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ORIGINAL ARTICLE

Regulation of fertilization in male rats by CatSper2 knockdown

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Interest in ion channels as drug targets for contraception has grown with the realization that certain ion channel subunits are located exclusively in sperm. Selective knockdown of ion channel subunits can lead to infertility without ill effects, and selective inhibitors and/or openers of these ion channels could interfere with sperm function. In this study, *in vivo* electroporation (EP) and rete testis microinjection-mediated plasmid DNA were adopted to silence CatSper2 expression, which is essential in sperm hyperactivation. The results showed that high transfection efficiency and expression were achieved by plasmid DNA that was directly injected into the rete testis. As a result of the expression of CatSper2 being blocked, the treatment group showed significantly lower ($P<0.05$) hyperactivation rate, fertilization rate *in vitro*, migration motility in viscoelastic solution and intracellular Ca^{2+} peak. The low hyperactivation and fertilization rates lasted for 60 days. Meanwhile, analysis of the sperm survival rate and testis histology indicated that *in vivo* EP had no significant effect on the function of the testis, spermatogenesis or sperm activity. The present study demonstrated that it was feasible to achieve male contraception by silencing the expression of CatSper2, the key protein involved in sperm hyperactivation.

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INTRODUCTION

Great advances in contraceptive research have been achieved in the past few decades, but there are still only a limited number of contraceptive measures or drugs available for men.[1,2] Such limitations may be due to various factors including the difficulty in suppressing the generation of large numbers of sperm. Hormonal contraception that blocks sperm production by disrupting the hypothalamic–pituitary–testicular axis is currently recommended, but this approach still has some obstacles to overcome.[2,3] One major hurdle is that complete hormonal suppression of spermatogenesis is necessary. Considering the drawbacks of hormonal methods, there is a growing interest in the development of non-hormonal methods for male contraception. Presently, several approaches are being pursued,[4] including the development of gene vaccines for sperm surface antigens[5] and drugs designed to cause retention of sperm in the epididymus.[6] Chinese medicines such as gossypol and *Tripterygium* and other active ingredients have also received increased international research interest.[4] The non-hormonal methods are appealing because of their minimal side effects, reversibility and because these methods work by means of agent or molecular regulation.[4] This study focused on a non-hormonal method of molecular regulation.

Ion channels regulate the permeability of the plasma membrane and maintain the proper intracellular ionic environment, which is closely related to regulation of cell excitability, potential changes, cell secretion, cell volume maintenance and epithelial membrane electrolyte fluidity.[7–10] With studies on ion channels progressing, researchers have generally agreed that ion channel regulation will be an important breakthrough of the next generation of contraceptives.[11,12] Clarification of the role and function of the CatSper ion channel protein family suggests that it may be a viable drug target, which supports this prediction.[11,13] As a unique ion channel that mediates Ca^{2+}, the CatSper proteins play an important role in sperm hyperactivation and fertilization capacity.[14] The CatSper family consists of four homologous proteins[14] whose expressions are exclusively located in the tissue of testis and sperm at different levels. CatSper1 and CatSper2 proteins form a homodimer that is mainly located at the principal piece of the sperm tail flagellar plasma membrane. Lack of either protein will accelerate the frequency of sperm flagellar beat and diminish the amplitude of the beat, which ultimately causes failure of fertilization.[13,15] The failure to achieve functional expression of CatSper1 and CatSper2 singly or in conjunction with heterogonous expression systems suggests that CatSper1 and CatSper2 proteins require additional subunits and/or interaction partners to function.[11,16] Previous studies have confirmed high expression of CatSper3 and CatSper4 in the sperm acrosome. Therefore, these proteins may play roles in regulating intracellular Ca^{2+} levels in the process of acrosome reaction.[17] However, recent studies have demonstrated a possibility of a formed tetramer by the four family members to mediate sperm hyperactivation.[18]

In contrast to higher homology between CatSper3 (mouse *vs.* rat 61.1%) and CatSper4 (mouse *vs.* rat 87.4%), the homology between Catsper1 and Catsper2 is low; however, the function and structure of

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CatSper1 and CatSper2 are very similar. These two proteins are expressed at different levels along the flagellum.[11,16] It has been shown that the levels of Catsper1 mRNA in ejaculated human spermatozoa could be a more feasible method for infertility screening than testis biopsy. Furthermore, it has been suggested that human CatSper1 could be a possible target for immunocontraception.[19]

In 1982, Neumann *et al.*[20] imposed short but powerful electricity pulses on cells to transiently enhance transduction and permeability of the cells. This electroporation-mediated transfection of nucleotides or drugs into cells has developed into a very effective biological technique.[21,22] In recent years, a number of studies in mice have shown that it is possible to use this approach to express reporter genes such as *lacZ* and green fluorescent protein (GFP) and its variants, under the control of constitutive or testis-specific gene promoters, in both spermatogenic cells[23–28] and testicular sperm.[25] These findings have led to the proposal that *in vivo* gene transfer into the testis provides an alternative way to study gene expression in testis and sperm, and to potentially explore male contraceptives.[29]

The purpose of this study was to verify the feasibility of using CatSper2—the critical protein in the sperm hyperactivation signalling pathway—as a target for male contraception. This was done by designing a target sequence according to the 785 sequence of the open reading frame of the CatSper2 subunit and inserting the target sequence into the U6 promoter expression vector. Electroporation-mediated transfection was performed in testis following rete testis microinjection *in vivo*. CatSper2 protein synthesis was blocked by continuous transcription of double-stranded short hairpin RNA (shRNA) in testis seminiferous tubules, which induced sterility in male rats.

MATERIALS AND METHODS

Experiment animals

Specific pathogen-free-class young male (Sprague–Dawley rats (licence No. SCXK-20080033) weighing between 300 and 400 g were purchased from the Experimental Animal Centre of Zhejiang Province, China, and were acclimatized in the Experimental Animal Centre of Nanjing Agricultural University, China. All the experimental procedures were in accordance with the rules of the Animal Experimental Committee of Nanjing Agricultural University. The experimental Sprague–Dawley rats were randomly divided into 3, 5, 9, 13, 22 and 60 days treatment groups, as well as the negative control (NC) GFP group and a control group.

Construction and synthesis of small interfering RNA (siRNA) plasmid vector

Three targeting sequences for Catsper2 in the open reading frame were designed *in vitro*, as described in our previous paper.[30] The results showed that Catsper2-785 was an optimal target sequence with the highest knockdown efficacy; therefore, Catsper2-785 was chosen as the only targeted sequence site in the *in vivo* electroporation (EP) and for plasmid construction (GeneBank accession No. NM_001012220.1). We designed Catsper2 shRNA oligonucleotides (sense, 5′-CACCGAATATTCCCGTTCAACTATTCAAGAGATAGTTGAACGGGAATATTCTTTTTTG-3′; antisense, 5′-GATCCAAAAAAGAATATTCCCGTTCAACTATCTCTTGAATAGTTGAACGGGAATATTC-3′) to test. The NC shRNA oligonucleotides were designed to have no significant homology with CatSper2 (sense, 5′-CACCGTTCTCCGAACGTGTCACGTCAAGAGATTACGTGACACGTTCGGAGAATTTTTTG-3′; antisense, 5′-GATCCAAAAAATTCTCCGAACGTGTCACGTAATCTCTTGACGTGACACGTTCGGAGAAC-3′). These oligonucleotides were annealed to generate double-stranded DNA and were ligated into the linearized empty vector pGPU6/GFP/Neo. The siRNA plasmids encoded shRNAs that specifically targeted CatSper2 mRNA. The shRNAs had no significant homology with other known genes. The nucleotide sequences of CatSper2 siRNAs (pGPU6/GFP/Neo-CatSper2) were verified by automated DNA sequencing. All of the plasmids were purchased from Genepharma (Shanghai, China), multiplied in the DH5α strain of *Escherichia coli* and purified using Qiagen Mega Kits (Qiagen, Shanghai, China). All other reagents were of analytical grade.

In vivo electroporation and detection of transgene expression

Male Sprague–Dawley rats were anaesthetized by intraperitoneal injection of pentobarbital sodium (75 mg kg^{-1}), and their testes were exposed under the dissecting microscope. The left testis was treated as the experimental group and the right testis remained completely untouched throughout the procedure to act as a control. The accuracy of the injection was monitored by adding 0.04% Trypan blue to the DNA solution. The DNA solution was injected into the rete testis using a glass injection pipette visualized with a microscope.[31] Injection into the rete testis was chosen over direct injection into the testis because it introduced the plasmid DNA directly into the seminiferous tubules. This method resulted in little damage to the testes.[24,28] To increase the injection volume, the injection pipette was connected to a polyethylene tube filled with the DNA solution, and the injection was performed until almost all of the surface tubules (80%–100%) became stained with Trypan blue (typically 40–70 μl, 25–50 μg). After the DNA injection, EP was performed with an Electro Square Porator ECM-2001 (BTX, San Diego, CA, USA). The testis was held in a tweezers-type electrode (BTX). Square electrical pulses were applied six times at 30–60 V with a time constant of 30–50 μs following the technique of Muramatsu *et al.*[23] The rats were subsequently sacrificed at 3, 5, 9, 13, 22 and 60 days ($n=4$) after surgery. Testes (left experimental and right negative control) from the animals killed on specified days were torn apart in phosphate-buffered saline (PBS) and analyzed *in vivo* using bioluminescence imaging. During the imaging, two consecutive scans of 2 min each were acquired (excitation wavelength, 480 nm; emission wavelength, 530 nm). The captured images were quantified using the Living Image software package (Xenogen; Model Animal Research Institute, Nanjing University, China). The images were represented using a pseudocolour scale, with the colors red and blue representing the most and least intense luminescence, respectively.

To evaluate GFP expression in epididymis-matured sperm (with the exception of the sperm viability assay, the sperm motility was maintained at greater than or equal to 72% in all of the experiments, and the decrease rate of the sperm motility was kept to within 5% during handling), rats were killed, and their epididymides were separated from their testes. The testes were snap-frozen in liquid nitrogen for other analyses. The epididymal sperm were obtained by partially macerating the epididymis in PBS and allowing the sperm to swim freely from the surrounding tissue. The epididymal sperm were then analyzed using a BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and Cell Quest software (Becton Dickinson).

Detection of spermatogenic CatSper2 knockdown efficiency

Quantitative polymerase chain reaction and Western blot analysis were performed to determine the efficiency of CatSper2 knockdown in the transfected testes and epididymal sperm samples collected from 3 to 60 days. Quantitative RT-PCR was performed with the SYBR PrimeScript RT-PCR Kit (TaKaRa, Dalian, China). Glyceraldehyde-3-phosphate dehydrogenase was used in parallel for each run as an

internal control. The 115-bp CatSper2 and 317-bp glyceraldehyde-3-phosphate dehydrogenase products that spanned 3–5 exons were amplified using the following primers: 5′-GGCTTGAGCCAAGCTGTACCA-3′ as the forward primer and 5′-AAGCGCACTAGCTGCTCCTGA-3′ as the reverse primer for CatSper2, and 5′-ATCACTGCCACTCAGAAG-3′ as the forward primer and 5′-AAGTCACAGGAGACAACC-3′ as the reverse primer for glyceraldehyde-3-phosphate dehydrogenase. All of the primers were synthesized by Invitrogen (Shanghai, China). After protein quantification, the cell extract was resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and was electroblotted onto nitrocellulose membranes (Bio-Rad, Shanghai, China). The membranes with the transferred proteins were incubated with goat anti-rat CatSper2 polyclonal antibody (1 : 800; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as the primary antibody, followed by incubation with horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin (IgG) (1 : 3000) as the secondary antibody. Chemiluminescence reaction was carried out with an ECL kit (Pierce, Rockford, IL, USA) for 1 min and then exposed to X-Omat radiographic film (Kodak, Shanghai, China). To assess sample loading, blots were stripped and reprobed using anti-β-actin (1 : 1000) and a goat anti-mouse IgG–HRP conjugate (1 : 3000). The amount of each protein sample was controlled by β-actin.

Epididymal sperm intracellular Ca^{2+} determinations and image analysis

Epididymal sperm cells (5×10^6 cells ml^{-1}) were stained with 5 μmol l^{-1} of fluo3-AM, a fluorescent Ca^{2+} indicator dye (in 0.5% DMSO) for 40 min at 37 °C in HEPES medium (containing 400 mmol l^{-1} sucrose, 50 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 20 mmol l^{-1} HEPES (pH 7.4), 1 mmol l^{-1} EGTA).[32] Extracellular dye was removed by centrifugation at 170*g* for 5 min. Sperm were resuspended in HEPES medium and incubated for an additional 20 min to allow for de-esterification of the dye into its charged and Ca^{2+}-sensitive form. Then, 0.5 ml of the sperm suspension (loaded with fluo-3 AM) was transferred to a 3 ml acrylic cuvette and held at a constant temperature of 37–39 °C with constant stirring A BD FACSCalibur flow cytometer running Cell Quest software was used to assess and analyze the data. Hyperactivation was then induced as previously described[33] by incubating sperm in Tyrode's albumin lactate pyruvate solution containing 50 μmol l^{-1} thimerosal (containing EGTA) and in Bigger–Whitten–Whittinghan medium containing 5 mmol l^{-1} procaine. Response calibration was carried out by measuring the fluorescence intensity of the tetracarboxylate form of the indicator in solutions with precisely known free Ca^{2+} concentrations. Calibration solutions were based on EGTA Ca^{2+} HEPES buffering. The following equation was used to determine the ion dissociation constant (K_d):

$$[Ca^{2+}]_{free}=K_d\times(F–F_{min})/(F_{max}–F)$$

where F_{min} was the fluorescence intensity of the indicator in the absence of calcium, F_{max} was the fluorescence of the calcium-saturated indicator and F was the fluorescence at intermediate calcium levels. When the K_d was known, the same equation was used to obtain $[Ca^{2+}]_{free}$ for experimental samples from the measured values of F. Fluorescence intensity was detected with a flow cytometer using a 480±40 nm excitation filter and a 535±50 nm emission filter. A side angle (SSC) F1 (fluo-3 AM) plot was used to identify the sperm. An F1 histogram was used to mark the mean fluorescence intensity. In each treated group, 10 000 sperm were detected. The ratio method by Grynkiewicz *et al.*[34] was used to calculate the sperm cell Ca^{2+} concentration.

The fluorescence of individual, motile sperm in an open microchamber at 39 °C was monitored for single-cell Ca^{2+} imaging before and after the treatments. Fluorescence intensity was detected with an epifluorescence microscope using a 480±40 nm excitation filter and a 535±50 nm emission filter with an oil immersion ×40 Fluar objective (Leica Inc., Oskar-Barnack-Straße, Germany).

Fertilizing capacity of epididymal sperm after *in vivo* EP

In order to compare the fertilizing capacity of spermatozoa with cauda epididymides, *in vitro* fertilization experiments were carried out with treated and control sperm. The procedure for *in vitro* fertilization was done as previously described.[35,36] Briefly, immature female rats (250–300 g) were given 600 IU of pregnant mare's serum gonadotropin and 600 IU of human chorionic gonadotrop(h)in (Ningbo Second Hormone Co., Ltd, Beijing, China) and were killed 68–70 h later by pentobarbitone sodium. Their ovaries and oviducts were dissected in PBS, and the oviducts were ruptured by needle to release the cumulus mass. Diluted sperm suspension (150 μl, 7×10^5 spermatozoa ml^{-1}) was transferred to the 450 μl *in vitro* fertilization dishes containing 5–10 oocytes (Fertilization Medium K-SIFM-20; Cook Medical, Brisbane Technology Park, Brisbane, Qld, Australia). In previous cases where the zona pellucida was removed, the ovulated mouse eggs were treated with hyaluronidase (100 units ml^{-1}, type I-S; Sigma, Shanghai, China) to remove zona pellucida, and then the eggs were washed and exposed briefly to acidic Tyrode's solution to dissociate the zona pellucida.[37] Gametes were cocultured at 37 °C under 5% CO_2 for 5 h (Cleavage Medium K-SICM-20; Cook Medical). Subsequently, all the oocytes and zygotes were transferred to a washing medium to remove excess spermatozoa. The oocytes and zygotes were examined 19–24 h after insemination as previously described.[35,36,38] Successful fertilization of an oocyte was scored as two-cell embryos 25–28 h after insemination.

Epididymal sperm cell motility and viscosity experiment assays

Spermatozoa were obtained by applying gentle pressure to excised caudal epididymal tissue nicked at four sites with scissors. Cells that dispersed into the modified Bigger–Whitten–Whittinghan solution at 37 °C were collected after 15 min.[18] Subsequently, sperm cells were observed on slides on a 37 °C stage of a Nikon 35 microscope and were videotaped using ×400 differential interference microscopy (Nikon Microscopy Inc., Tokyo, Japan) Stroboscopic illumination at 30 Hz was provided by a xenon flash tube (Chadwick-Helmuth Co., El Monte, CA, USA). Computer-assisted semen analysis (CASA) was performed to analyze the sperm by a ×4 Nikon negative-phase objective. Video images were digitized (30 frames at 60 Hz) and analyzed using HTM-IVOS, ver. 10 (Hamilton-Thorne Research, Beverly, MA, USA). Sperm motility was assessed by video capture of the swimming pattern and by CASA analysis (WLJY-9000) of path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH) and linearity (LIN). From each treated sample, 400–450 motile sperm were analyzed. Each experiment was repeated three times using three males from each treatment. Because the movement of the sperm heads depended on flagellar bending and beating patterns, increased VCL and ALH, and decreased LIN were indicative of hyperactivation.[39,40]

Sperm cells that dispersed into 1 ml prewarmed (37 °C) modified Bigger–Whitten–Whittinghan medium were assessed for viability using a live-to-dead count ratio. After the sperm were incubated for 3 min, 100 μl of sperm were mixed with 9 mmol l^{-1} 5-carboxyfluorescein diacetate (Sigma) and 24 μmol l^{-1} of the nuclear stain

propidium iodide (Sigma). After a 5-min incubation, samples were analyzed using fluorescence microscopy. At least 200 sperm were counted per epididymis, and the number of live sperm versus dead sperm was determined. In the viscosity experiments, long-chain polyacrylamide (Invitrogen, Shanghai, China) was dissolved directly in the medium by stirring overnight at room temperature, resulting in a 2% stock solution (viscosity of 4000 cP, according to the manufacturer's instructions; 1 P=0.1 Pa s). This medium was diluted to 0.75% with freshly prepared medium and 18 μl of the medium was overlain onto 2 μl of spermatozoa in medium on a prewarmed 80 μm chamber slide. A coverslip was then placed over the sample, and an analysis was performed with the WLJY-9000 Sperm Analyzer, as described above. In the assay, means were obtained from the 20 sperm measured for each group (mock, negative control and treatment).

Statistical analysis

All of the data were obtained from one independent experiment carried out in triplicate and were presented as mean±s.d. The main and interactive effects were analyzed by a one-way ANOVA using SPSS13 software (SPSS Inc., Chicago, IL, USA). When justified by one-way ANOVA, differences between individual groups were analyzed by Fisher's PLSD test. Differences were considered statistically significant if $P<0.05$.

RESULTS

Transfection efficiency and cellular targets of plasmid DNA in the testis

The bioluminescence imaging signal data (**Figure 1**) from this study demonstrated that there was a strong positive correlation of the plasmid GFP signal with the amount of plasmid injected when the injection amount ranged from 25 to 45 μg. The testicular GFP signal was the

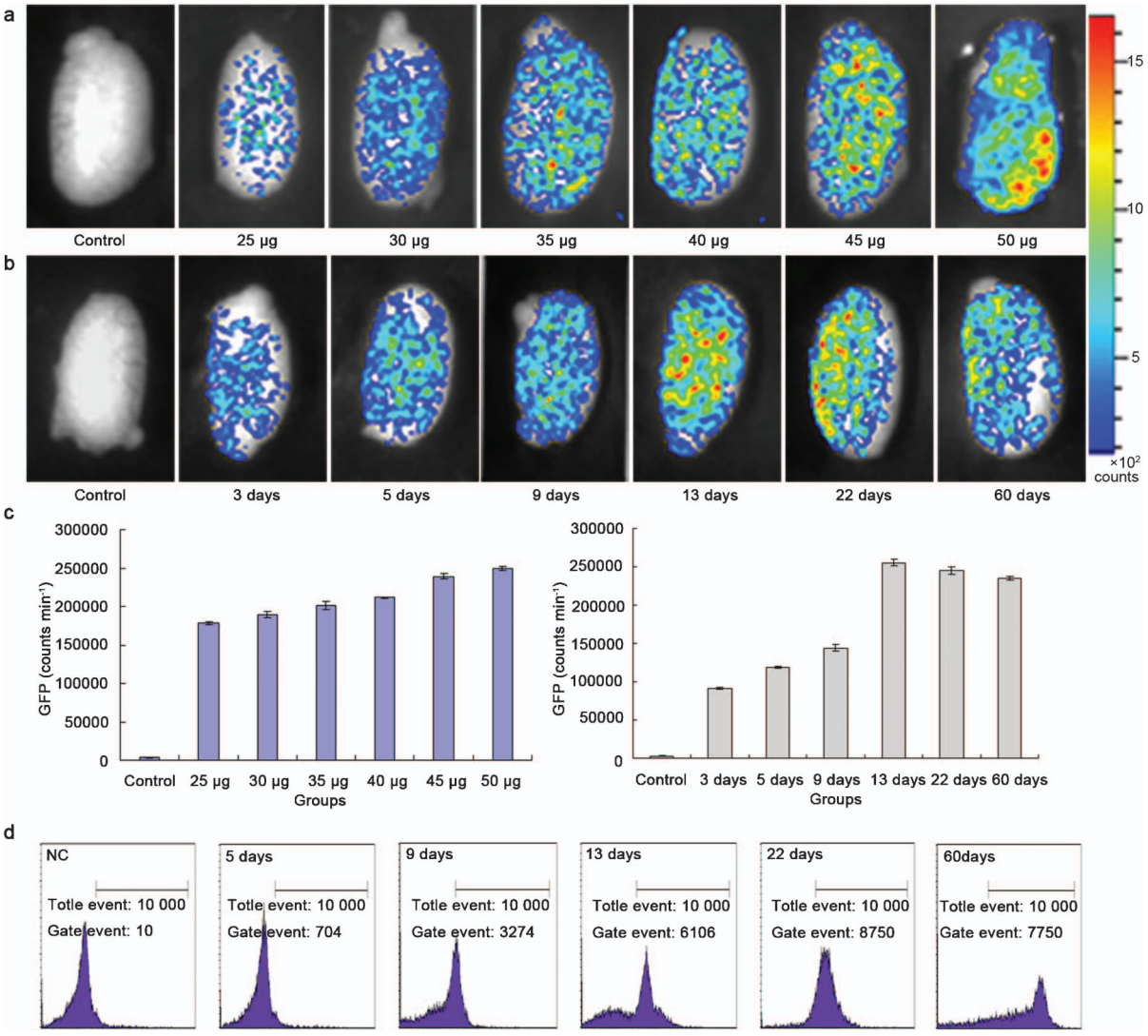

Figure 1 The detection of the sperm transfection rate using *in vivo* Imaging-200 System (IVIS-200 System) and flow cytometry. (**a**) The intensity of GFP expressed in rat testis as a function of the plasmid dose. (**b**) The intensity of GFP expressed in rat testis observed up to 60 days following plasmid transfection *via in vivo* testis electroporation. The GFP fluorescence intensity was detected by the IVIS-200 System (Xenogen; Caliper Life Sciences, Hopkinton, MA, USA). The pseudocolor image of the rat testis represents the relative expression of GFP. (**c**) The exactitude fluorescence intensity histogram description of **a** and **b** ($n=3$). (**d**) The GFP-positive epididymal-derived sperm, as detected by flow cytometry: 0.7% (704/10 000) GFP-positive sperm are detected at 5 days, while 32.7% (3274/10 000) and 61.1% (6106/10 000) are shown at 9 days and 13 days, respectively, after transfection. The maximum percentage of positive cells is observed at 22 days, which is 87.5% (8750/10 000), and 77.5% (7750/10 000) of positive cells are found at 60 days in rat epididymis ($n=3$). GFP, green fluorescent protein; NC, negative control.

strongest when each side of the testicle was injected with 70 μl PBS solution containing 50 μg pGP/U6/GFP/Neo-siRNA-NC. The signal was weaker when the 70 μl PBS contained 45 μg pGP/U6/GFP/Neo-siRNA-NC, but the difference between the two signals was not statistically significant. In the 60-day test period, the testicular fluorescence signal peaked at 13 days and diminished thereafter; however, the strong fluorescence signal could still be detected on 60 days and showed no statistical difference from the signal on 13 days.

The flow cytometric analysis of epididymal sperm from the initial transfection (**Figure 1d**) showed that few cells with a fluorescence signal were detected. As the expression of shRNA in the testis increased, the number of GFP-positive sperm cells in the epididymis increased to 87.5% 22 days after transfection and decreased to 77.5% by 60 days, but no significant difference was found between the two numbers of GFP-positive sperm. The sperm morphology test was not repeated in this experiment because the sperm morphological analysis in our previous *in vitro* EP-mediated siRNA transfection experiments confirmed that both sperm morphology and physiology were not severely affected by EP.[30]

As shown in **Figure 2a**, the knockdown effect of shRNA specific to CatSper2 mRNA in testis and epididymal sperm was markedly time-dependent. The maximum knockdown to testicular CatSper2 mRNA reached 88.0% 13 days after transfection and decreased afterwards. However, the knockdown was still at 70.1% at 60 days, a value not much different from the value at 13 days, which indicated a long period of effective knockdown. The CatSper2 mRNA knockdown in epididymal sperm reached the peak value at 22 days, later than in the testis. The CatSper2 mRNA knockdown was 83.4% at 22 days after transfection *in vivo*, and it decreased very little afterwards.

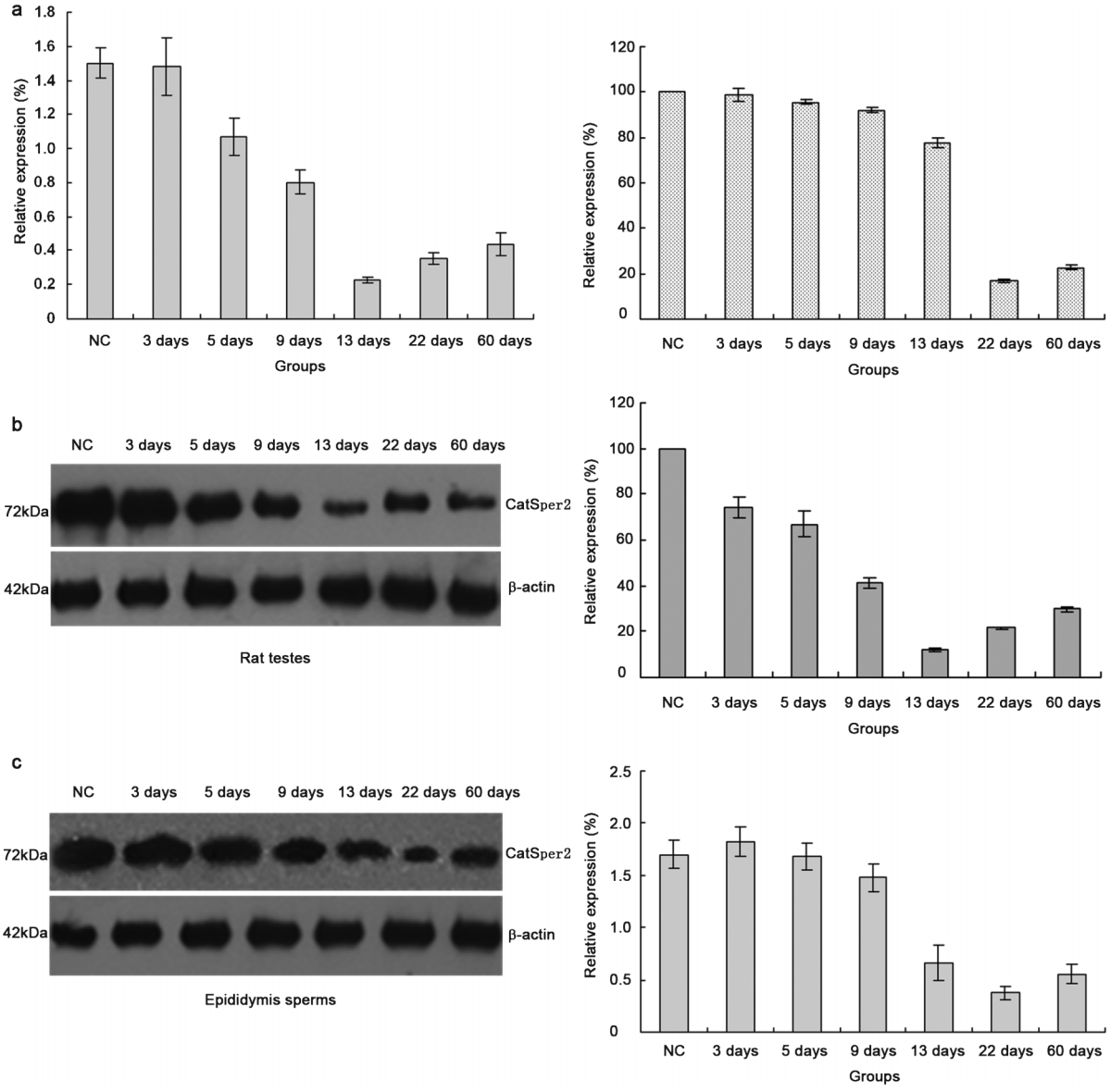

Figure 2 Knockdown efficiency of plasmid on CatSper2 ion channel proteins in testis and epididymis. (**a**) The relative expression of CatSper2 mRNA in testis (left) and epididymal sperms (right). (**b**) The knockdown of CatSper2 protein in testis. The Western blot analysis of shRNA-CatSper2 knockdown in testis (left) and the exactitude histogram description of relative expression of CatSper2 after interference in testis (right). (**c**) The knockdown of CatSper2 protein in epididymal sperm. The Western blot analysis of shRNA-CatSper2 knockdown in epididymal sperm (left) and the exactitude histogram description of relative expression of CatSper2 after interference in epididymal sperm (right). NC, negative control; shRNA, short hairpin RNA.

The anti-rat CatSper2 antibody Western blot analysis showed that shRNA significantly blocked Catsper2 protein expression in testis and epididymal sperm in a strongly time-dependent manner. **Figure 2b and c** shows that, after electrotransfection *in vivo*, the highest knockdown appeared in testicular tissue (84.87%) on 13 days and in epididymal sperm (77.89%) on 22 days. With shRNA transcribed in testis, the relative expression of Catsper2 in testis and epididymal sperm did not appear to recover within a 60-day period. The knockdown of CatSper2 proteins in testis and epididymis was still at 70.87% and 67.49%, respectively, at the end of the 60-day test.

cAMP-induced Ca^{2+} influx and hyperactivation in epididymal sperm

As shown in **Table 1**, the intracellular Ca^{2+} concentration of the treated and control sperm increased markedly when sperm were incubated in the Tyrode's albumin lactate pyruvate medium containing thimerosal, independent of the presence of extracellular Ca^{2+} in the medium. Meanwhile, fluorescent imaging of fluo3-loaded individual sperm demonstrated that thimerosal raised the Ca^{2+} concentration in the head and flagellum within 25 s (**Figure 3**). However, the high concentration was maintained for only a very short time without supplementing with extracellular Ca^{2+}. In contrast, a high intracellular Ca^{2+} concentration also appeared in control groups when the sperm were incubated in procaine with a 5 mmol l^{-1} final concentration for 4 h. The Ca^{2+} concentration of the treated groups was higher than that in the normal group, but no significant difference was seen between the two groups (**Table 1**). The Ca^{2+} concentration of the treated groups was far below the normal concentration of 200–1000 nmol l^{-1} required for triggering hyperactivation.

Sperm were incubated for 1 min in Tyrode's albumin lactate pyruvate with 50 μmol l^{-1} of Ca^{2+}-free thimerosal (containing EGTA). Critical velocity or track speed (VCL), amplitude of lateral head displacement (ALH) and beat cross-frequency (BCF) detected by CASA are listed in **Table 2.** The results indicated that more than 90% of the sperm were hyperactivated in both the control and treated groups. This was much higher than in the normal sperm, whose hyperactivation rate was only 5%–10%. Subsequently, the sperm were incubated for 5 min in capacitation solution with procaine at a final concentration of 5 mmol l^{-1} CASA analysis indicated that the hyperactivation rate of treated sperm dropped to 11.1%. The swimming pattern of the sperm was characterized by helical motility with narrow amplitude and high frequency of flagellar beating, as previously reported.[13] However, the control group maintained a high hyperactivation rate of 95.7% ($P<0.05$) (**Table 2**) for a longer time when no blocking agents were added.

Fertilization efficiency suppressed by CatSper2 expression decline

The feasibility of the CatSper protein family being a target for male contraception depends on the fertilizing capacity of the sperm after RNA interference. In this study, mature sperm from rat cauda epididymis were collected and used to fertilize eggs with an intact zona pellucida *in vitro*. After 19–24 h of fertilization cleavage, no significant difference was shown in the sperm fertilization rate among the treated groups (from 0 to 13 days, 45.4%, $n=10/22$), the NC group (46.7%, $n=14/30$) and the normal group (45.8%, $n=11/24$) (**Figure 4a**). With the increased transcription of shRNA *in vivo* and the constant renewal of sperm in the epididymis, only 12.5% ($n=4/32$) of oocytes were fertilized by epididymal sperm from the 22 days treated group, evidently lower than 46.4% ($n=13/28$) from the NC group and 45.8% ($n=11/24$) from the normal group. However, when the sperm from rat cauda epididymis fertilized eggs with the extracellular matrix removed, no significant difference in cleavage rate was shown among the three groups ($P>0.05$).

Table 1 Flow cytometer (FCM) detection of sperm intracellular $[Ca^{2+}]$

Groups	*Control (nmol l^{-1})*		*siRNA-785 (nmol l^{-1})*	
	0 h	4 h	0 h	4 h
Capacitation	42.6±0.8[a]	56.6±0.5[a]	42.5±0.4[a]	41.1±1.1[a]
Thimerosal	235.1±3.8[b]	62.7±0.7[a]	232.0±3.2[b]	57.3±0.9[b]
Procaine	241.8±1.7[b]	216.7±1.7[b]	57.2±1.1[c]	61.5±0.8[b]

The results are presented as means±s.d. The different small letters in the same column indicate significant difference ($P<0.05$).

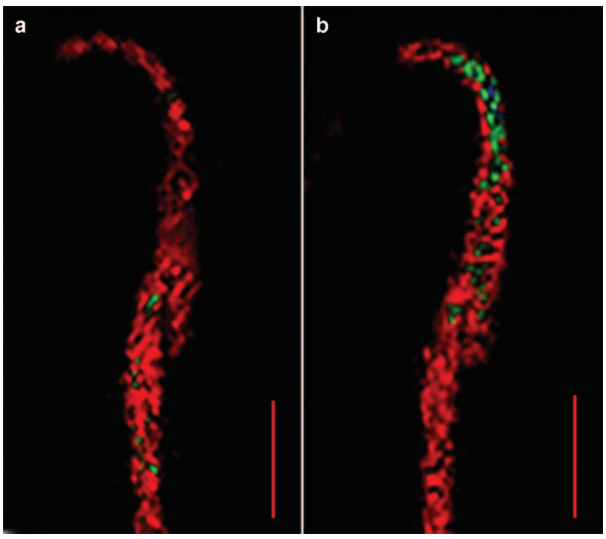

Figure 3 Ca^{2+} imaging of fluo3-loaded sperms. Pictures were taken within 25 s of applying medium without thimerosal (**a**) and with thimerosal (solution containing EGTA) (**b**). The green spots indicated higher fluorescent intensities and increased intracellular Ca^{2+} (scale bars=10 μm).

Viability and motility of transfected sperm in viscoelastic solution

Sperm quality was further investigated. The sperm cells were assessed for viability using a live-to-dead count ratio with propidium iodide staining. Compared with the control, the treated groups showed differences in fertilizability, hyperactivation rates and intracellular Ca^{2+} peak, but no differences were seen in the survival rate (**Figure 5**). To further reveal the motility status of sperm with low CatSper2 expression, a migration assay was performed in viscoelastic solution. As shown in **Figure 5**, in normal cell medium containing 0.75% (w/v) long-chain polyacrylamide, the treated spermatozoa were immobilized, whereas the spermatozoa of the control and normal groups remained progressively mobile, albeit with their forward velocity reduced by about 50%.

DISCUSSION

The CatSper protein family plays an important role in sperm hyperactivation and fertilizing capacity.[14] For the spermatozoa, abnormal expression of the CatSper protein subunit will not only lead to failure in acquiring hyperactivation, but also in the capability of penetrating the zona pellucida. In this study, CatSper2 expression was suppressed by microinjection of plasmid DNA into the rete testis combined with EP, which indicated that male fertility could be manipulated by blocking CatSper2 expression in order to suppress the signal channel of sperm hyperactivation. Furthermore, microinjection of EP-mediated

Table 2 CASA analysis of the treated and control sperm

Groups		*The non-treated groups*	*The thimerosal-treated groups*	*The procaine-treated groups*
Motile (%)	Control	65.4±0.9[a]	66.8±1.0[a]	63.5±0.4[b]
	siRNA-785	64.3±0.7[a]	60.6±0.4[c]	57.6±0.4[d]
VCL (μmol l^{-1} s^{-1})	Control	303.6±1.8[a]	345.3±1.4[b]	362.1±0.5[c]
	siRNA-785	210.4±3.1[d]	285.3±2.8[e]	212.5±2.9[d]
ALH (μmol l^{-1})	Control	17.3±0.6[a]	20.8±0.9[b]	19.1±0.4[b]
	siRNA-785	12.2±0.6[c]	20.7±0.4[b]	11.3±0.4[c]
BCF (Hz)	Control	19.7±0.3[a]	13.9±0.3[b]	23.6±0.3[c]
	siRNA-785	25.6±0.3[d]	14.8±0.2[b]	28.8±0.2[e]
Hyperactivation (%)	Control	5.5±0.5[a]	99.2±0.2[b]	95.7±0.6[b]
	siRNA-785	5.0±0.1[a]	97.5±0.6[b]	11.1±0.5[c]

The results were represented as mean±s.e. The different small letters in the same parameter of different treatment method between the treated and control groups indicated marked difference ($P<0.05$).

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; CASA, computer-assisted semen analysis; VCL, curvilinear velocity.

plasmid DNA did not affect the function of testis and the survival rate of sperm.

In non-hormonal or genetic contraceptive research, the testis is a good target because cellular renewal and biochemical reaction of spermatogenesis takes place in the seminiferous epithelium and results in the daily production of 100–200 million spermatozoa.[23,29] Potential targets for fertility intervention might be identified by elucidating the mechanisms of the events of spermatogenesis. *In vivo* gene transfer into the testis by EP has been put forward as a non-hormonal approach to study the testis and sperm function, and as a novel way of creating transgenic animals.[41–43] Selective inhibitors of ion channels could, in principle, inhibit sperm function and prevent fertilization. By applying the patch-clamp technique to mature human spermatozoa, Lishko *et al.*[44] and Strunker *et al.*[45] independently found that the Catsper ion channel of human spermatozoa is synergistically activated by elevation

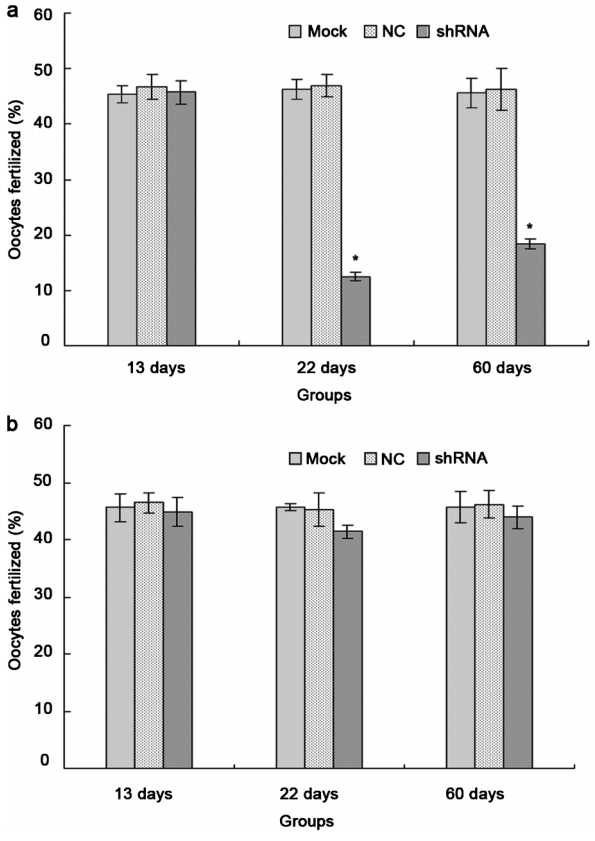

Figure 4 Fertilizing capacity of rat epididymal sperm after electrotransfection *in vivo*. The motility of sperm before and after treatment was unchanged (72%). (**a**) The oocyte fertilization rate of rat sperm following interference *in vivo*, using unaltered mature oocytes. The fertilization capacity of treated sperm decreased compared to the control group at 22 and 60 days (*$P<0.05$). (**b**) The oocyte fertilization rate of rat sperm following interference *in vivo*, using oocytes without zona pellucida ($n=3$). There are no marked differences in the cleavage rate between the control group and the experimental group. NC, negative control; shRNA, short hairpin RNA.

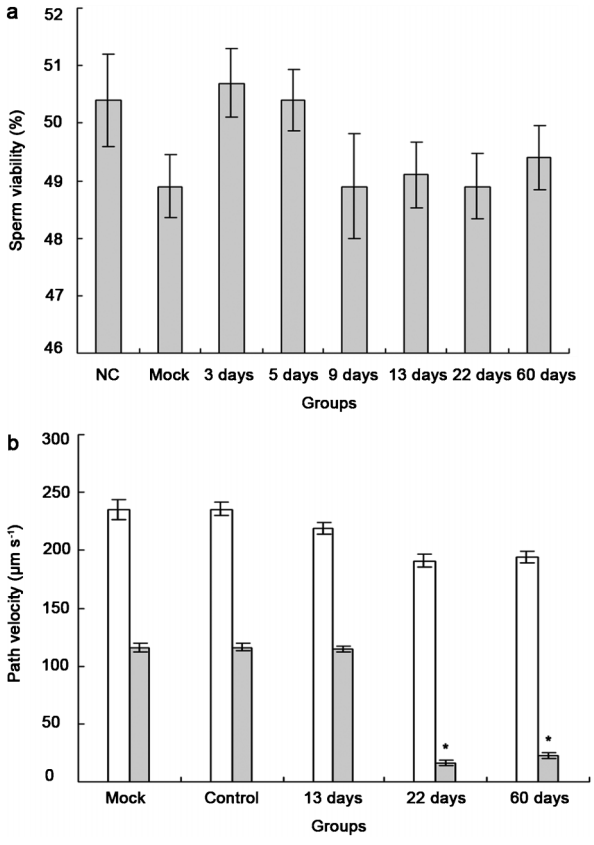

Figure 5 Viability and motility of transfected sperm in viscoelastic solution. (**a**) Sperm viability of the treated and control groups at different time points after transfection. (**b**) The open bars represent normal medium and the filled bars represent medium containing 0.75% (w/v) long chain polyacrylamide (*$P<0.05$). NC, negative control.

of the intracellular pH and extracellular progesterone, which is different from the genomic progesterone receptor. The CatSper ion channels represent a promising target for the development of a new class of non-hormonal contraceptives.[46] Nifedipine, a commonly prescribed Ca^{2+} channel blocker in the treatment of high blood pressure and migraine, was found to have reversible contraceptive effects.[25,47] In this study, we successfully blocked fertility in rats by knocking down CatSper2 ion channels with the help of EP-mediated plasmid DNA.

For sperm hyperactivation, a relatively high concentration of cellular Ca^{2+} is essential and must be sustained.[48] Ca^{2+} not only plays a role in motility but is also a key regulator in the initiation and maintenance of sperm hyperactivation.[49,50] Our study showed that thimerosal induced hyperactivation in more than 90% of sperm (**Table 2**) because there was sufficient intracellular Ca^{2+} to initiate the hyperactivation, even without extracellular Ca^{2+}. This result was similar to that seen by Ho and Suarez.[33]

Intracellular Ca^{2+} initiates sperm hyperactivation, but extracellular Ca^{2+} sustains hyperactivation. The availability of extracellular Ca^{2+} can be achieved by using procaine as an inducer.[48] It was reported that, when induced by procaine, sperm almost immediately reverted to activated motility upon transfer into medium in which extracellular Ca^{2+} was buffered below 50 nmol l^{-1}.[49,51] In this study, the control sperm acquired high hyperactivation motility when incubated in 5 mM procaine for 5 min, while the treated sperm did not (**Table 2**). The hyperactivation (11.1%) of the treated sperm was higher than in the normal sperm (5%–10%) because we did not completely block the expression of CatSper2 protein. However, the concentration of the cytoplasmic Ca^{2+} still remained low and could not evoke hyperactivation in treated sperm. Procaine failed to induce hyperactivation of treated sperm, which supported the statement that procaine evokes sperm hyperactivation by triggering Ca^{2+} influx, which is mediated by the Ca^{2+} channel on the cytolemma.[40,52]

In one mouse study, treated sperm showed relatively poor motility and progressive path velocity, but they had the same survival time as control sperm. The changes to flagellar beat increased the velocity of spermatozoa (path and track) and the frequency of abrupt turning.[48] In this rat study, the results of CASA and confocal microscopic analysis indicated that both the forward velocity parameters (**Table 1**) and percentage of motility (**Table 2**) were slightly decreased in normal sperm and treated spermatozoa. However, the treated sperm significantly increased their flagellar beat. The experiment on the viability of sperm (**Figure 5**) showed no difference between the normal, control and treated groups. These results may also support the observation that although the treated sperm failed to fertilize the eggs with integrated zona pellucida, they did fertilize the naked eggs lacking zona pellucida. It has been suggested that CatSper2 is a unique cation channel protein required for normal sperm motility and particularly for sperm penetration of the zona pellucida.[13]

Removal of the egg zona pellucida enabled treated spermatozoa to fertilize the egg, suggesting that the zona pellucida of the egg represented an absolute barrier to spermatozoa lacking CatSper2.[18] Compared to the untreated group, we found that treated spermatozoa failed to penetrate the zona pellucida of intact eggs (**Figure 4**). This finding suggests that, despite normal forward velocity and percentage of motile cells, the treated spermatozoa lack the power to penetrate the zona pellucida. To further verify the change of fertilization capability of the treated sperm, we tested the effects of elevating the viscosity of the medium on the motility of control and treated spermatozoa. We found that, although the mobility of all of the sperm was reduced in the higher viscosity media, the treated group showed a total loss of forward mobility, while the sperm of the control group still possessed 48.8% mobility (**Figure 5b**). This result was consistent with previous research.[53] CatSper2 was essential for generation of the hyperactivated form of motility, which was necessary for successful fertilization.[13,18]

Owing to a concern that male contraception induced by blocking CatSper2 could produce side effects, we observed the function of testis tissue and the duration of sperm survival. The results showed that microinjection of DNA plasmid and EP did not cause significant impact on the spermatogenesis cycle and testicular function (data not shown). Propidium iodide analysis indicated that the viability of the treated sperm was similar to that of the control sperm (**Figure 5a**).

In conclusion, this study showed that CatSper2 knockdown by EP-mediated plasmid DNA could effectively interfere in male fertility, thus providing a prospective candidate for contraceptive drug development. However, plasmid DNA-mediated RNAi could not completely block protein expression as well as traditional gene knockout technology does. Furthermore, *in vivo* electroporation could only be realized through complicated surgery, which might influence the effect of blocking. Therefore, further study is necessary.

AUTHOR CONTRIBUTIONS

ZZ conceived and designed of the study, participating in whole experiment and draft the manuscript. GLW conceived of the study, coordinated the experiments performed by the members of the research team and helped to draft the manuscript. HXL and LL participated in the design of the study and *in vivo* electroporation. QWC and CBW performed the statistical analysis. FZ participated in the IVIS assay and helped with the statistical analysis.

COMPETING FINANCIAL INTERESTS

The authors declare that they do not have any competing financial interests.

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