Supplementary materials

Materials and methods

Preparation of PS-NP particles

Two types of PS-NPs were employed in this study. The fluorescence-labeled PS-NPs particles of 50 nm with emission wavelengths range from 460 to 750 nm, and excitation wavelengths range from 460 to 750 nm. These particles were purchased from Suzhou Nanomicro Technology Co., Ltd (Suzhou, China; www.nanomicrotech.com) and used to visualize the accumulation of PS-NPs in mice and cells. Other pristine PS-NP particles of 50 and 90 nm were purchased from Suzhou Nanomicro Technology Co., Ltd and used for the gavage exposure assay *in vivo* and toxicological tests *in vitro*. Both cases, PS-NPs particles were dispersed in water at 50 mg/mL concentrations. The morphology of PS-NPs was determined using a transmission electron microscope (TEM, HC-1, Hitachi, Japan).

Animals and PS-NP exposure

Four-week-old specific pathogen-free (SPF) inbred male ICR mice (mean body weighting 20±2 g) were obtained from the Experimental Animal Center of Ningxia Medical University. All animals were fed a commercial diet and sterile water *ad libitum* at a temperature of 22 ± 2 °C and in a 12-h light/dark cycle environment. After a week of accommodation, all mice were randomly divided into various concentrations of exposure groups. In biodistribution experiments, the fluorescence-labeled PS-NPs were performed daily by intravenous tail injection at 200 μL 17 mg/kg doses for two days (n=3). Two days later, the mice of control and labeled PS-NPs treatment groups were anesthetized with chloral hydrate and scanned by a fluorescence imaging system (Carestream MS FX Pro, Dongsheng Innovative Biotechnology Co., LTD, China) with excitation and emission wavelengths at 470 and 530 nm, respectively. The mice were euthanized after fluorescence-labeled PS-NPs exposing 2 days, and the blood was removed by a systemic infusion of paraformaldehyde (4%). Then, the testicular tissue was embedded with OCT for frozen sections and photographed under a fluorescence confocal microscope (Nikon,

Japan) (Li et al., 2022). In the toxicological assessment *in vivo*, 50 mg/mL of pristine 50 and 90 nm PS-NPs particles were suspended in 200 μL deionized water at a final dosage of 3, 15, 75 mg/kg, and daily given to mice by gavage for 60 days, the control group only received the same volume of distilled water, and body weights were recorded weekly. The mice were anesthetized at the corresponding time points (30, 45, and 60), and the testicles were immediately fixed in glutaraldehyde for hematoxylin-eosin staining. Simultaneously, the related reproduction indicators, including testosterone, survival rate and deformity rate of sperm, and the expression of spermatogenic genes, were evaluated. All animal experiments were conducted following the National Institute of Health guidelines and approved by the Medical Ethical Committee of Ningxia Medical University, China (NXMU-2022-G073).

Histology of testes

After mice were anesthetized, testes of mice from the PS-NPs treated and control groups were immediately excised and weighed. Then, testicular tissues were fixed with 4% paraformaldehyde for 24 h, embedded in paraffin, sectioned at a 5 μm thickness, stained with hematoxylin-eosin (H&E), and examined under a microscope. The images were captured using a Micro Image (Olympus, Japan).

Sperm quality analysis

Sperm samples obtained from the cauda epididymidis of sacrificed mice were examined for morphology, sperm concentration, survival rate, and deformity rate. Briefly, the epididymis of each animal was cut and gently squeezed to 1 mL saline, and then the epididymal pieces were placed in a 37 °C incubator for 10 min for sperm dispersing by itself. The sperm suspension was filtered into a 1.5 mL EP tube with a 200 mesh sieve. Then, a 10 μL filtered sperm suspension was dropped in a Makler sperm counting plate for sperm quality (including sperm concentration, viability and abnormality) detection (Wu et al., 2023).

Testosterone analysis

Blood samples for sera were collected from the control and PS-NPs exposed groups (n=3 in each group) using cardiac puncture. The testosterone was tested using

a Kit from Jianglai Biotechnology Co., LTD (China). According to the manufacturer's instruction, the testosterone level was determined by enzyme-linked immunosorbent assay (ELISA).

Testis organ coefficient measure

Before euthanasia, the mice were weighed, the testicles were harvested, and the excess adipose tissue around the testicles was removed. The organ coefficient was calculated after weighing the testicles. Organ coefficient = organ weight (mg)/body weight (g) (Guan et al., 2020).

Cell culture and treatment

The Mouse spermatocyte cell line GC-2 spd was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. GC-2 was cultured in DMEM medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and 1% penicillin/streptomycin (Gibco BRL, Grand Island, NY, USA) in an atmosphere of a 37 °C, 5% CO_2. When the cell fusion rate reached 80%, it was digested with 0.25% trypsin (Gibco BRL, Grand Island, NY, USA) and seeded in new dishes for culturing 12 h, and then exposing to various concentrations of PS-NPs. For some assays, 10 μmol/L Ferrostatin-1 (Fer-1, Selleckchem, USA), 10 μmol/L Erastin (MedChemExpress, Shanghai, China), 50 μmol/L Ferric ammonium citrate (FAC, Sangon Biotech, Shanghai, China), or 5 μmol/L RSL3 (Med Chem Express, Shanghai, China) was incubated for 24 h with PS-NPs or independently.

Cell viability assay

The cell viability of GC-2 cells was assayed using a Cell Counting kit-8 (CCK-8, APExBIO Technology, USA) according to the manufacturer's instruction. Briefly, GC-2 cells (5000 cells/well) were seeded in 96-well plates for 24 h and then replaced with a DMEM medium containing a series of concentrations PS-NPs for viability determination experiments. After exposure to PS-NPs at indicated time points, cells were replaced with fresh medium and incubated with a 10% kit reagent at 37 °C for 4 h. Subsequently, each well's absorbance (O.D value) was measured at 450 nm using a microplate reader (Thermo Scientific, USA), and the mean of three independent tests

was calculated to determine the viability of PS-NPs on GC-2 cells.

RNA-sequencing and differential gene annotation

The total RNA was extracted from GC-2 cells of control, 12.5 μg/mL 50 nm PS-NPs treated, and 25 μg/mL 90 nm PS-NPs treated groups (n=3) using Trizol reagent (Takara, China) according to the manufacturer's instructions. The sequencing service was provided by Novogene Technology Co., Ltd (Beijing, China). Briefly, RNA purity and integrity were detected using Nano Photometer1 spectrophotometer (IMPLEN, CA, USA) and Bioanalyzer 2100 system (Agilent Technologies, CA, USA), respectively. Then, the mRNA was extracted from the total RNA using poly-T oligo-attached magnetic beads. Next, the first strand of cDNA was synthesized using a random hexamer primer and a Reverse Transcriptase (RNase H). The second strand of cDNA was subsequently synthesized using DNA Polymerase I and RNase H. After PCR operation, the library quality of PCR products was assessed using the Agilent Bioanalyzer 2100 system. Finally, the library fragments were sequenced on an Illumina Novaseq platform of Novogene Technology Co., Ltd according to the manufacturer's instructions. The raw RNA-sequencing data was processed by removing the containing adapter and low-quality reads. Subsequently, all clean reads were annotated through reference genomes of the Genebank Database. The RPKM (Reads Per Kilo bases per Million reads) was used to calculate the number of expression tags and the differentially expressed genes on an adjusted p-value < 0.05. Finally, the pathways of differentia genes were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Du et al., 2022).

Western blotting

First, 400 μL of RIPA lysis buffer (Beyotime, Jiangsu, China) supplemented with protease inhibitors (Beyotime) was added to the 1.5 mL EP tube of testes and homogenized with a homogenizer. For GC-2 cells, 200 μL of lysis buffer supplemented with protease inhibitors was added to the 1.5 mL EP tube of cells and then placed on ice for 10 min. All samples were centrifuged at 4 °C for 10 min at 12,000 rpm, and the supernatant was collected in a new 1.5 EP tube for protein

concentration determination. The protein concentration was quantified using BCA Protein Quantification Kit (Beyotime, Jiangsu, China). The Nuclear and Cytoplasm Extraction Kit (Ambion) was used in the nucleus and cytoplasmic isolation experiment. The experiments in the co-immunoprecipitation assay were carried out using the Co-IP kit (Absin, Shanghai, China) according to the manufacturer's instructions. Briefly, cell lysate supernatants were harvested and incubated with a Keap1 antibody overnight. Meanwhile, the nonspecific homologous IgG antibody was used to eliminate interference. The agarose gel beads combined Protein A and Protein G were incubated for 3 h, and then the precipitate was collected by centrifugation. The Western blotting was performed as previously described (Du et al., 2022). The expression of the proteins was visualized using an ECL detection kit (Amersham Biosciences) on a ChemiDoc MP imaging system (Bio-Rad, USA), and the intensities of the Western blotting bands were quantified using Image J software (NIH, USA). Assays were independently performed in triplicate for each sample. Antibodies against Nrf2 (Proteintech, USA), Keap1 (Cell Signaling, USA), GPX4 (Abcam, USA), TFR (Abcam, USA), TF (Santa Cruz, USA), DMT1 (Proteintech, USA), FPN1 (Novus, Germany), HO-1 (Proteintech, USA), β-actin (Beyond Biotech, China), and Tubulin (Beyond Biotech, China) were used in this study.

Real-time quantitative PCR (RT-qPCR) analysis

Total RNA was isolated from the testes by vigorous homogenate in TRIzol reagent (Invitrogen, Thermo Scientific, USA). The concentration and quality of total RNA were measured using a NanoDrop 2000 (Thermo Scientific, USA). Reverse transcription and RT-qPCR quantification of RNAs were performed using a Quantscript RT Kit (Tiangen, Beijing, China) and 2× SYBR Green qPCR Master Mix (TaKaRa, China), respectively. The expression levels of *pizf*, *ddx4*, *dazl*, *stra8,* and *sycp3* were analyzed using a Real-time quantitative PCR instrument (BD, USA). Gene-specific primers are showed in Table S1. Gene expression levels from three independent experiments were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak, 2001).

Transmission electron microscopy

The mitochondrial morphology of GC-2 was assessed by transmission electron microscope (Hitachi, Tokyo, Japan). Briefly, after treating with 12.5 μg/mL, 50 nm PS-NPs and 25 μg/mL 90 nm PS-NPs for 24 h, 1×10^6 GC-2 cells were harvested and fixed with 2.5% glutaraldehyde for 24 h at 4 °C and treated with 1% OsO_4 for 1 h. Then, the cells were dehydrated with absolute ethyl alcohol before embedding them in epon resin. Finally, the ultrathin sections were subjected to a transmission electron microscopy at 80 kV.

Mitochondrial membrane potential assay

The mitochondrial membrane potential (MMP) was detected using a JC-1 fluorescent probe assay kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, 1×10^5 GC-2 cells were seeded in 12-well plates and exposed to PS-NPs for 12 h. Then, the culture medium was replaced with a fresh medium, and the JC-1 working solution was added and incubated for 5 min at 37 °C. Finally, cells were washed thrice with PBS and visualized using a fluorescence microscope (Leica, Wetzlar, Germany).

Lipid ROS determination

The C11-BODIPY 581/591 molecular probe was used following the manufacturer's instructions to assess lipid peroxidation of PS-NPs on GC-2 cells. Briefly, GC-2 cells were incubated in a working concentration of 2 μM in HEPES buffer for 20 min, washed thrice with PBS, and visualized under a fluorescence microscope at 590 and 510 nm for imaging.

Intracellular free divalent iron analysis

The free divalent iron assay kit (Jiancheng Biotech., Nanjing, China) measured intracellular iron. Briefly, 1×10^6 cells were harvested and washed thrice with PBS and then lysed with lysis buffer on ice for 20 min. After centrifugation for 15 min at 12,000 rpm, 4 °C, the supernatant was collected and detected intracellular free divalent iron depending on the 2 mg/L iron standard solution of kit. Next, the total protein concentration in samples was quantified using a BCA protein assay kit

(Beyotime, Jiangsu, China). Finally, the concentration of the intracellular iron was calculated, and the unit was converted into μmol/g protein following the manufacturer's instructions.

RNA interference

Three double strands of RNA fragments (si-2, si-7, si-9) of Nrf2 were synthesized by GenePharma Co. (Shanghai, China). These siRNAs were designed according to the mRNA (AH006764.2). After optimal experiment, si-2 fragment (sense: 5’-GCAGGACAUGGAUUUGAUUTT-3'; antisense: 5’-AAUCAAAUCCAUGUCCUGCTT-3') was used in the subsequent experiment. Briefly, a mixture containing the Nrf2 interference fragment and control fragment (target for *gapdh*) was transfected into GC-2 cells with 60% confluence using X-tremeGENE HP DNA transfection reagent. After 48 h, GC-2 cells were used for exposure and treatment experiments.

Statistical analysis

All data were analyzed using GraphPad Prism 6.0 software (https://www.graphpad.com/scientific-software/prism). The data were visualized based on the means±standard deviation (SD). Student's t-tests and analysis of variance (ANOVA) were used to determine the significant differences. Comparative analysis of various factors was performed using two-way ANOVA, and multiple samples were compared by one-way ANOVA using the least significant difference (LSD) method, while Student’s *t*-test was used to evaluate whether there were significant differences between the two groups. The value of *P* less than 0.05 was considered significant. Each experiment was repeated thrice.

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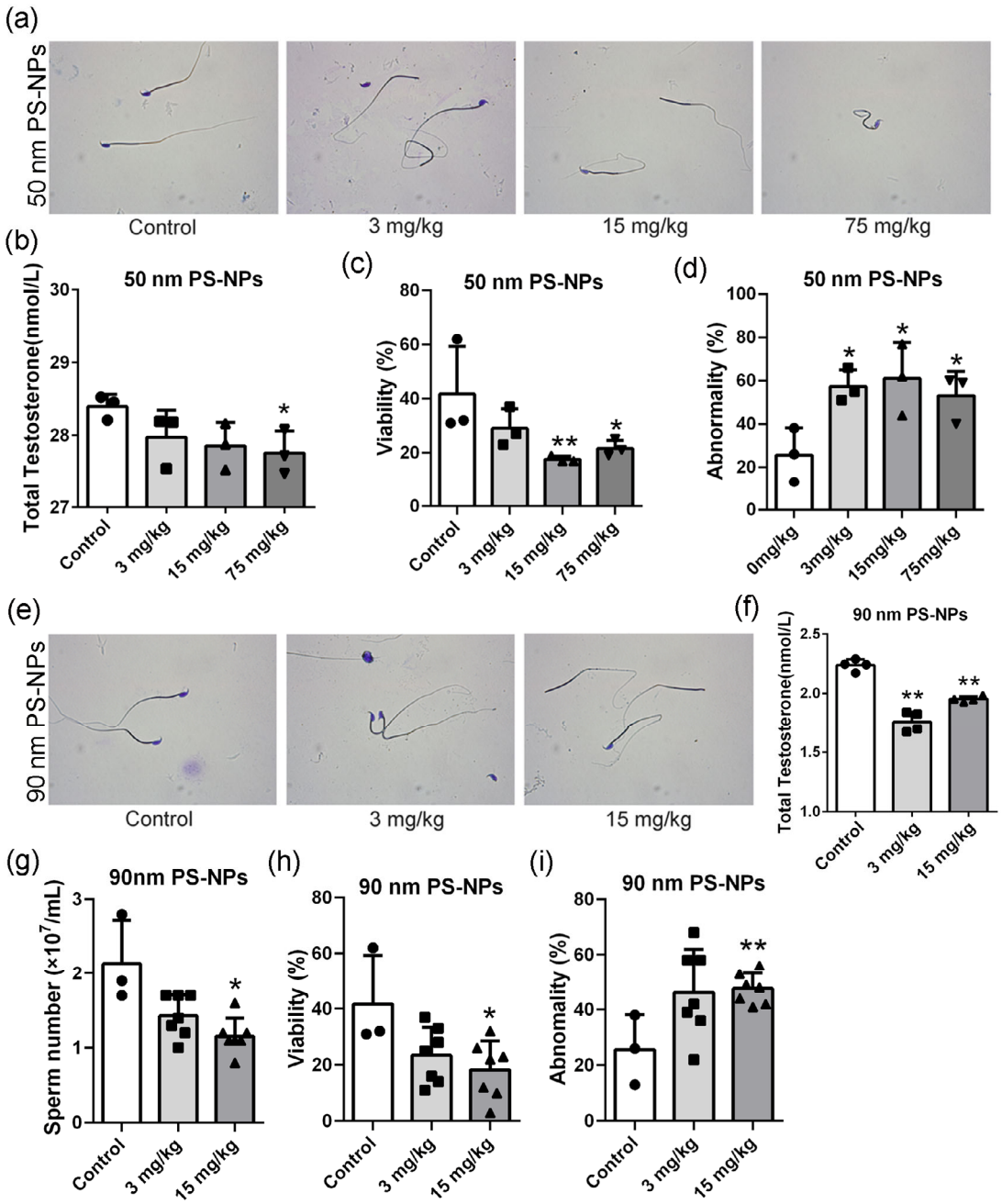

Fig. S1. The sperm of mice were influenced by 50 and 90 nm PS-NPs for 30 days. The sperm morphology (a), testosterone level (b), sperm viability (c), and abnormality (d) after 50 nm PS-NPs exposure for 30 days. The sperm morphology (e), testosterone level (f), sperm concentration (g), sperm viability (h), and sperm abnormality (i) after 90 nm PS-NPs exposure for 30 days.

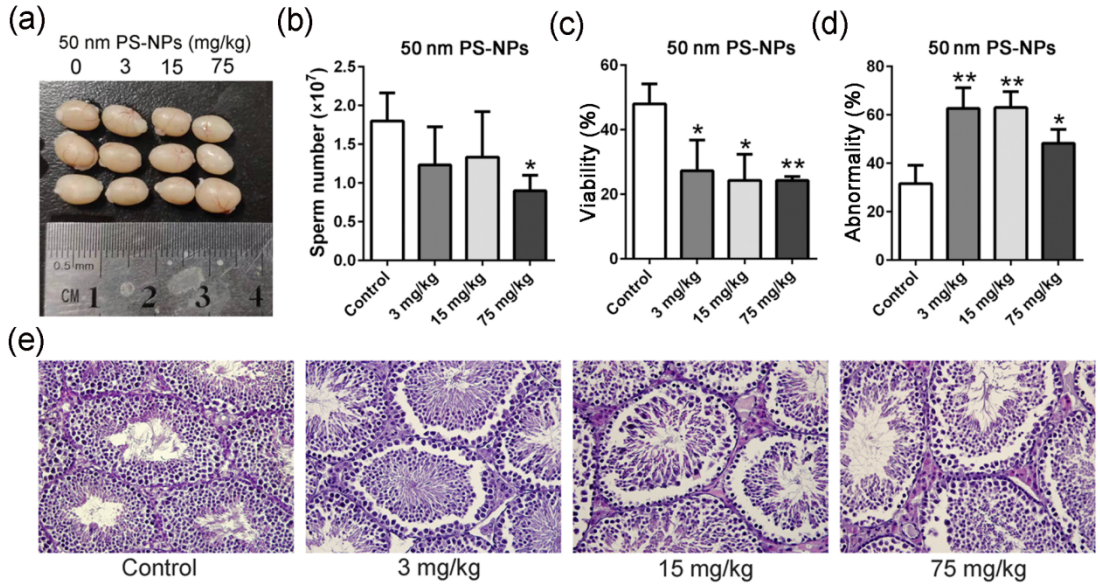

Fig. S2. The male reproductive characteristics were changed after 50 nm PS-NPs exposure for 45 days. (a) The testicular morphology of 50 nm PS-NPs exposure for 45 days. The sperm concentration (b), viability (c), and abnormality (d) after 50 nm PS-NPs exposure for 45 days (n=3). (e) The testicular histology of 50 nm PS-NPs exposure for 45 days. * denotes P<0.05, ** denotes P<0.01.

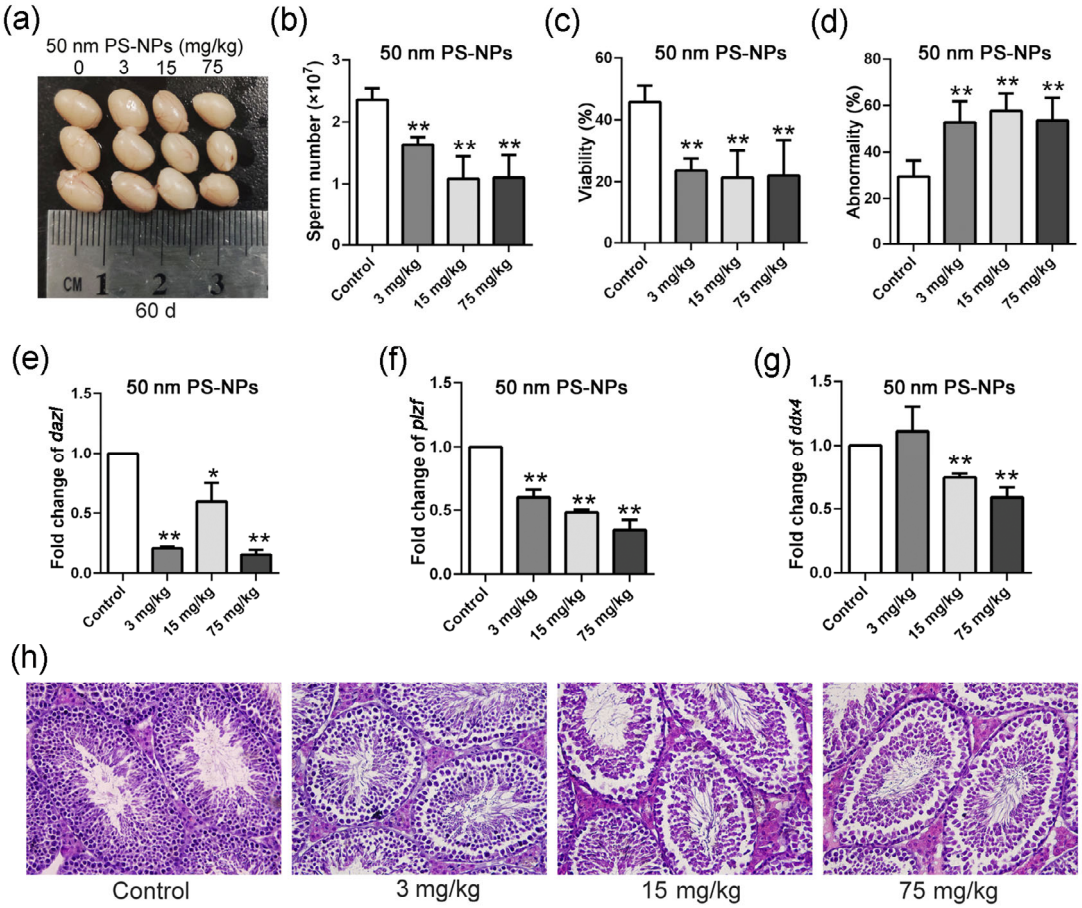

Fig. S3. The male reproductive characteristics were changed after 50 nm PS-NPs exposure for 60 days. (a) The testicular morphology by 50 nm PS-NPs exposure for 60 days. The sperm concentration (b), viability (c), and abnormality (d) after 50 nm PS-NPs exposure for 60 days (n=3 per group). The gene expression of *dazl* (e), *plzf* (f), and *ddx4* (g) in testicular tissue after 50 nm PS-NPs exposure for 60 days (n=3 per group). (h) The testicular histology of 50 nm PS-NPs exposure for 60 days. * denotes P<0.05, ** denotes P<0.01.

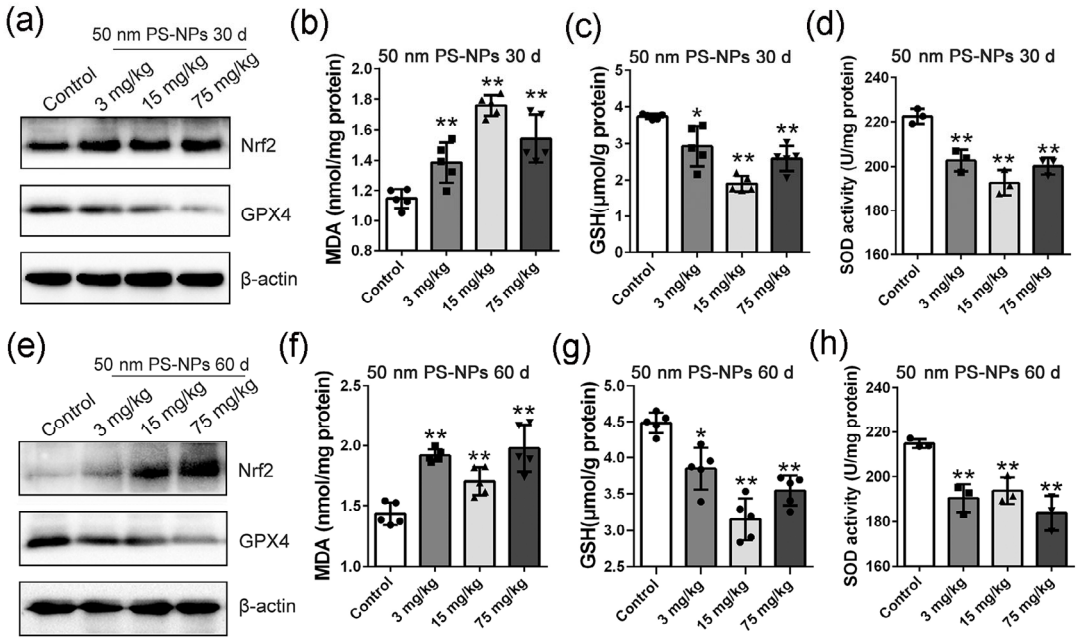

Fig. S4. PS-NPs induce ferroptosis occurrence in testis. (a) Nrf2 and GPX4 expressions in testis after 50 nm PS-NPs exposure for 30 days. The levels of MDA (b), GSH (c), and SOD (d) in testis after 50 nm PS-NPs exposure for 30 days. (e) Nrf2 and GPX4 expressions in testis after 50 nm PS-NPs exposure for 60 days. The levels of MDA (f), GSH (g), and SOD (h) in testis after 50 nm PS-NPs exposure for 60 days. * denotes $P<0.05$, ** denotes $P<0.01$.

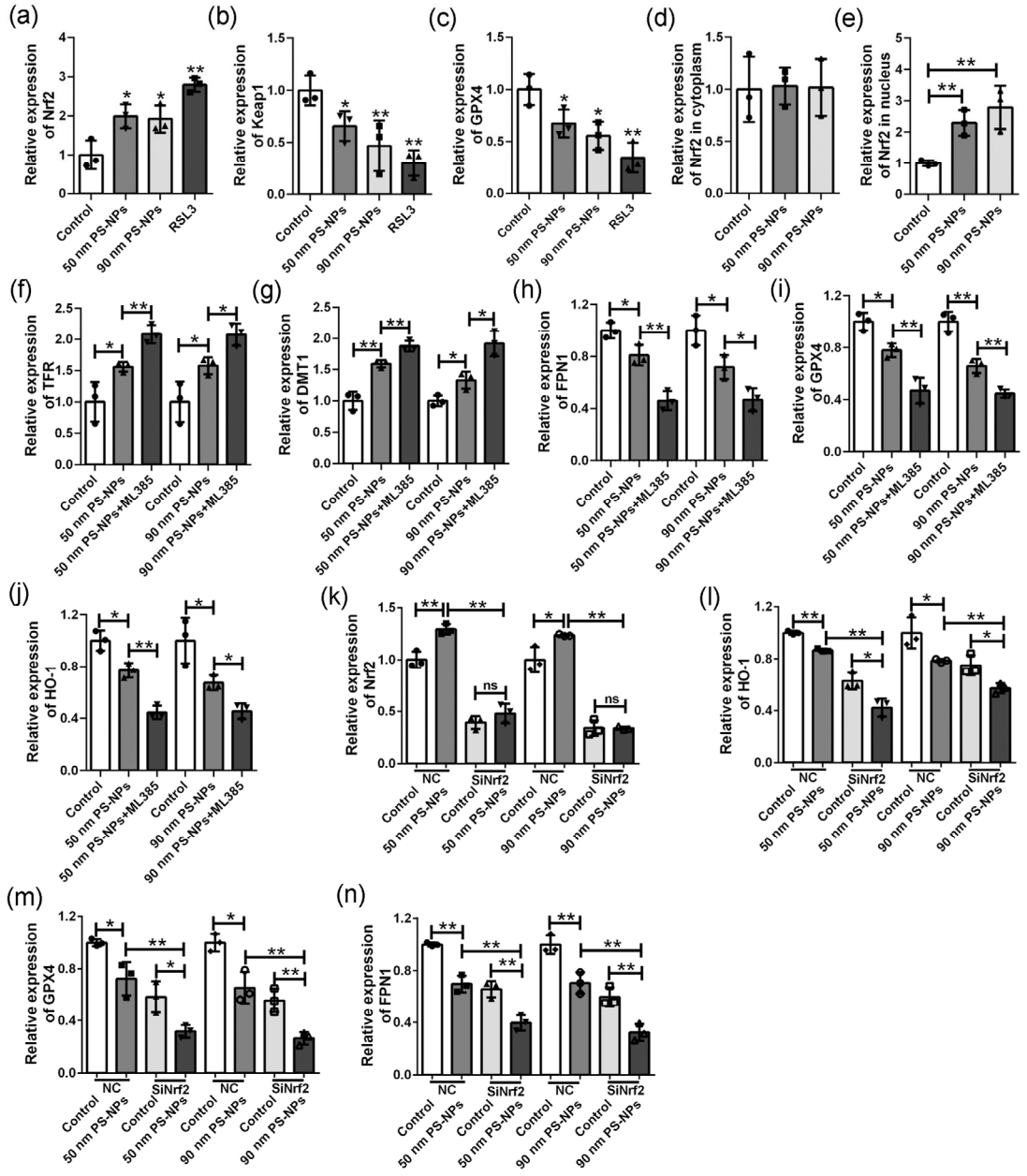

Fig. S5. The Western blotting quantification of **Fig. 5**. The quantification of Nrf2 (a), Keap1 (b), and GPX4 (c) expression in **Fig. 5a**. The quantification of Nrf2 expression in the cytoplasm (d) and nucleus (e) in **Fig. 5b**. The quantification of TFR (f), DMT1 (g), FPN1 (h), GPX4 (i), and HO-1 (j) expression in **Fig. 5d**. The quantification of Nrf2 (k), HO-1 (l), GPX4 (m), and FPN1 (n) expression in **Fig. 5i**. * denotes $P<0.05$, ** denotes $P<0.01$.

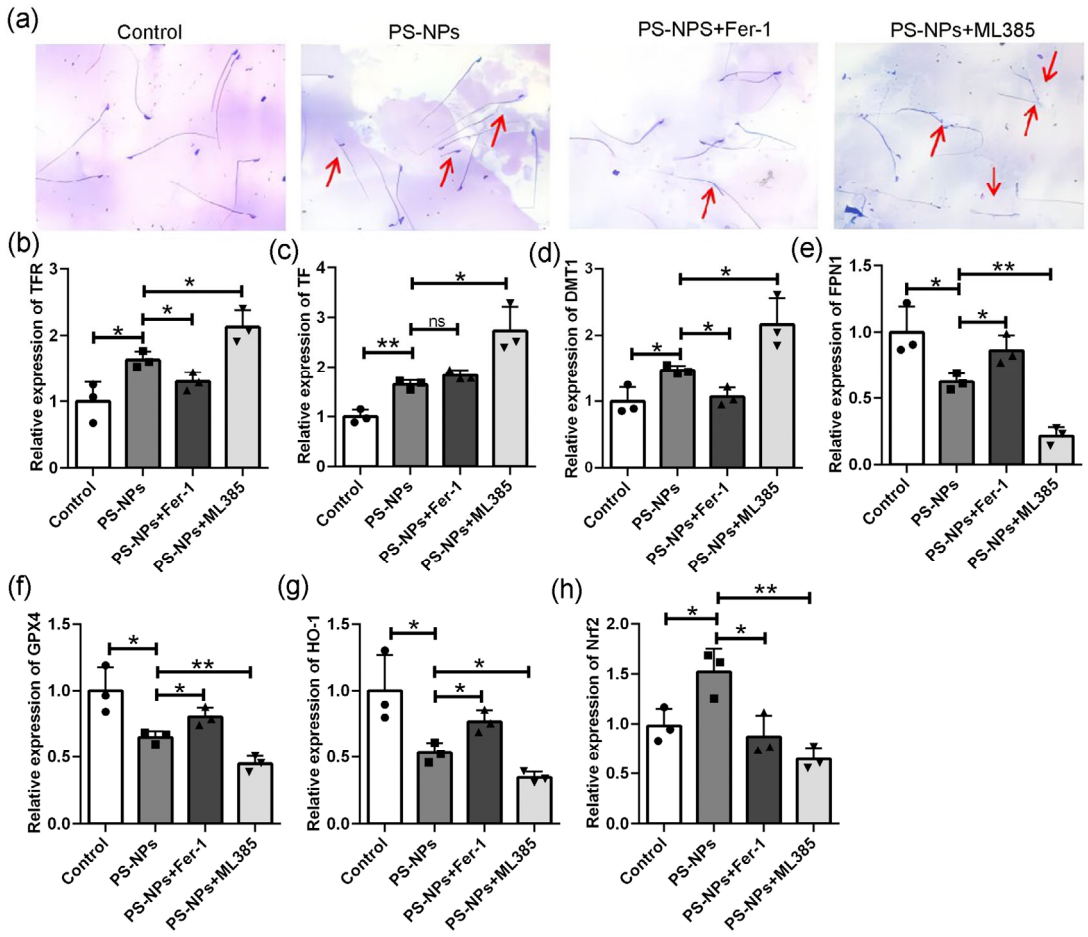

Fig. S6. (a) The sperm morphology of PS-NPs exposure as well as Fer-1 and ML385 treatment, the red arrow is the damaged sperm. The quantification of TFR (b), TF (c), DMT1 (d), FPN1 (e), GPX4 (f), HO-1 (g), and Nrf2 (h) expression in **Fig. 6i**. * denotes $P<0.05$; ** denotes $P<0.01$.

Table S1 Gene-specific primers

Gene	Forward primer	Reverse primer
plzf	5′GCATTTACTGGCTCATTCA3′	5′GTATGGGTCTGTCTGTGT3′
ddx4	5′CAGCTTCAGTAGCAGCACAAG3′	5′CATGACTCGTCATCAACTGGA3′
sycp3	5'GCTTCTTTCAAAGCCAGTAACC3'	5′CACTGCTGCAACACATTCATAA3′
stra8	5′CTCCTCCTCCACTCTGTTGC3′	5′GCGGCAGAGACAATAGGAAG3′
dazl	5′ATGTTGTACCTCCGGCTTATTCA3′	5′CCATTTCCAGAGGGTGGAGTA3′
β-actin	5'AGCCATGTACGTAGCCATCC3'	5'GCTGTGGTGGTGAAGCTGTA3'