

# Silica nanoparticles inducing the apoptosis of spermatocyte cell through microRNA-450b-3p targeting MTCH2-mediating mitochondrial signaling pathway

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## Research

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# Abstract

**Background:** Silica nanoparticles (SiNPs) are found in environmental particulate matter and are proven to have adverse effects on fertility. The relationship and underlying mechanisms between miRNAs and apoptosis induced by SiNPs during spermatogenesis is currently ambiguous.

**Experimental design:** The present study was designed to investigate the role of miRNA-450b-3p in the reproductive toxicity caused by SiNPs. In vivo, 40 male mice were randomly divided into control and SiNPs groups, 20 per group. The mice in the SiNPs group were administrated 20 mg/kg SiNPs by tracheal perfusion once every 5 days, for 35 days, and the control group were given the equivalent of a normal luminal saline. In vitro, spermatocyte cells were divided into 0 and 5 µg/mL SiNPs groups, after passaged for 30 generations, the GC-2spd cells in 5 µg/mL SiNPs groups were transfected with miRNA-450b-3p and its mimic and inhibitor.

**Results:** In vivo, the results showed that SiNPs damaged tissue structures of testis, decreased the quantity and quality of the sperm, reduced the expression of miR-450b-3p, and increased the protein expressions of the MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3 in the testis. In vitro, SiNPs obviously repressed the viability and increased the LDH level and apoptosis rate, decreased the levels of the miR-450b-3p, significantly enhanced the protein expressions of the MTCH2, BID, BAX, Cytochrome C, Caspase-9, Caspase-3; while the mimic of miR-450b-3p reversed the changes induced by SiNPs, but inhibitor further promoted the effects induced by SiNPs.

**Conclusion:** The result suggested that SiNPs could induce the spermatocyte apoptosis by inhibiting the miR-450b-3p expression to target promoting the MTCH2 resulting in activating mitochondrial apoptotic signaling pathways in the spermatocyte cells.

## Background

Silica nanoparticles (SiNPs) are found in environmental particulate matter (PM), and it is the major inorganic component of PM<sub>2.5</sub>(1). SiNPs are also one of the most widely used in engineered nanomaterials and listed as one of the top five nanomaterials in consumer products(2). The unique physicochemical properties of SiNPs are extensively utilized in biomedical and biotechnological fields, such as drug delivery, gene therapy, diagnosis, and imaging(3–5). With increasing environmental pollution and the rapid application of SiNPs, their toxicity and toxic mechanisms have raised significant concerns, which will be helpful for risk assessment.

Environmental pollution has been proven to have an adverse effect on fertility. It was reported that atmospheric pollution was connected to the clinical and sub-clinical symbols of infertility, such as poor sperm quality(6). Infertility remains a major clinical problem that occurs in 10 to 15% of couples worldwide, and male factor infertility accounts for 40 to 50% of all infertility cases(7). Previous studies have shown that SiNPs could penetrate the blood-testis barrier(8) and decrease semen quality and count of sperm in mice(9). However, many of these studies focused on the pathways and proteins related to

apoptosis and autophagy(10, 11), and there are relatively few studies that focus on the epigenetic regulations of the toxic mechanisms of SiNPs on reproduction.

MicroRNAs (miRNAs) are noncoding RNAs with nearly 22-nucleotide-long(12). miRNA expression during spermatogenesis could regulate the expressions of mRNAs in the testis(13). It was reported that miR-34b/c and miR-449a/b/c clusters in the sperm are essential for fertilization and pre-implantation development in vivo(14). Mir-141, miR-429, and miR-7-1-3p increased in the seminal plasma of patients with non-obstructive azoospermia(15). SiNPs could induce apoptosis via loss of mitochondrial membrane potential and Caspase-3 activation, while miR-98 plays a key role in modulating this effect(16). Silica nanoparticles and MeHg co-exposure could induce expressions of miRNAs in zebrafish significantly(17). Exposure to Silica nanoparticles also exerted altered the expression of apoptosis-associated proteins in HaCaT cells(18). Our Previous study showed that SiNPs altered the miRNA profile, and there were five upregulated miRNAs and ten down-regulated miRNAs in spermatocyte cells, and miR-138 and miR-2861 were related to the death receptor pathways of apoptosis(10). However, the underlying mechanisms behind some miRNA in SiNPs induced male reproductive toxicity are still unclear.

It was reported that mitochondrial carrier homolog 2 (MTCH2) is a mitochondrial outer membrane protein that is essential for embryonic development(19). Recent research has shown that MTCH2 is highly expressed in rat testis and primarily related to the apoptosis of spermatocytes(20). Based on the above studies, the present study was designed to firstly investigate the effects of SiNPs on the expressions of miR-450b-3p, MTCH2 and mitochondrial apoptosis pathway in mice, further study the relationship between the miRNA and MTCH2 and the MTCH2 role in mitochondrial apoptosis pathway of mouse spermatocytes via transfecting mimic and inhibitor of miR-450b-3p in vitro, so as to clarify the role of miRNA in the reproductive toxicity caused by SiNPs.

## Results

### Characterization of the silica nanoparticles

The shape of SiNPs was near-spherical, and the size was uniform (Fig. 1). The average diameter of the SiNPs was  $65.72 \pm 7.29$  nm. The SiNPs exhibited remarkable monodispersity and stability in ultrapure water, DMEM, and saline. Our data demonstrated that SiNPs have good dispersibility in testing medium. The characterization of SiNPs was performed, which is similar to our previous study(10). The results of Zeta potential and surface area of the SiNPs in the ultrapure water, DMEM, and saline are shown in Table 1.



Table 1

Hydrodynamic size and zeta potential of SiNPs in different medium at different time points(Means  $\pm$  S.D.)

TIME	Ultrapure water		DMEM medium		Saline	
	Hydrodynamic size(nm)	Zeta potential (mV)	Hydrodynamic size(nm)	Zeta potential (mV)	Hydrodynamic size(nm)	Zeta potential (mV)
0 h	79.70 $\pm$ 5.39	-34.71 $\pm$ 6.41	72.67 $\pm$ 10.3	-20.48 $\pm$ 1.47	75.86 $\pm$ 0.92	-36.45 $\pm$ 4.37
6 h	81.12 $\pm$ 4.21	-33.3 $\pm$ 7.62	86.89 $\pm$ 13.1	-25.81 $\pm$ 5.00	74.34 $\pm$ 0.69	-30.7 $\pm$ 0.65
12 h	92.65 $\pm$ 14.99	-28.59 $\pm$ 4.49	157.92 $\pm$ 12	-23.96 $\pm$ 2.94	75.45 $\pm$ 1.46	-27.6 $\pm$ 0.29
24 h	112.71 $\pm$ 14.54	-32.20 $\pm$ 6.46	325.07 $\pm$ 50.37	-22.47 $\pm$ 2.21	74.85 $\pm$ 1.13	-28.3 $\pm$ 3.02

### Changes of the quality and quantity of sperm caused by the SiNPs.

The results showed that sperm morphology in the control group is normal (Fig. 2a), while in the SiNPs group had different morphological defects in the head, neck, and tail of the sperm (Fig. 2a). Meanwhile, the sperm abnormalities in the SiNPs group obviously increased when compared with the control group ( $p < 0.05$ )(Fig. 2b). The sperm motility and sperm concentrations in the SiNPs groups were significantly lower than those of the control groups ( $p \leq 0.05$ )(Fig. 2c, d).

### Effects of SiNPs on the tissue structure and spermatogenic cell apoptosis in testis

The tissue structures of testis in the control groups were normal morphology, the seminiferous tubules with spermatogenic cells arranged regularly, the lumens were filled with amounts of sperms; while in the SiNPs groups, the damages of the seminiferous tubules were observed, there were the depletion and exfoliation of spermatogenic cells and vacuolation in lumens (Fig. 3a).

The data from the TUNEL staining showed that the relative fluorescence intensity in the SiNPs group was increased compared with their control group, which means spermatogenic cell apoptosis in lumens of testis was obviously higher than that of control group ( $p \leq 0.05$ )(Fig. 3b, 3c).

### The protein expressions of changes of MTCH2 and mitochondrial apoptotic signaling pathway in testis

The present results showed that SiNPs significantly affected the protein expressions of MTCH2 and mitochondrial apoptotic signaling pathways in testes of mice ( $p \leq 0.05$ )(Fig. 4). The protein expression level of MTCH2 in the SiNPs group was higher than that of the control group ( $p \leq 0.05$ )(Fig. 4a, b). The protein expressions of BID, BAX, Cytochrome C, Caspase-9, and Caspase-3 in mitochondrial apoptosis

signaling pathway in the SiNPs group significantly increased when compared to control group ( $p \leq 0.05$ ) (Fig. 4a, b).

### **Prediction and verification of the miR-450b-3p and verification of the target genes of the miR-450b-3p in vivo and in vitro**

Results from the document searching showed that the MTCH2 might be the target genes of miR-450b-3p (Fig. 5a, Table S1). Starbase was used to predict the relationships between the miR-450b-3p and MTCH2 (Fig. 5a). We found that the combination of the mir-450b-3p and MTCH2 is 6mer and identified its binding region on the chromosome. Bottini et al. verified the targeting relationship between the mir-450b-3p and MTCH2, through the Ago-CLIP experiment(21). To verify the relationship between the miR-450b-3p and MTCH2, we firstly examined the levels of miR-450b-3p and MTCH2 mRNA in testis by RT-PCR. The results showed that the levels of miR-450b-3p in the SiNPs group was lower than that of the control group ( $p \leq 0.05$ ), while the level of MTCH2 mRNA was higher than that of the control group ( $p \leq 0.05$ ) (Fig. 5b, c).

To further verify the relationship among the miR-450b-3p, MTCH2, and mitochondrial apoptotic signaling pathway, the mimic and inhibitor of miR-450b-3p were used in vitro via transfection. The results showed that the level of the miR-450b-3p was downregulated after exposure of the GC-2spd to the SiNPs for 30 generations when compared to that in the control group ( $p \leq 0.05$ ) (Fig. 5d). While the inhibitor of miR-450b-3p further decreased the expression level of miR-450b-3p, and the mimic of miR-450b-3p antagonized the expression decrease of miR-450b-3p caused by SiNPs when compared to those in 5  $\mu\text{g/mL}$  SiNPs group ( $p \leq 0.05$ ) (Fig. 5d). Besides, there were no significant differences in miR-450b-3p level among the 5  $\mu\text{g/mL}$  SiNPs group and 5  $\mu\text{g/mL}$  SiNPs with two negative control groups (Fig. 5d), which means that the solvents of mimic and inhibitor had no significant effect on the levels of miR-450b-3p (Fig. 5d). The mRNA levels of the MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3 in the SiNPs group significantly increased when compared to control group ( $p \leq 0.05$ ); while in the mimic of miR-450b-3p group, the mRNA levels of MTCH2, BAX, Cytochrome C, Caspase-9, and Caspase-3 obviously decreased when compared to the 5  $\mu\text{g/mL}$  SiNPs group, but BID had no significant difference than that in the 5  $\mu\text{g/mL}$  SiNPs. In addition, the mRNA levels of MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3 in the inhibitor of the miR-450b-3p group were higher than those in the 5  $\mu\text{g/mL}$  SiNPs ( $p \leq 0.05$ ) (Fig. 5e).

### **Cytotoxicity of the SiNPs on the spermatocyte cells in vitro**

To determine the cytotoxicity of the SiNPs on the spermatocyte cells through modified expression levels of the miR-450b-3p, cell viability, and LDH activity were determined. The results showed that the viability of the GC-2spd cell in the 5  $\mu\text{g/mL}$  SiNPs group was lower than that in the control group ( $p \leq 0.05$ ) (Fig. 6a), the LDH level and apoptosis rate in 5  $\mu\text{g/mL}$  SiNPs group were higher than those in the control group ( $p \leq 0.05$ ) (Fig. 6b, c d). While the mimic of the miR-450b-3p group significantly reversed the decrease of cell viability and relieved the increases of LDH level and apoptosis rate caused by SiNPs when compared to the 5  $\mu\text{g/mL}$  SiNPs group ( $p \leq 0.05$ ); but the inhibitor of the miR-450b-3p further repressed the decrease

of cell viability, and promoted the increases of LDH level and apoptosis rate induced by SiNPs compared to the 5 µg/mL SiNPs group ( $p \leq 0.05$ ) (Fig. 6).

### Changes of protein expressions of in the MTCH2 signaling pathways in vitro

The protein expression of the MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3 were measured by western blot analysis. In vitro, compared with the control group, the expression of the MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3 were significantly increased in the SiNPs group ( $p \leq 0.05$ ), while the mimic of miR-450b-3p relieved the increases induced by SiNPs ( $p \leq 0.05$ ). The inhibitor of miR-450b-3p further promoted the protein expression increases of MTCH2, BID, BAX, Caspase-9, and Caspase-3 caused by SiNPs ( $p \leq 0.05$ ) compared to 5 µg/mL SiNPs group, while Cytochrome C has no significant change between the 5 µg/mL SiNPs group and 5 µg/mL SiNPs group with inhibitor group although there was an increasing tendency ( $p \leq 0.05$ ) (Fig. 7a-c).

## Discussion

This investigation has shown that SiNPs destroyed the histological structures of the testis, decreased sperm numbers and motility, increased sperm malformation rates, and led to spermatogenic cell apoptosis of the testis in mice; the results in vitro also showed that the SiNPs caused the apoptosis and a viability decrease in the GC-2 spermatocyte line. The current results from both the in vivo and in vitro suggested that the SiNPs exposure could affect the sperm quality and quantity by damaging the histological structures of the testis and inducing the adverse effects on spermatogenesis. The present results are similar to the study from Xu et al. (22), who found that SiNPs could cause reversible damages to the sperms and testicular structures. The apoptosis of the sperm cells induced by the SiNPs was also previously reported by Özgür ME(16, 23).

To further explore the mechanisms of reproduction toxicity induced by SiNPs, we measured the protein expressions of the MTCH2 mitochondrial apoptosis signaling pathway in the testicular tissues of mice. The results showed that the SiNPs significantly increased the protein expression of the MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3, which suggested that the SiNPs activated the MTCH2 and mitochondrial apoptosis signaling pathway in vivo. MTCH2, a mitochondrial outer membrane protein(24), was mostly related to the apoptosis of spermatocytes(20). It was reported that MTCH2 could facilitate the recruitment of the truncated BID to mitochondria(24). BID could indirectly activate BAX/BAK and thus allowing them to undergo unimpeded, spontaneous activation in the mitochondrial outer membrane, leading to apoptosis initiation(25). Studies have suggested that, when activated, Bak and Bax, create discontinuity or pores in the outer mitochondrial membrane, to mediate the release of Cytochrome C(26). Caspase-9 is a vital apoptosis initiator, while Caspase-3 is an apoptosis executioner(27). Therefore, SiNPs could activate the MTCH2-mediating the mitochondrial signaling pathway and therefore lead to apoptosis of the spermatocyte.

miRNAs play a crucial role in SiNPs -induced male reproduction toxicity. A previous study from Xu et al. showed that miR-98 exerts an important role in the apoptosis of germ cells caused by SiNPs(16). Our

previous study found that SiNPs could change the miRNA profile of the spermatocytes exposed to the SiNPs for 30 generations and lead to the expression changes of 15 miRNAs, including 5 upregulated miRNAs and 10 downregulated miRNAs. Among the 15 miRNAs, the expressions of the miRNA-450b-3p were downregulated(10). The miRNA database prediction tool Starbase was used to predict the relationships between the miR-450b-3p and MTCH2(21, 28), which showed that the target gene of the miR-450-3p is MTCH2. The present results showed that the MTCH2 could mediate the mitochondrial apoptosis pathway of the spermatocytes. The above results indicated that the effects of the SiNPs on reproduction toxicity might be induced by miRNA and that miRNA-450b-3p might play an important role in the apoptosis of the spermatocytes via the mitochondrial apoptosis pathway. Therefore, the miRNA-450b-3p was chosen for further investigation of its functions in the toxicity of the spermatocytes caused by SiNPs.

The GC-2 spermatocyte cells were transfected for studying the function of the miRNA-450b-3p in these cells after exposure to the SiNPs. The present results showed that SiNPs downregulated the expression of the miR-450b-3p both in vivo and in vitro, and upregulated the mRNA and protein expression levels of the MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3 in the GC-2spd cells. In contrast, mimic of the miR-450b-3p upregulated the expression of the miR-450b-3p, and significantly antagonized the upregulated levels of the mRNA and protein expression in the MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3, and relieved both the decrease of the viability and the increase of the apoptosis in the GC-2spd cells induced by the SiNPs. Conversely, inhibition of the miR-450b-3p further downregulated the expression of the miR-450b-3p, and upregulated the mRNA and protein expression levels of the MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3, and further decreased the cellular viability and increased the apoptosis of the GC-2spd cells when compared with the levels induced by SiNPs. The results indicated that the SiNPs could induce the apoptosis of the spermatocyte cells by inhibiting the expression of the miR-450b-3p to target improving MTCH2 expression resulting in the activation of the mitochondrial apoptosis pathway. This investigation had, for the first time, focused on the effects of SiNPs on male reproductive toxicity by verifying the relationship between the miR-450-3p and MTCH2 in the spermatocyte cells. Poorly studies have reported the expression of miR-450b-3p and the relationship among miR-450b-3p, MTCH2, and apoptosis. Zhao et al. found that miR-450b-3p could inhibit cell proliferation by targeting HER3(29). Arigoni found that miR-135b could control cancer cell growth by targeting MTCH2(30). Li et al. (31) demonstrated that miR-125b-2 regulated testosterone secretions by targeting Papolb, and increased the DNA copy number of sperm mitochondria to influence the semen quality. In male mice, miR-34b/c and - 449a/b/c led to sperm aggregation and agglutination, luminal obstruction, and sperm granulomas in male mice(32). In Sertoli cells, the miR-202-3p controls the apoptosis via targeting LRP6 and Cyclin D1 of the Wnt/ $\beta$ -catenin signaling pathway(33). Humans found that several kinds of environmental chemicals, including SiNPs, could alter the expressions of miRNAs(34–36). Xu et al. found that SiNPs could affect the expression of mir-98 in GC-2, and mir-98 regulated the expression of the Caspase-3 in GC-2(16). The results showed that the toxicity of the SiNPs on the reproductive function might be induced by miRNA.

Moreover, miRNAs are thought to be functionally important in regulating apoptosis. Their abnormal expression could alter the expression in target genes. miR-450b-3p exists in abundance in the testis in mice. Therefore, miRNA-450b-3p might be considered as the biomarker of the SiNPs-induced apoptosis of the spermatocyte cells, as determined by the mitochondrial signaling pathways in this investigation, which may be useful for the early detection and early warning of the reproductive toxicity of SiNPs.

## Conclusion

SiNPs reduced the expressions of miR-450b-3p, increased the protein expressions of the MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3 in testis and GC-2spd cell in vitro, while the mimic of miR-450b-3p reversed the changes induced by SiNPs, but inhibitor further promoted the effects induced by SiNPs. The results suggested that miR-450b-3p could target and regulate the expression of MTCH2, SiNPs induce the spermatocyte apoptosis by inhibiting the expression of the miR-450b-3p to target promoting the expression of MTCH2, thereby resulting in activating the mitochondrial apoptosis signaling pathways of the spermatocyte cells. This suggested that miR-450b-3p might play important roles in the apoptosis of spermatogenic cells via MTCH2-mediating the mitochondrial signaling pathways induced by the SiNPs. The miRNA-450-3p might be considered as the biomarker for male reproductive toxicity induced by SiNPs.

## Methods

### Animals and treatment

Forty-five-week-old clean grade male ICR mice whose weights ranged from 20-22g were obtained from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). The mice put into standard plastic boxes (26 cm ×15 cm ×15 cm), 5 mice per box, with hygienic laboratory conditions at  $20 \pm 2$  °C with a 12 h photoperiod and relative humidity of  $60 \pm 10\%$ , with a 12:12 hour light/dark cycle. Animals were fed pellets food and water ad libitum, following the procedures of the Animal Experiments and Experimental Animal Welfare Committee of Capital Medical University (ethical review number: AEEI-2019-003).

After adapting to the lab conditions for one week, 40 healthy adult male mice were randomly divided into either a saline control group or SiNPs group and there were 20 mice per group. The mice in the SiNPs group were administrated with 20mg/kg SiNPs by tracheal perfusion once every 5 days for a total of 35 days. The dosage of 20 mg/kg was based on previous studies of acute toxicity studies(22). The mice in the saline control group were given equivalently volumes of normal saline. After 35 days, the mice were sacrificed using tribromoethanol anesthesia, and the blood, testis, and epididymides collected from each animal for analysis. After 1 h at room temperature, the blood samples were centrifuged for 15 min at 3000 rpm (Eppendorf, 5415D, USA), then the serum samples were amassed and stored at -80°C for future analysis, one side of the testis was fixed for the histopathological experiment, and the other side was stored at -80°C and liquid nitrogen.

## **Determination of the SiNPs characterization**

The configuration of the SiNPs was verified using the transmission electron microscope (TEM) (JEOL JEM 2100, Japan), and their hydrodynamic size and zeta potential of SiNPs were detected in distilled water, saline, and Dulbecco's modified eagle's medium (DMEM) using a Zetasizer (Nano ZS90; Malvern, UK). Before addition of the SiNPs to the culture medium, a sonicator (160 W, 20 kHz, 5 min) (Bioruptor UDC-200, Belgium) was used to suspend SiNPs to minimize their aggregation.

## **Analysis of the sperm quality and quantity**

Sperms were extracted from the epididymides and immediately incubated in Dulbecco's Modified Eagle Medium (2 mL) at 37 °C for 5 min, then their concentrations and motility were measured using semen analyzer (Hamilton Thorne IVOS-II; Hamilton Thorne Research, Beverly, MA, USA). A smear of the sperm suspension was made on the glass. To determine the sperm deformation, the number 1000 sperms under a high magnification microscope (Olympus BX53, Japan) by counting. Sperm were scored as normal or abnormal using the Kruger strict criteria. Sperm morphology was observed and imaged under a light microscope (Olympus BX53, Japan).

$$\text{The sperm abnormality} = \frac{\text{The abnormality sperm number}}{1,000} \times 100\%$$

## **Histopathological analysis of the testis**

After animals were sacrificed with tribromoethanol anesthesia, the testis from a side of the mice in all groups were fixed in 4 % paraformaldehyde, embedded in paraffin blocks, sectioned and stained with hematoxylin and eosin (HE) for histological examination. The testis sections were observed using the Qupath (The University of Edinburgh, Scotland) software.

## **Detection of spermatozoa cell apoptosis in the testis**

Cell apoptosis in the testis was analyzed using the method of terminal deoxyribonucleotide transferase-mediated nick end labeling (TUNEL). Each group of testis sections were stained using the TUNEL assay kit (KeyGen, Nanjing, China). In apoptotic cells, Biotin-labeled dUTP could be linked to the 3'-OH end of the DNA fragments with the TdT enzyme. Fluorescence microscopy can detect fluorescein-isothiocyanate (FITC), which the labeled streptavidin binds to biotin during the biotin-streptavidin amplification. The testis sections were first dewaxed, and then the sections were dealt with Proteinase-K working fluid for permeabilization and then reacted with the TdT working solution (mixture of dUTP and the TdT-enzyme). The testis sections were stained with streptavidin fluorescein; meanwhile, the nucleus were stained by DAPI (ThermoFisher, USA). The testis sections of each group were imaged using laser scanning confocal microscopy (A1R HD25, Nikon, Japan). Ten visual fields from each group were selected randomly to examine the relative fluorescence intensity and count the number of TUNEL-positive cells per tubule. The relative fluorescence intensity was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

## Protein expressions detection of MTCH2 and apoptotic signaling pathway in the testis

To expound the influence of the SiNPs on the expression of the cellular factors involved in the MTCH2 and apoptotic signaling pathway, the protein levels of MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3 in the testis were determined with western blot analysis.

The total protein of the testis tissues was extracted using a bicinchoninic acid (BCA) protein assay. Equal amounts of lysate proteins (40 µg per testis tissue) were electrophoresed in SDS-polyacrylamide gels (12% separation gels) and transferred to nitrocellulose membranes (Millipore, USA). After being blocked with 5% bovine serum albumin (BSA) (Epsilon, USA) and being dissolved in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) for 1 h at room temperature, the membranes were incubated with rabbit polyclonal antibodies of MTCH2 (1:500, Abcam, UK), BID (1:1000, proteintech, USA), BAX, Cytochrome C, Caspase-9, and Caspase-3 (1:1000, CST, USA) overnight at 4 °C, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000, CST, USA) was used as an internal control. The membranes were then washed with TBST and incubated with fluorescent anti-rabbit Ig G secondary antibody (Amyjet, China) for 1 h at room temperature. After washing with TBST three times, the antibody-bound proteins were detected with Li-Cor Odyssey system (Li-Cor Biosciences). The densitometric values of the protein bands were analyzed using Image Studio Lite Software (Li-Cor Biosciences).

## Cell cultures and experimental design

Primary mouse spermatocyte cells (GC-2spd) were purchased from Guangzhou Jennio Biotech Company Limited. The cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Genviue, USA) furnished with 10 %fetal bovine serum (Gibco, USA), 100 mg/mL streptomycin, 100 U/mL penicillin, and cultured at 37 °C in a 5 % CO<sub>2</sub> humidified environment. The cells were seeded into 10-cm (diameter) dishes at a density of  $1 \times 10^5$  cells/mL. The spermatocyte cells were divided into two groups, those with 0 mg/mL SiNPs and those with 5 mg/mL SiNPs and the cells in each group were serially passaged for 30 generations. At each generation, the cells in the 5 mg/mL SiNPs group were exposed to SiNPs in a culture medium for 24 h after cell attachment, and the cells in the 0 mg/mL SiNPs group were exposed to an equivalent volume of culture medium without the SiNPs. To evaluate the function of the key miRNAs associated with apoptosis, the spermatocyte cells were transfected with mimic and inhibitor. The mimic can increase the activity of the miRNAs, whereas the inhibitor can restrain its activity. The transfection experiment of the mimic and inhibitor involved 6 groups: control group, SiNPs group, mimic transfection group, mimic negative control group, inhibitor transfection group, and inhibitor negative control group. The mimic and inhibitor were synthesized by Sangon Biotech Company, Ltd, Shanghai. Each group had three replicate wells. All experiments were carried out at least three times.

## Prediction of target genes of the miRNA

Data about the prediction of the target genes of the miRNA were obtained from MiRWalk (<https://www.umh.uni-heidelberg.de/apps/zmf/mirwalk>), RNA22 (<https://cm.jefferson.edu/rna/22>), RNA-hybrid (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>), Micro T ([Page 11/24](http://diana.imis.athena-</a></p></div><div data-bbox=)

innovation.gr/DianaTools/index.php?r=microT\_CDS/index), miRMap (<https://mirmap.ezlab.org/>), and PITA (<https://genie.weizmann.ac.il/pubs/mir07/>), RNAhybrid (<http://alk.ibms.sinica.edu.tw/cgi-bin/RNAhybrid/RNAhybrid.cgi>) from CapitalBio Technology Corporation (Beijing, China). Starbase (<http://starbase.sysu.edu.cn/>) was then used to verify the target genes of the miRNA.

## Transfection

Spermatocyte cells in the 5µg/mL group were transfected with four groups of the vectors: miRNA-450b-3p mimic group, mimic negative control group, inhibitor group, and inhibitor negative control group. The mimic of the miRNA-450b-3p (AUUGGGAACAUUUUGCAUGCAU), the mimic of the negative control of miRNA-450b-3p (GGAUAUUCAAGUGAUCUCAUGU), the inhibitor of miRNA-450b-3p (AUGCAUGCAAAAUGUCCCAAU), and the inhibitor of the negative control of miRNA-450b-3p (CAGUACUUUUGUGUAGUACAA) were devised and synthesized by Sangon Biotech Company. Ltd (Shanghai). After exposure to the SiNPs for 30 generations, cells at a density of  $1 \times 10^5$  were allowed to adhere to a six-hole plate for 24 h. After the cells were washed thrice with PBS, they were cultured in 1750 µL DMEM for transfection. The 117.5 µL of the Opti-MEM (Gibco, USA) and 7.5 µL of the mimic, mimic negative control, inhibitor, and inhibitor negative control were first mixed for 5 min, respectively, and 117.5 µL of the Opti-MEM and 7.5 µL of the EndoFectin<sup>TM</sup>-MAX transfection array (GeneCopocia, Inc., Guangzhou, China) were mixed for 5 min. The two mixed solutions described above, were again mixed together for 20 min. Then the cells were cultured for 24 h, and finally, the cells were collected for detection.

## Detection of the expression levels of the miRNA and mRNA

The total RNA was extracted from the GC-2spd cells and testis by the Trizol method according to the manufacturer's instructions (Trizol; Applygen Technologies Inc., Beijing, China). RT-PCR of the miRNA was conducted using the All-in-One<sup>TM</sup> miRNA qRT-PCR Detection Kit (GeneCopocia, Inc., Guangzhou, China). The RT-PCR of the mRNA was conducted using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) and KAPA SYBRR FAST Universal qPCR Kit (Kapa Biosystems, USA), separately. U6 and GAPDH were regarded as the references. The relative expression levels of the miRNA and mRNA were represented as  $2^{(-\Delta\Delta Ct)}$ .

## Detection of cell viability and lactate dehydrogenase

After being exposed to 5µg/mL of SiNPs for 30 generations, the GC-2spd cells were seeded into 96 wells at a density of 7000 cells per well. Then, the mimic, mimic NC, inhibitor, and inhibitor NC of the miRNA-450b-3p were transfected for 24 h. After culturing for 24 h, 10 mL of Cell Counting Kit-8(CCK8) (Dojindo, Japan) was added to the cells, and then, they were incubated for 4 h. The absorbance was detected at 450nm using a microplate reader (Thermo Multiskan MK3, USA). In order to indicate whether the cell membrane was damaged, lactate dehydrogenase (LDH) leakages were detected using the LDH Kit (KeyGEN BioTECH, Nanjing, China), as its instructions. After GC-2spd cells were transfected, we assessed



the supernatants by detecting their absorbance at 440nm using a microplate reader (Thermo Multiskan MK3, USA).

### **Detection of the GC-2spd cells apoptosis in testis**

After being exposed to 5µg/mL of SiNPs for 30 generations, the GC-2spd cells were seeded into 6 wells at a density of  $1 \times 10^5$  cells per well. Then, we transfected the mimic, mimic NC, inhibitor, and inhibitor NC of the miRNA-450b-3p for 24 h. Using the PBS washing GC-2spd cells for three times and then centrifuging the cells at 1500 rpm for 5 min and then they were suspended in 500 µL of binding buffer. There was 5 µL Annexin V-FITC and 5 µL PI added to the cells in the dark. Analysis apoptosis of the cells by flow cytometry.

### **Protein expression analysis of MTCH2 and apoptotic signaling pathway in the GC-2spd cells in vitro**

To expound the influence of the SiNPs and the miRNA-450b-3p on the expression of the cellular factors involved in the MTCH2 and apoptotic signaling pathway, the protein levels of the MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3 in GC-2spd cells were determined through western blot analysis.

After transfect of the mimic, mimic NC, inhibitor, and inhibitor NC of the miRNA-450b-3p, the proteins in the GC-2spd cells were extracted and quantified using a bicinchoninic acid (BCA) protein assay (Dingguo Biotechnology, China). The equal amount of lysate proteins (20 µg per cells group) were electrophoresed in SDS-polyacrylamide gels (12 % separation gels) and transferred to nitrocellulose membranes (Millipore, USA). After being blocked with 5% bovine serum albumin (BSA) (Epsilon, USA) and being dissolved in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) for 1 h at room temperature, the membranes were incubated with rabbit polyclonal antibodies of MTCH2 (1:500, Abcam, UK), BID (1:1000, proteintech, USA), BAX, Cytochrome C, Caspase-9, and Caspase-3 (1:1000, CST, USA) overnight at 4 °C, rabbit-anti-β-Actin (1: 1000; Santa Cruz, USA) was used as an internal control. The membranes were then washed with TBST and incubated with fluorescent anti-rabbit Ig G secondary antibody (Amyjet, China) for 1 h at room temperature. After washing with TBST three times, the antibody-bound proteins were detected with Li- Cor Odyssey system (Li-Cor Biosciences). The densitometric values of the protein bands were analyzed using Image Studio Lite Software (Li-Cor Biosciences).

### **Statistical analysis**

All the experimental data were analyzed by using the SPSS 17.0 software. Independent-sample t tests was used to analyze the data in the experiments. The values of  $p < 0.05$  was considered as statistically significant. Data were expressed as means  $\pm$  standard deviation (S.D.).

## **Declarations**

### **Funding**

This study was supported by the National Natural Science Foundation of China (Grant No. 31770441).

### **Availability of data and materials**

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

### **Authors' contributions**

All authors read and approved the manuscript. GQZ did most of the experiments and prepared tables and figures; JHL, XYL, YJS, YZ, LQG performed animal experiments; GQZ and JHL performed cells experiments; JW and YY prepared for the experiment; WG reviewed the manuscript; ZWS coordinated the laboratory work, and XQZ designed the study and reviewed the manuscript; All authors read and approved the final manuscript.

### **Ethics approval**

All animal procedures described in this study were conducted in accordance with the procedures of the Animal Experiments and Experimental Animal Welfare Committee of Capital Medical University (ethical review number: AEEI-2019-003).

### **Consent for publication**

Not applicable

### **Competing interests**

The authors have no conflict of interest to declare.

## **Abbreviations**

SiNPs: Silica nanoparticles; GC-2spd cells: spermatocyte cells ;MTCH2: mitochondrial carrier homolog 2; BID: BH3-only proteins; Caspase-9: cysteinyl aspartate-specific proteinase- 9; Caspase-3: cysteinyl aspartate-specific proteinase-3; PM: particulate matter; miRNAs: microRNAs; RT-PCR: Reverse Transcription-Polymerase Chain Reaction; Ago-CLIP: Argonaute-crosslinking and immunoprecipitation; LDH: lactate dehydrogenase; CCK8: Cell Counting Kit-8; TEM: transmission electron microscope; DMEM: Dulbecco's modified Eagle's medium; HE: hematoxylin and eosin; TUNLE: terminal deoxyribonucleotide transferase-mediated nick end labeling; FITC: fluorescein-isothiocyanate; DAPI: 4',6-diamidino-2-phenylindole; BSA: bovine serum albumin; TBS: Tris-buffered saline; BCA: bicinchoninic acid; TBST: Tris-buffered saline containing 0.05% Tween-20;

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## Figures

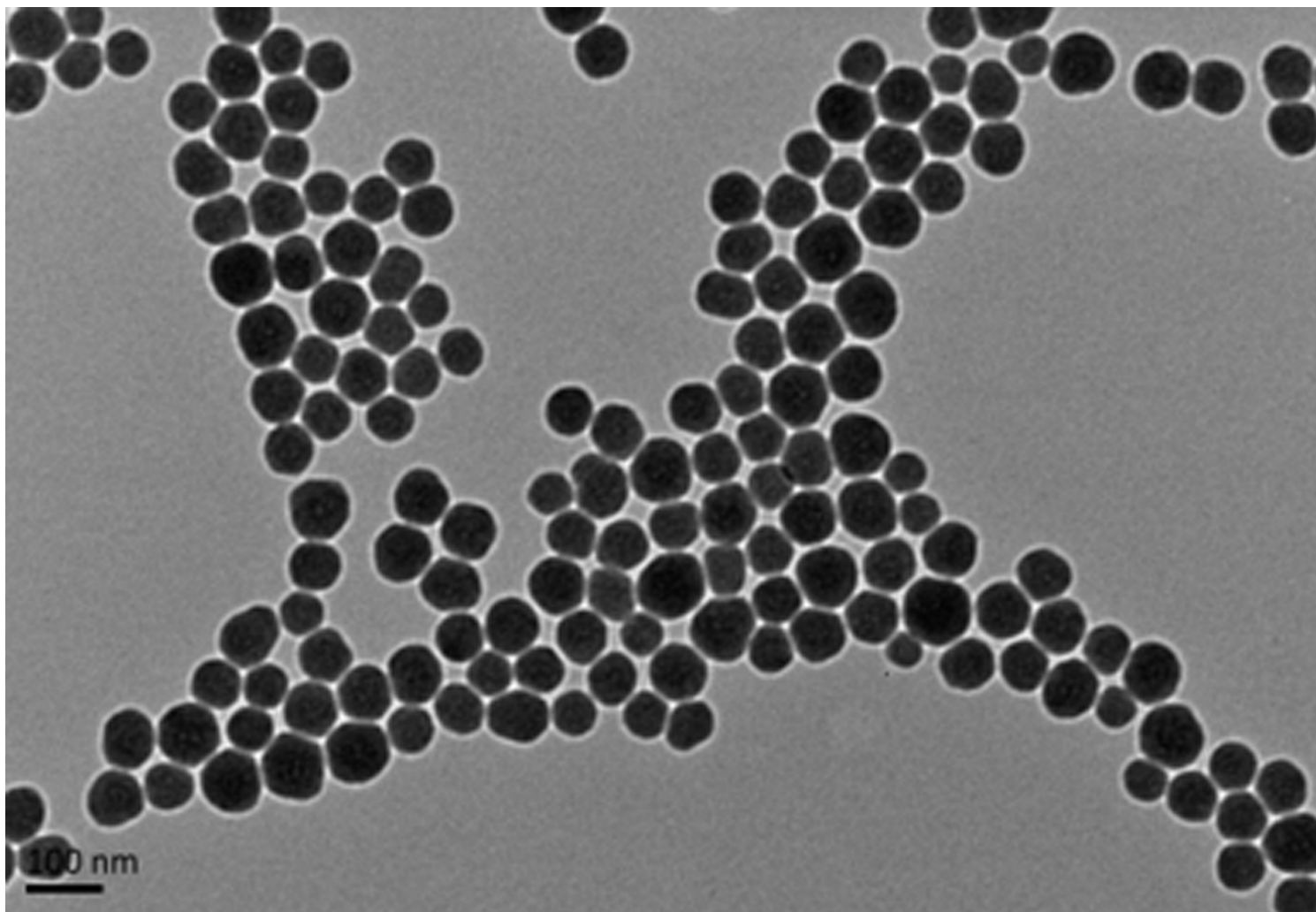
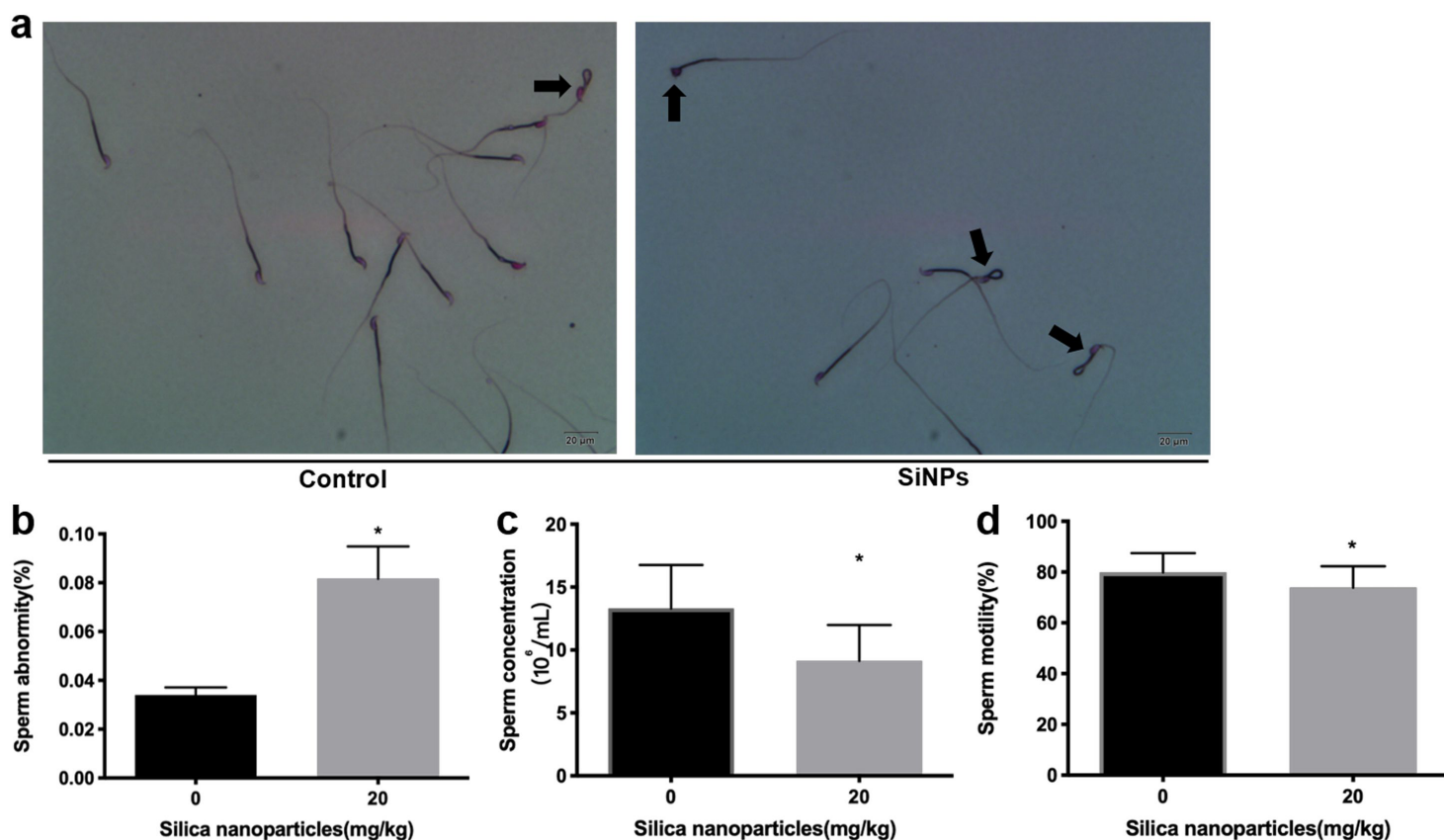


Figure 1

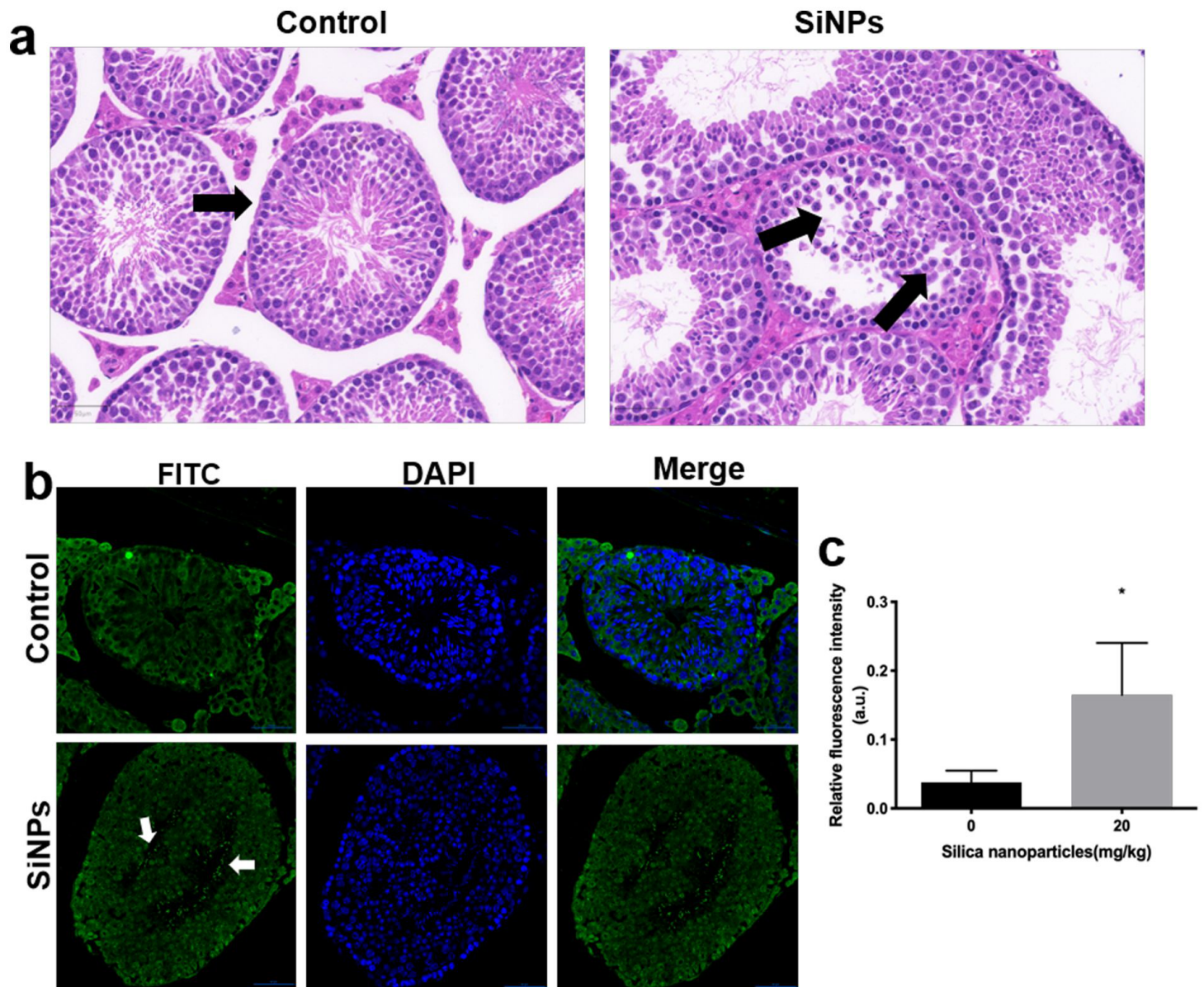
Characterization of SiNPs.



**Figure 2**

The effects of the SiNPs on the quality and quantity of sperm in mice (Mean  $\pm$  S.D.). a Spermatozoa smears stained with eosin were observed under a light microscope. Black arrows denoted the sperm with deformity. b Sperm abnormality. c Sperm concentration. d Sperm motility. \* $p < 0.05$  vs control group.

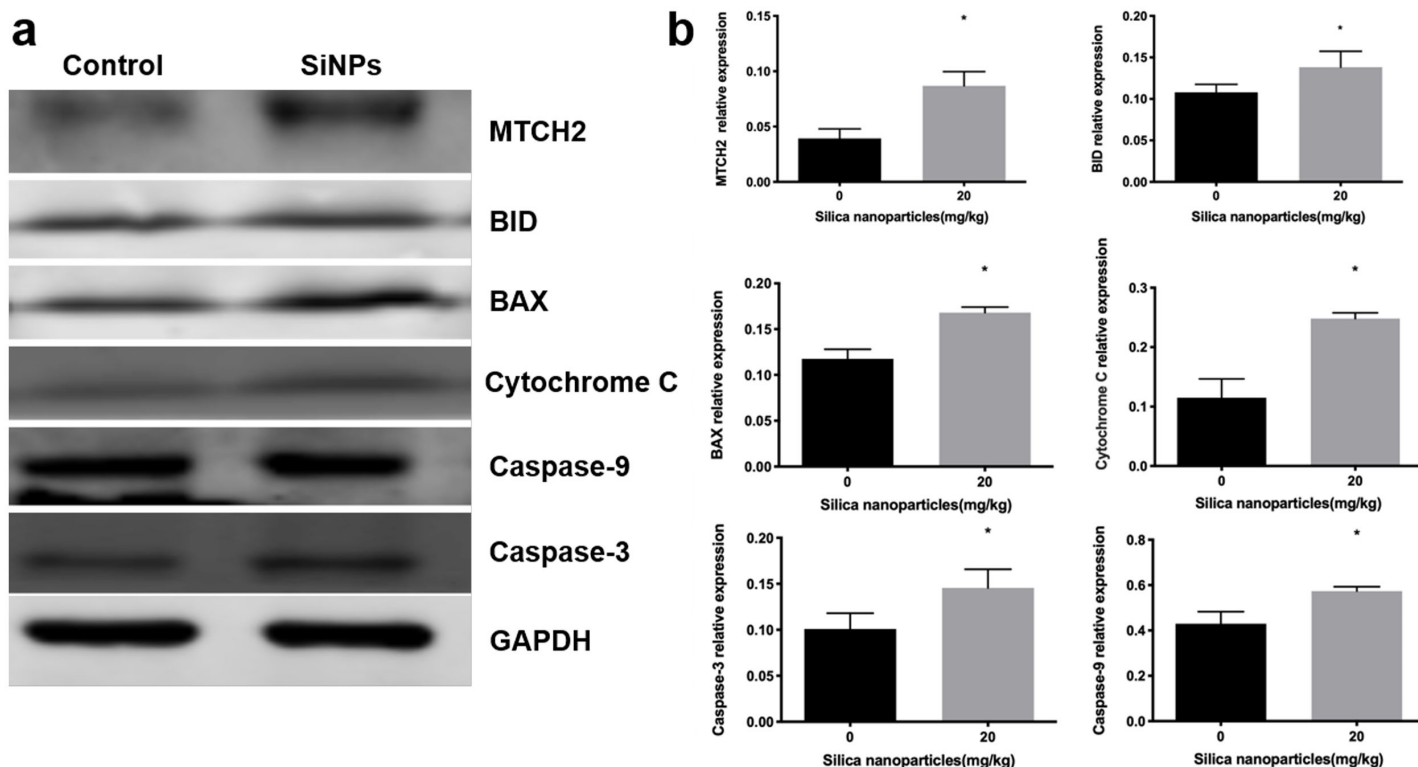




**Figure 3**

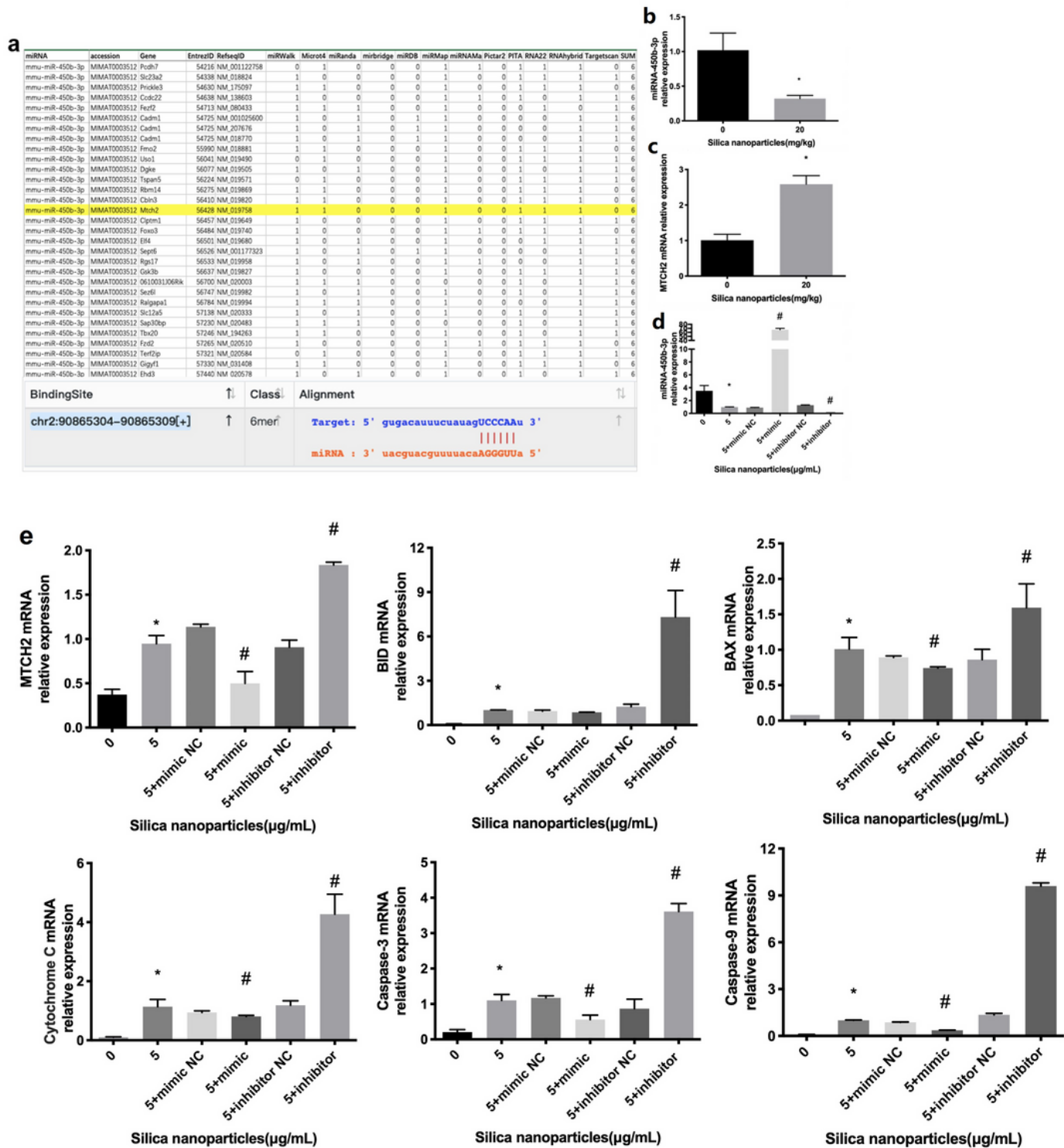
Effects of the SiNPs on the tissue structure and spermatogenic cell apoptosis of testes in mice (Mean  $\pm$  S.D.). a The tissue structure of testis. b The apoptotic cells in testis sections were stained by TUNEL and DAPI and observed under LSCM. c The relative fluorescence intensity analyzed by image J analysis software. \* $p < 0.05$  vs control group.





**Figure 4**

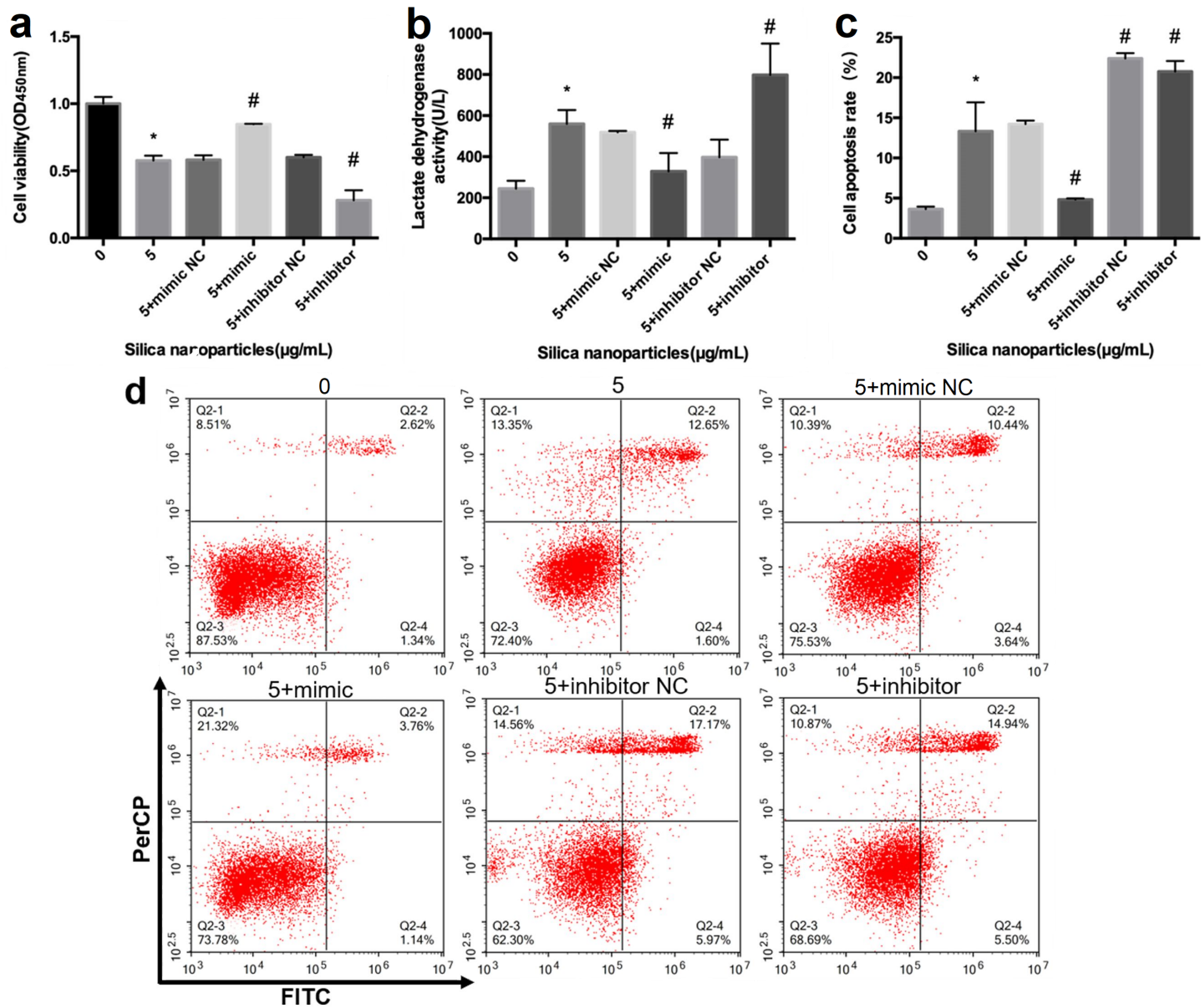
Effects of the SiNPs on the tissue structure and spermatogenic cell apoptosis of testes in mice (Mean  $\pm$  S.D.). a The tissue structure of testis. b The apoptotic cells in testis sections were stained by TUNEL and DAPI and observed under LSCM. c The relative fluorescence intensity analyzed by image J analysis software. \* $p < 0.05$  vs control group.



**Figure 5**

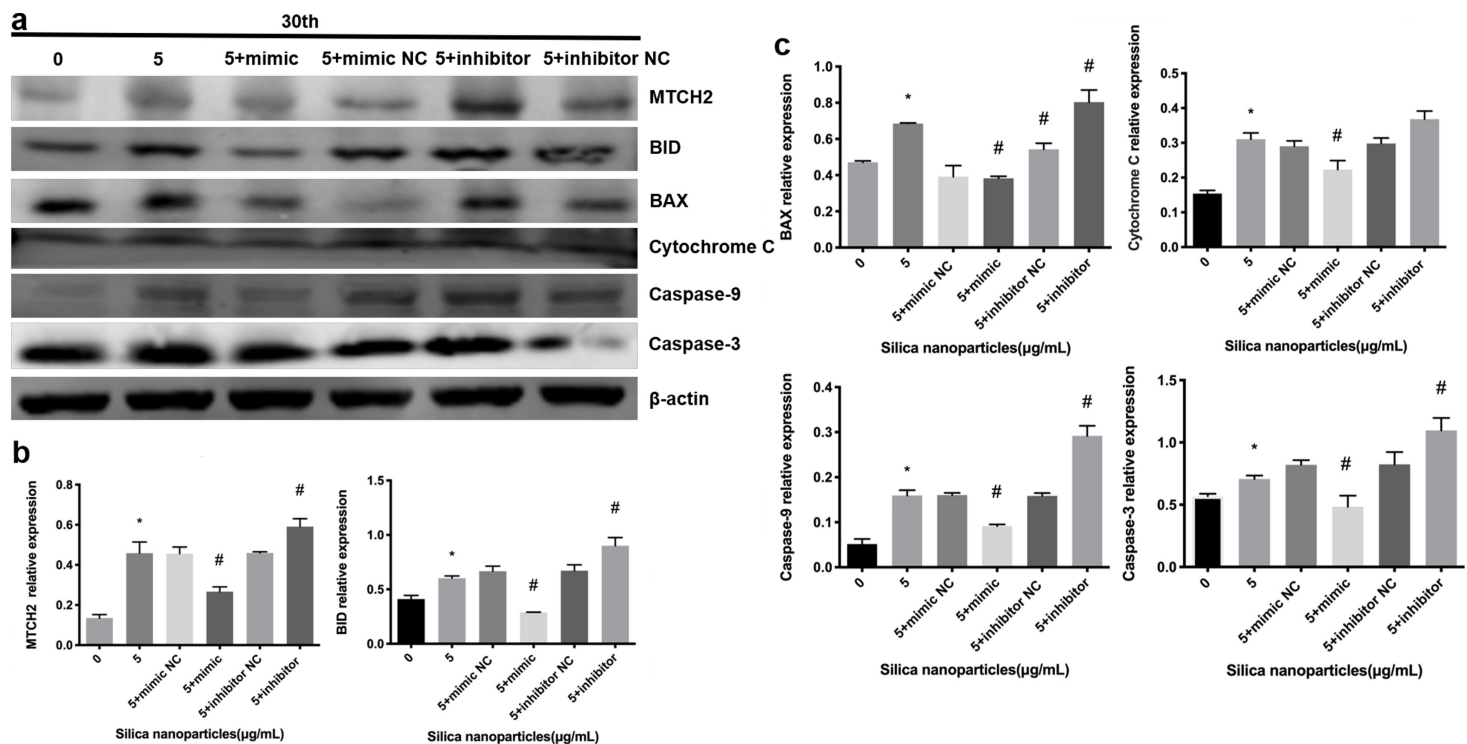
Prediction and verification of the miR-450b-3p target gene in mice and GC-2spd cells (Mean  $\pm$  S.D.). a The target gene prediction of the miRNA-450b-3p. b The expressions of the miRNA-450b-3p in testis. c The mRNA expression of the MTCH2 in testis. d The expression changes of the miRNA-450b-3p in the GC-2spd cell after exposure to the SiNPs for 30th generations with miR-450b-3p mimic and inhibitor. e mRNA levels MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3 of after exposure to silica nanoparticles

for 30 generations with miR-450b-3p mimic and inhibitor. \* $p \leq 0.05$  vs control group. # $p \leq 0.05$  vs 5  $\mu\text{g/mL}$  SiNPs group.



**Figure 6**

Prediction and verification of the miR-450b-3p target gene in mice and GC-2spd cells (Mean  $\pm$  S.D.). a The target gene prediction of the miRNA-450-3p. b The expressions of the miRNA-450b-3p in testis. c The mRNA expression of the MTCH2 in testis. d The expression changes of the miRNA-450b-3p in the GC-2spd cell after exposure to the SiNPs for 30th generations with miR-450b-3p mimic and inhibitor. e mRNA levels MTCH2, BID, BAX, Cytochrome C, Capase-9, and Caspase-3 of after exposure to silica nanoparticles for 30 generations with miR-450b-3p mimic and inhibitor. \* $p \leq 0.05$  vs control group. # $p \leq 0.05$  vs 5  $\mu\text{g/mL}$  SiNPs group.



**Figure 7**

Effects of the SiNPs, miR-450b-3p mimic and inhibitor on the protein expression of the MTCH2 and apoptotic signaling pathway in GC-2spd cells in vitro (Mean  $\pm$  S.D.). a The expressions of MTCH2, BID, BAX, Cytochrome C, Caspase-9, and Caspase-3 in GC-2spd cells. b Densitometric analysis about the MTCH2 and BID protein bands.  $\beta$ -actin was internal control protein. c Densitometric analysis about the BAX, Cytochrome C, Caspase-9, and Caspase-3 protein bands.  $\beta$ -actin was internal control protein. \* $p < 0.05$  vs control group. # $p < 0.05$  vs 5  $\mu$ g/mL SiNPs group.

## Supplementary Files

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