (Fig. 4G). Interestingly, METTL3 reduced GPX4 mRNA stability, and silencing of METTL3 had the opposite effect (Fig. 4F). These results demonstrated that SQ could regulate METTL3-mediated GPX4 m^{6}A modification.

SQ inhibit ferroptosis and improve asthenozoospermia through METTL3/GPX4 signaling axis

To demonstrate SQ's impact on sperm quality through modulation of the METTL3/GPX4 axis, connecting molecular changes to improvements in sperm count and motility. The results showed that knockdown of METTL3 signifi-

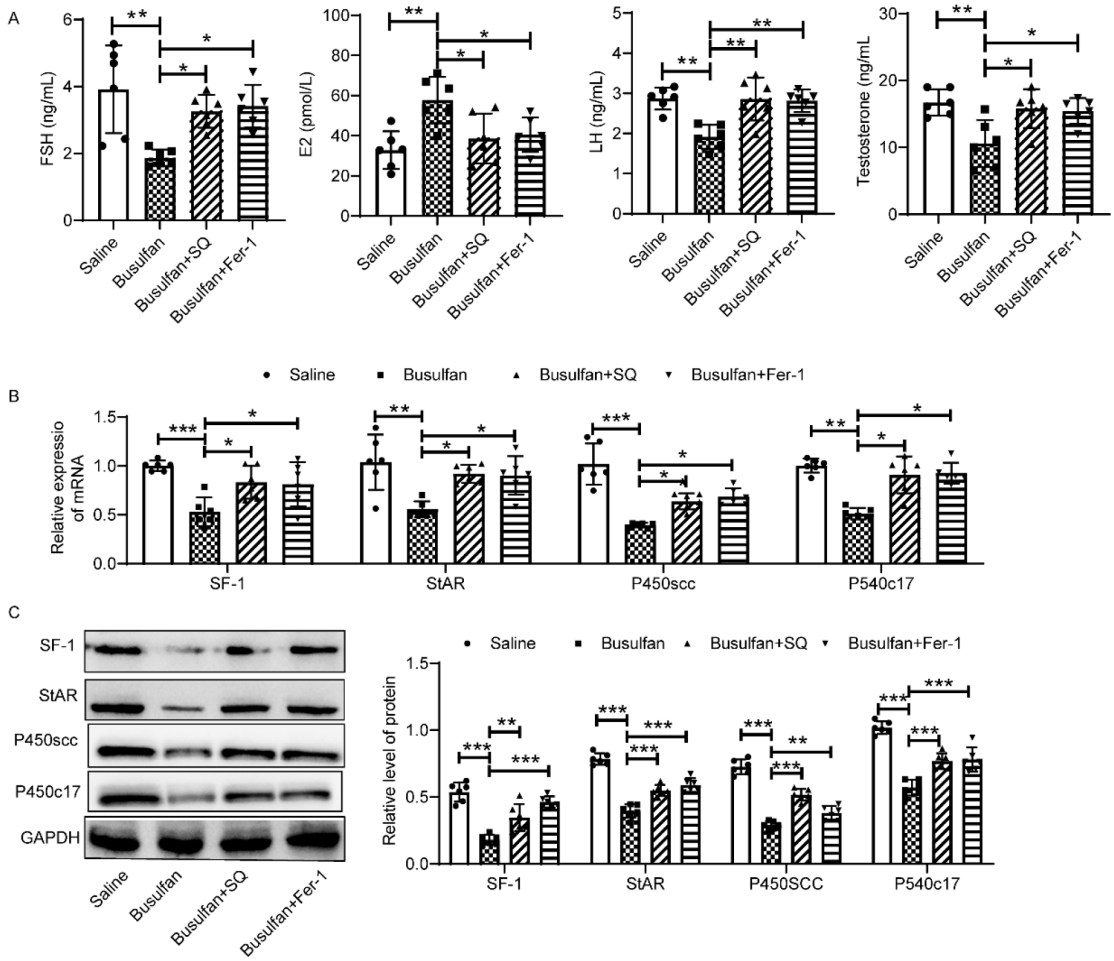

Fig. 2. Effects of SQ on sex hormone and steroidogenic enzyme levels.
A: ELISA was used to detect serum FSH, LH, E_2, and testosterone levels in mice of Saline, Busulfan, Busulfan + SQ, and Busulfan + Fer-1 groups. B: qPCR was used to detect SF-1, StAR, P450scc, and P450c17 mRNA expression. C: Western blot was used to detect SF-1, StAR, P450scc, and P450c17 protein levels. *p < 0.05, **p < 0.01, ***p < 0.001.

cantly upregulated GPX4 mRNA and protein levels, and overexpression of METTL3 partially inhibited the effects of SQ on GPX4 mRNA and protein levels (Fig. 5A, B). Kit assays indicated that METTL3 knockdown increased GSH level and decreased iron levels, and the effects of SQ on their levels were suppressed by METTL3 upregulation (Fig. 5C, D). ELISA assay results showed that METTL3 inhibition increased serum FSH and testosterone levels, and overexpression of METTL3 decreased the upregulation caused by SQ in serum FSH and testosterone levels (Fig. 5E). Furthermore, repression of METTL3 upregulated SF-1 and StAR mRNA expression and protein levels, and the effects of SQ on SF-1 and StAR mRNA expression and protein levels were suppressed by increasing METTL3 (Fig. 5F, G). Knockdown of METTL3 significantly increased sperm count and motility, and overexpression of METTL3 clearly repressed the effects of SQ on sperm count and motility Fig. 5H, I). The above results indicated that SQ could inhibit ferroptosis and ameliorate asthenozoospermia through the METTL3/GPX4 signaling axis.

Discussion

Asthenozoospermia is defined as less than 50% progressive motility or less than 25% grade a motility in semen parameters (World Health Organization 2001). Studies have shown that 81% of male infertility is associated with sperm motility disorders, and about 20% of them are closely related to low sperm motility (Curi et al. 2003), therefore the use of drugs to improve asthenozoospermia has gradually become a research hotspot. In this study, we established a mouse model of asthenozoospermia and intervened with SQ, revealing that SQ could improve testicular pathological damage, serum sex hormone and steroidogenic enzyme levels, thereby improving asthenozoospermia, and the mechanism may be through regulating ferroptosis mediated by the METTL3/GPX4 signaling axis.

Busulfan, chemically known as 4-butanediol dimesylate, is mainly used to treat malignant tumors, but long-term administration can lead to deposition in the body to inhibit reproductive stem cell differentiation, and lead to abnormal

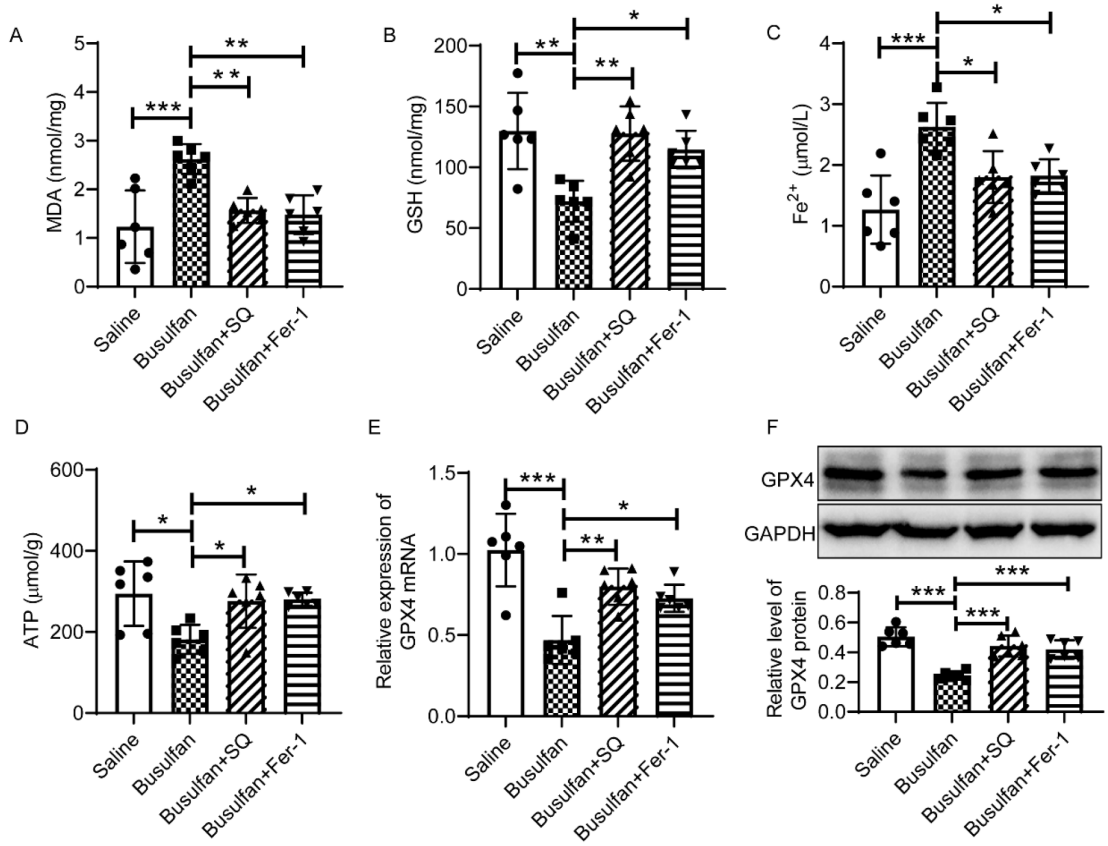

Fig. 3. Ferroptosis is involved in the anti-testicular injury process of SQ.
A-D: MDA (A), GSH (B), iron (C) and ATP (D) levels in testicular tissues of mice detected by kits. E: GPX4 mRNA expression was detected by qPCR; F: GPX4 protein level was detected by Western blot. *p < 0.05, **p < 0.01, ***p < 0.001.

spermatogenic function (Ben-Barouch et al. 2016). Related studies have found that intraperitoneal injection of fixed doses of busulfan in male mice can cause abnormal spermatogenic function, mainly manifested as decreased testosterone secretion, reduced testicular volume and changes in the structure of seminiferous tubules (Mobarak et al. 2022; Pu et al. 2023). In this study, after busulfan treatment, reproductive system toxicity and testicular atrophy was observed in mice, with significant decrease in various parameters of semen analysis and impaired sperm motility, manifested as asthenozoospermia. However, related indicators were evidently improved after SQ treatment, including sperm motility parameters, testicular pathological status, serum sex hormones, and steroidogenic enzyme levels (including SF-1, StAR, P450scc, and P450c17), suggesting that SQ have a significant therapeutic effect on asthenozoospermia. Shenqi Qiangjing Capsule originated from Qiangjing Decoction, and based on which we developed SQ to treat oligoasthenozoospermia. Among them, Cuscuta chinensis, Dipsacus, Lycium barbarbarum, Milkvetch, staghorn cream and Schisandra chinensis can tonify the kidney and generate sperm; Astragalus membranaceus and Codonopsis pilosula can replenish qi and invigorate the spleen; Raw oyster can solidify the essence and astringent essence; Angelica sinensis can nourish blood and promote blood circulation; Leonurus heterophyllus can promote blood circulation, clear heat Combined use of various drugs has the effects of invigorating the spleen and tonifying the kidney, nourishing blood and activating blood circulation, clearing away heat and dampness, and generating sperm and strengthening essence. Our findings corroborate previous research implicating ferroptosis in the pathogenesis of asthenozoospermia. Notably, the observed improvements in testicular pathology, serum sex hormone levels, and sperm quality following SQ treatment are consistent with studies demonstrating the therapeutic potential of targeting ferroptosis in various pathological conditions (Cantrell et al. 2024).

Because of their low antioxidant capacity and high concentrations of unsaturated fatty acids, germ cells are vulnerable to disruption of the oxidation-antioxidant balance and damage of free radicals and their oxidative products (de Lamirande et al. 1997; Koksal et al. 2003). Therefore, we then detected MDA and GSH, indicators of oxidative stress, in asthenozoospermic mice, and the results showed that busulfan increased MDA levels and decreased GSH levels, which indicated that busulfan induced lipid peroxidation and decreased antioxidant capacity in the tes-

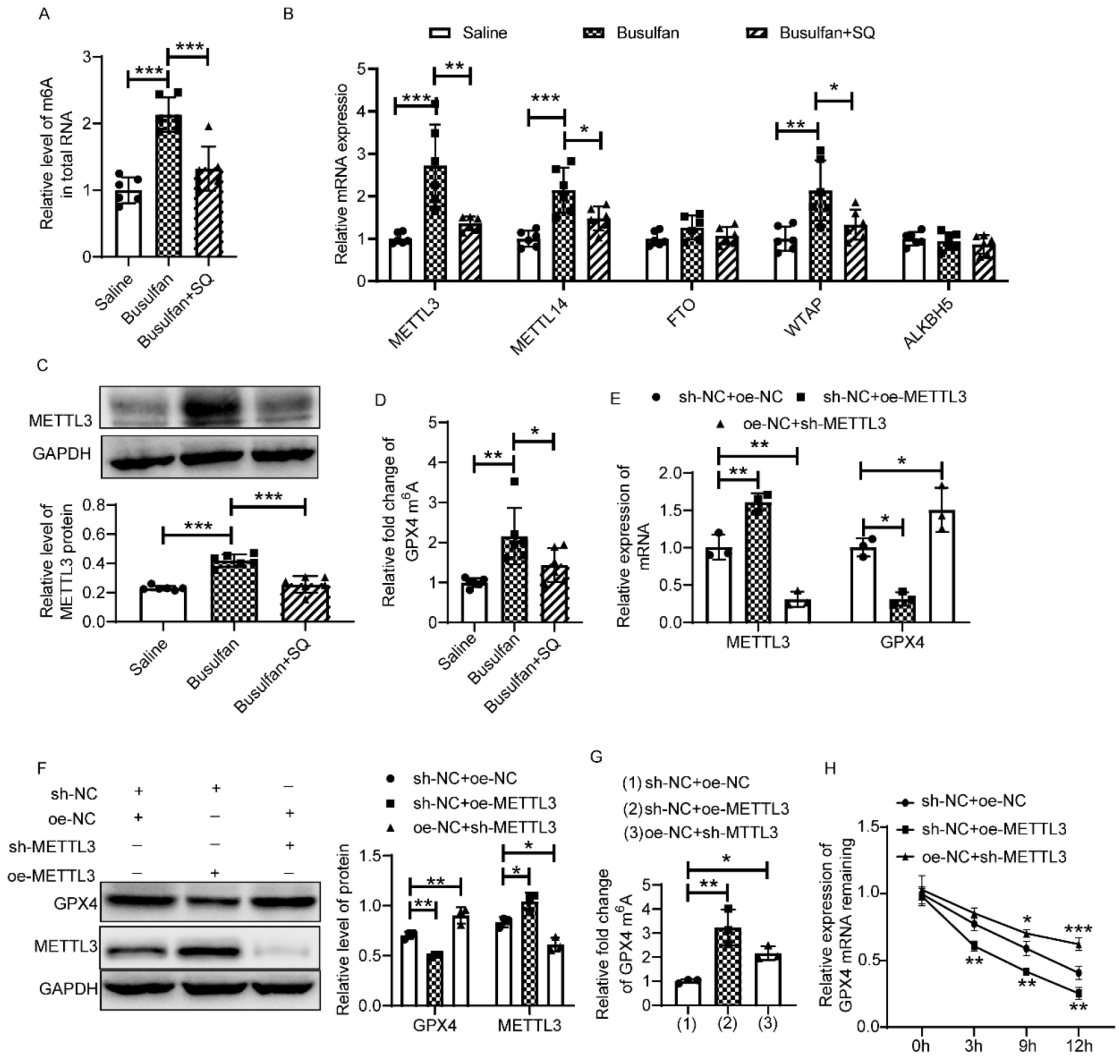

Fig. 4. SQ regulate METTL3-mediated GPX4 m^{6}A modification.
A: m^{6}A RNA methylation quantification kit detected m^{6}A levels in mice. B-C: qPCR/western blot detected the mRNA expression and protein levels of m^{6}A methyltransferases and demethylases. D: RIP detected GPX4 m^{6}A level in mice. E-F: qPCR/western blot measured METTL3 and GPX4 mRNA expression and protein levels in GC-1 spg cells transfected with sh-METTL3 or oe-METTL3. G: RIP detected the effect of METTL3 on GPX4 m^{6}A level in GC-1 spg cells transfected with sh-METTL3 or oe-METTL3. H: qPCR determined GPX4 mRNA stability in GC-1 spg cells transfected with sh-METTL3 or oe-METTL3. *p < 0.05, **p < 0.01, ***p < 0.001.

tis. Ferroptosis differs biochemically, morphologically, and genetically from apoptosis, autophagy, and necrosis. It plays a major role in acute tissue injury, oxidative stress injury, and neurotoxicity (Garg and Vucic 2016; Xie et al. 2016). The main mediators of ferroptosis are lipid peroxidation and iron metabolism signaling (Yang and Stockwell 2016). GPX4 is an important antioxidant enzyme in mammals that regulates iron-dead cell death by protecting cells from deleterious lipid peroxidation (Yang et al. 2014). Importantly, GPX4 is strongly expressed in testes and spermatozoa, and a clinical study found an evident decrease of GPX4 expression in spermatozoa from 30% of infertile men diagnosed with oligoasthenozoospermia (Imai et al. 2001). We also found that busulfan significantly induced an increase in bivalent iron levels and a significant decrease in ATP and GPX4 mRNA levels, but SQ intervention was able to reverse the above changes. Previous research has highlighted the critical role of ATP in sperm motility and function, with disruptions in ATP production contributing to impaired sperm quality (Costa et al. 2023). Consistent with our results, studies have demonstrated the dysregulation of GPX4 expression in various disease states, underscoring its significance in maintaining cellular homeostasis (Chu et al. 2023). Over all, the correlation between MDA, glutathione concentration, and GPX4 is pivotal in understanding ferroptosis. MDA reflects oxidative damage, while glutathione acts as an antioxidant, and GPX4 regulates lipid peroxidation. Dysregulation of these factors can lead to ferroptosis (Czyżowska et al. 2023). Specifically, decreased glutathione or impaired GPX4 can exacerbate lipid peroxi-

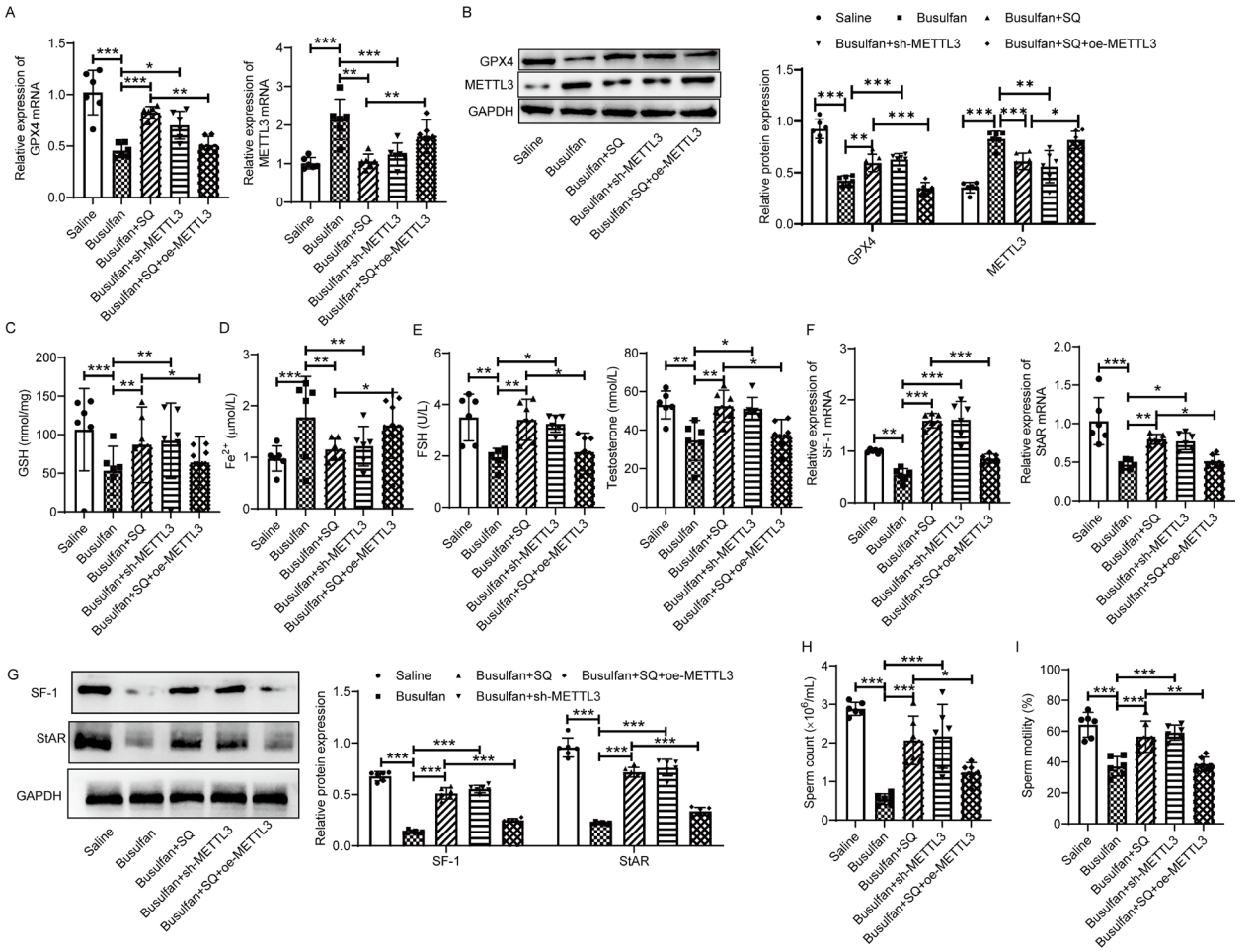

Fig. 5. SQ inhibit ferroptosis and improve asthenozoospermia through METTL3/GPX4 signaling axis.
A-B: qPCR/western blot measured METTL3 and GPX4 mRNA expression and protein levels in mice. C-D: kits were used to detect GSH (C) and iron (D) levels. E: ELISA was performed to detect serum FSH and testosterone levels; F-G: qPCR/western blot detected SF-1 and StAR mRNA expression and protein levels. H-I: statistics of sperm count and motility. *p < 0.05, **p < 0.01, ***p < 0.001.

dation, promoting ferroptotic cell death. Conversely, enhanced glutathione or GPX4 expression can mitigate ferroptosis. Thus, the interplay between MDA, glutathione, and GPX4 highlights their role in ferroptosis-related diseases like asthenozoospermia (Li et al. 2024). Sperm tail mitochondria support sperm motility mainly by producing ATP through glycolysis, and only intact mitochondrial function ensures that sperm can move long enough to enter the egg cell to complete fertilization (Zhou et al. 2015; Pang et al. 2022). In addition, we observed that Fer-1, an antioxidant small-molecule ferroptosis inhibitor, could also improve sperm count and motility in asthenozoospermic mice, which suggests that ferroptosis is involved in the development of asthenozoospermia. Our results suggest that SQ may improve asthenozoospermia by regulating GPX4 to affect ferroptosis to improve germ cell antioxidant effect, but its specific mechanism needs further study.

m^6A is the most common modification in epigenetic changes and drives multiple biological functions, including promoting tissue development and stem cell differentiation, as well as repairing DNA damage responses (Ma and Ji 2020). METTL3 is a member of the m^6A methyltransferase complex and catalyzes m^6A modification (Hao et al. 2019). METTL3-mediated m^6A modification has been reported to be involved in cancer progression. However, the role of METTL3 in asthenozoospermia remains to be elucidated. Increasing studies have revealed that m^6A methylation is specifically associated with obesity and testicular reproductive function (Wang et al. 2020; Yang et al. 2021). It has been suggested that increased expression of METTL3 is associated with m^6A methylation level (Xu et al. 2022). Also, m^6A methylation and its regulators are aberrantly expressed in testicular diseases, which leads to reproductive dysfunction, such as m^6A methylation level as well as METTL3 expression that are upregulated in semen samples from asthenozoospermic patients (Yang et al. 2016; Zhao et al. 2020a). In our study, we found that both m^6A methylation and METTL3 levels were significantly increased in asthenozoospermic mice, and overexpression of METTL3 was found to significantly reduce GPX4 mRNA and protein

levels, while knockdown of METTL3 had the opposite effects, suggesting that METTL3 can modulate ferroptosis by mediating GPX4 m^6A modification in asthenozoospermic mice. In addition, we found that knockdown of METTL3 significantly decreased iron level and significantly upregulated SF-1 and StAR mRNA expression and protein levels in mice, and overexpression of METTL3 partially reversed the effects of SQ on SF-1 and StAR mRNA expression and protein levels. These findings all suggest that SQ can inhibit ferroptosis and ameliorate asthenozoospermia in mice through the METTL3/GPX4 signaling axis.

Conclusions

In conclusion (Supplementary Fig. S2), this study demonstrated that SQ could ameliorate asthenozoospermia in mice through regulating ferroptosis mediated by the METTL3/GPX4 signaling axis, elucidating the role and possible mechanism of SQ in asthenozoospermia, and providing a reference program for the development of treatment options for asthenozoospermia. However, this study remains subject to several limitations. This study is a basic study and still needs to be further validated in clinical treatment, and we will further conduct clinical studies to verify our conclusions.

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Author Contributions

Guarantor of integrity of the entire study: Aicun Tang. Study concepts: Bin Bin. Study design: Aicun Tang. Definition of intellectual content: Qiuyu Lu. Literature research: Xin Li, Bingyu Xia. Clinical studies: Luying Wei. Experimental studies: Qiuyu Lu, Jiabao Ma. Data acquisition: Jing Fu, Xiaoxia Li. Data analysis: Kedao Lai, Luying Wei. Statistical analysis: Xiaoxia Li, Jing Fu. Manuscript preparation: Qiuyu Lu. Manuscript editing: Jiabao Ma. Manuscript review: Aicun Tang, Bin Bin.

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Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Files

Please find supplementary file(s);
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