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A novel copy number variation in *CATSPER2* causes idiopathic male infertility with normal semen parameters

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STUDY QUESTION: Are genetic abnormalities in *CATSPER* (cation channel of sperm) genes associated with idiopathic male infertility with normal semen parameters and, if so, how do they affect male fertility?

SUMMARY ANSWER: A novel copy number variation (CNV) in *CATSPER2* causes idiopathic male infertility with normal semen parameters by disrupting the ability of sperm to penetrate viscous media, undergo hyperactivation and respond to progesterone.

WHAT IS KNOWN ALREADY: CATSPER is the principle Ca^{2+} channel mediating extracellular Ca^{2+} influx into spermatozoa. Although several case reports have suggested a causal relationship between CATSPER disruption and human male infertility, whether genetic abnormalities in *CATSPER* genes are associated with idiopathic male infertility with normal semen parameters remains unclear.

STUDY DESIGN, SIZE, DURATION: Spermatozoa were obtained from men attending the reproductive medical center at Jiangxi Provincial Maternal and Child Health Hospital, Nanchang, Jiangxi, China between January 2014 and June 2016. In total, 120 men from infertile couples and 20 healthy male donors were selected to take part in the study, based on their normal semen parameters.

PARTICIPANTS/MATERIALS, SETTING, METHODS: CATSPER and KSPER currents were assessed using the whole-cell patch-clamp technique. Whole-genome sequencing and TaqMan® CNV assays were performed to identify genetic variations. The expression levels of genes encoding the CATSPER complex were measured by quantitative real-time PCR and Western blot. Sperm motion characteristics and hyperactivation were examined with a computer-aided sperm analysis (CASA) system. Sperm responses to progesterone, assessed as increases in CATSPER current and intercellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), as well as inducement of penetration ability and acrosome reaction, were examined by means of whole-cell patch-clamp technique, single-sperm $[Ca^{2+}]_i$ imaging, penetration into methylcellulose assay and chlortetracycline staining, respectively.

MAIN RESULTS AND THE ROLE OF CHANCE: An infertile man with complete disruption of CATSPER current was identified. This individual has a novel CNV which disrupts one gene copy in the region 43894500–43950000 in chromosome 15 (GRCh37.p13 Primary Assembly, nsv3067119), containing the whole DNA sequence of *CATSPER2*. This CNV affected the expression of *CATSPER2*, resulting in dramatically reduced levels of CATSPER2 proteins in the individual's spermatozoa. Although this individual exhibited normal semen parameters, his spermatozoa showed impaired penetration ability, deficient hyperactivation, and did not respond to progesterone, in terms of monovalent current potentiation, $[Ca^{2+}]_i$ increase, penetration ability enhancement and acrosome reaction inducement, which may explain the individual's idiopathic infertility.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: Our novel findings require more cases to support the *CATSPER2* CNV identified in this study as a common cause of idiopathic male infertility in patients with normal semen parameters. Therefore, caution must be taken when extrapolating the use of this CNV as a potential biomarker for idiopathic male infertility.

WIDER IMPLICATIONS OF THE FINDINGS: The findings from the unique human CATSPER 'knockout' model in this study not only confirm the essential roles of CATSPER in mediating progesterone response and regulating hyperactivation in human spermatozoa but also reveal that disruption of CATSPER current is a significant factor causing idiopathic male infertility.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by National Natural Science Foundation of China (81771644 and 31400996 to T.L.; 31230034 to X.Z.); National Basic Research Program of China (973 Program, 2015CB943003 to X.Z.); National Key Research and Development Program of China (2016YFC1000905 to T.L.); Natural Science Foundation of Jiangxi, China (20121BBG70021 and GJJ12015 to X.Z.; 20161BAB204167 and 20171ACB21006 to T.L.) and the open project of National Population and Family Planning Key Laboratory of Contraceptives and Devices Research (No. 2016KF07 to T.L.). The authors have no conflicts of interest to declare.

Key words: CATSPER2 / copy number variation / idiopathic male infertility / progesterone response / whole-genome sequencing

Introduction

Calcium signaling in spermatozoa is essential for successful fertilization. It regulates mammalian sperm functions, such as capacitation, hyperactivation and the acrosome reaction (Publicover, *et al.*, 2007, 2008; Alasmari, *et al.*, 2013; Sánchez-Cárdenas, *et al.*, 2014). The vital source of sperm intracellular free Ca^{2+} ($[Ca^{2+}]_i$) is Ca^{2+} influx from the extracellular environment, which is predominantly mediated by cation channel of sperm (CATSPER), a pH-dependent voltage-gated Ca^{2+}-selective channel (Kirichok, *et al.*, 2006; Lishko, *et al.*, 2011). CATSPER is a heterotetrameric channel composed of four separate pore-forming α subunits (CATSPER 1–4) and five reported auxiliary subunits (CATSPER β, γ, δ, ε and ζ) (Chung, *et al.*, 2017; Sun, *et al.*, 2017). Knockout models of murine *CATSPER* genes exhibit CATSPER-current deficiency and male infertility (Ren, *et al.*, 2001; Quill, *et al.*, 2003; Jin, *et al.*, 2007; Qi, *et al.*, 2007; Chung, *et al.*, 2011, 2017), indicating that CATSPER is the predominant sperm Ca^{2+} channel and is crucial for male fertility in mice. However, scientific understanding of the importance of CATSPER in human sperm relies heavily upon the clinical identification of *CATSPER*-abnormal cases. Although a few case reports have identified *CATSPER1* and *CATSPER2* genetic abnormalities in infertile men (Hildebrand, *et al.*, 1993, 2010; Avidan, *et al.*, 2003; Zhang, *et al.*, 2007; Avenarius, *et al.*, 2009; Jaiswal, *et al.*, 2014), comprehensive analyses have not been performed in these cases sufficient to reveal the functional significance of CATSPER in human spermatozoa.

While not significantly altering spermatogenesis, disruption of *CATSPER* genes in mice leads to a lack of CATSPER current and a decrease in sperm hyperactivation (Ren, *et al.*, 2001; Quill, *et al.*, 2003; Jin, *et al.*, 2007; Qi, *et al.*, 2007; Chung, *et al.*, 2011, 2017). In humans, the importance of CATSPER has been emphasized by the finding that the rapid influx of calcium into the spermatozoon induced by progesterone occurs via CATSPER (Lishko, *et al.*, 2011; Strunker, *et al.*, 2011), a result confirmed in a *CATSPER2*-deletion patient whose spermatozoa had no progesterone-potentiated CATSPER current (Smith, *et al.*, 2013). However, sperm functional analyses were not carried out in that study because most spermatozoa were immotile and morphologically abnormal in that case. Thus, the roles of human CATSPER played in sperm hyperactivation and the acrosome reaction were shown by the results from the pharmacological experiments (Alasmari, *et al.*, 2013; Tamburrino, *et al.*, 2014). Recently, it was reported that a CATSPER current-deficient man who suffers from a homozygous in-frame deletion in *CATSPERε* showed normal sperm quality but his spermatozoa failed to respond to progesterone (Williams, *et al.*, 2015; Brown, *et al.*, 2018), indicating the functional significance of auxiliary subunit of CATSPER in human spermatozoa. However, whether the abnormality in pore-forming subunits of CATSPER affects spermatogenesis and sperm functions in humans remains obscure.

In this study, we screened the men from infertile couples who had normal semen parameters by sperm patch-clamp recording and identified a novel *CATSPER2*-abnormal man to characterize the functional role of the pore-forming subunit of CATSPER in spermatogenesis and sperm functions in humans.

Materials and Methods

Study participants

Potential cases were screened from men attending the reproductive medical center at Jiangxi Provincial Maternal and Child Health Hospital, Nanchang, Jiangxi, China between January 2014 and June 2016. Before inclusion in this study, donors underwent a standardized clinical and laboratory evaluation including measurements of the size and volume of the testes, assessment for the presence of hydrocele, varicocele, secondary sexual characteristics and routine semen analysis. The details of the donors' lifestyle, habits and family history were also recorded. In total, 120 men from infertile couples were included based the absence of any other significant illnesses and their normal semen parameters (Supplementary Table SI). We also recruited 20 healthy fertile men who had a successful reproductive history during the most recent 2 years and normal sperm quality according to the World Health Organization (WHO) laboratory manual for the examination and processing of human semen (http://www.who.int/reproductivehealth/publications/infertility/9789241547789/en/).

Ethical approval

The donors participating this study provided signed informed consent. The collection of semen and blood samples and the performance of experiments in this study were approved by the Institutional Ethics Committee on human subjects of Jiangxi Maternal and Child Health Hospital.

Sperm patch-clamp recordings

Sperm in semen samples were precipitated by centrifugation and washed with HS solution (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES, 5 mM glucose, 10 mM lactic acid and 1 mM Na-pyruvate at pH 7.4 with NaOH). The washed sperm were loaded on an HS-washed cell culture dish (Φ, 3.5 cm, Corning Inc., Corning, NY, USA) and allowed to attach for 30 min. The whole-cell patch-clamp technique was applied to record the currents of CATSPER and pH-sensitive potassium channel of sperm (KSPER), as previously described (Kirichok, *et al.*, 2006; Lishko, *et al.*, 2011; Zeng, *et al.*, 2015). Seals were formed at the sperm cytoplasmic droplet with a 20–30 MΩ pipette in HS solution. Then, transition into whole-cell mode was achieved via application of short (1 ms) voltage pulses (400–650 mV) combined with light suction. The currents were stimulated by 1 s voltage ramps from −100 to +100 mV from a holding potential of 0 mV. Divalent-free (DVF) solution (150 mM NaCl, 20 mM HEPES, and 5 mM EDTA, pH 7.4) was used to record basal CATSPER monovalent currents. Then, 1 μM progesterone (Sigma Chemical Co., St. Louis, MO, USA) was perfused to assess progesterone-induced CATSPER currents. For KSPER recordings, cells were perfused with high-K^+ HS (160 K^+) solution (160 mM KOH, 10 mM HEPES, 150 mM methanesulfonic acid (MES), and 2 mM $Ca(MES)_2$, adjusted to pH 7.4 with MES) to record the KSPER currents. Data were analyzed with Clampfit software (v10.4, Axon, Gilze, Netherlands).

Assessment of intracellular pH (pH_i)

Sperm were harvested by direct swim-up and adjusted to a concentration of 1×10^7 cells/ml with HS solution, then 1 ml aliquots were labeled with 1 μM 2′–7′-bis-(2-carboxyethyl)–5-(and-6-)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Molecular Probes) for 30 min at 37°C in a 5% CO_2 incubator. Two 90 μl aliquots of stained sperm were loaded in two wells of a Corning 96-well microplate. Each well was first recorded for 60 s, and then 10 μl HS solution (HS sample) and 250 mM NH_4Cl (NH_4Cl sample) in HS solution were added to the sperm suspensions, respectively, and recorded for an additional 90 s. BCECF fluorescence was detected at 2-s time intervals at 535 nm, exciting at 440 and 495 nm, with an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). The 495-to-440 ratios were examined at the end of each experiments by adding Triton X-100 to a final concentration of 0.12% (v/v) to permeabilise the cell, then by modifying the pH of the sperm suspensions with sequential additions of HCl. At each step, the pH was determined with a Micro pH Electrode (Cole-Parmer, Vernon Hills, IL, USA). A calibration curve was constructed based on the sigmoid relationship between the 495-to-440 ratio and pH. The 495-to-440 ratio values were converted to pH_i via the calibration curve. The changes in sperm pH_i (ΔpH_i) were calculated as pH_i (NH_4Cl sample) − pH_i (HS sample).

Routine semen analysis

Semen samples were obtained from donors via masturbation after 3–5 days of sexual abstinence. Semen characteristics, such as vitality (using the eosin-alone staining method) and morphology (using a Diff-Quik rapid staining kit, BRED Life Science Technology Inc., Shenzhen, China), were examined according to the World Health Organization laboratory manual for the examination and processing of human semen (http://www.who.int/reproductivehealth/publications/infertility/9789241547789/en/). To assess sperm motility, the semen samples were diluted with sperm-free seminal plasma for a final sperm concentrations between 2×10^6 and 50×10^6 cells/ml. Aliquots of 6 μl semen samples were introduced into a 20-μ chamber (Leja, Nieuw-Vennep, Netherlands) maintained at 37°C by a MiniTherm slide warmer (Hamilton Thorne Biosciences, Beverly, MA, USA) placed on a negative phase contrast trinocular microscope (Zeiss Axio Lab A1, Carl Zeiss Jena GmbH, Jena, Germany) connected to a Hamilton Thorne CEROSII computer-aided sperm analysis (CASA) system. At least 200 spermatozoa in 12 fields were assessed in each chamber. Motion characteristics were analyzed by Human Motility II software (version 1.7, Hamilton Thorne Biosciences).

Whole-genome sequencing

Whole-genome sequencing was conducted by Novogene Corporation (Beijing, China). Genomic DNA was extracted from blood samples using TIANamp Blood DNA Midi Kit (TIANGEN Biotech, Co., Ltd., Beijing, China). A genomic DNA library was prepared using a TruSeq DNA PCR-Free Library Preparation Kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The genomic DNA library was sequenced on the Illumina HiSeq X Ten using the HiSeq X Reagent Kits.

TaqMan® CNV assay

A TaqMan® CNV Assay with predesigned *CATSPER2* primers and probe (Assay ID: H s04452005_cn, Thermo Fisher Scientific, Waltham, MA, USA) was used to validate the *CATSPER2* CNV identification using quantitative real-time PCR according to the manufacturer's instructions in a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). The Ct values of *CATSPER2* and *RNASEP* (a reference gene with two copies in the human genome, GenBank: NM_001104546.1) were calculated using the StepOne™ Software (Thermo Fisher Scientific). The ΔCt was calculated by subtracting the *RNASEP* Ct from the *CATSPER2* Ct for each replicate. The average ΔCt from four replicates was then calculated. A normal control (a fertile male, NC) was used as a calibrator for the *CATSPER2* gene dosage assay: ΔCts from all other DNA samples were normalized to the NC to determine the ΔΔCt. Relative quantity (RQ) was assessed as $2^{-\Delta\Delta Ct}$ and copy number was assessed as 2 × RQ.

Examination of CATSPER1–4 transcripts and proteins in spermatozoa

CATSPER1–4 transcripts and proteins was examined by quantitative real-time PCR analysis in a StepOnePlus™ Real-Time PCR System and by western blot as described previously (Luo, *et al.*, 2016). Primers and primary antibodies are shown in Supplementary Tables SV and SVI.

Single-sperm $[Ca^{2+}]_i$ imaging

Sperm were harvested by direct swim-up and adjusted to a concentration of 1×10^7 cells/ml with HS solution. Aliquots of 200 μl sperm were stained with 5 μM Fluo-4 AM (Molecular Probes, Eugene, OR, USA) and monitored by single-sperm $[Ca^{2+}]_i$ imaging as previously described (Luo, *et al.*, 2015). The stained sperm were first recorded for 40 s before the addition of 1 μM progesterone, and then 300 s was recorded before the addition of 10 μM A23187 (a positive control) for a final 24 s recording. Images were captured at 100 ms exposure time and 2 s time intervals. Sperm $[Ca^{2+}]_i$ was calculated by the formula $\triangle F/F_0$ (F_0, the mean fluorescent intensity before application of progesterone; F, the fluorescent intensity at every time point; $\triangle F = F - F_0$).

Penetration into an artificial viscous medium

Sperm were harvested by direct swim-up in human tubal fluid (HTF) medium (93.8 mM NaCl, 4.69 mM KCl, 0.2 mM $MgSO_4$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_2$, 21.4 mM lactic acid, 2.78 mM glucose, 21 mM HEPES, 4 mM $NaHCO_3$, and 0.33 mM Na-pyruvate, pH 7.35 with NaOH), adjusted to a concentration of 30×10^6 cells/ml with HTF medium and incubated in HTF++ medium (HTF plus 25 mM $NaHCO_3$ and 0.4% HSA) for 2 h at 37°C in a 5% CO_2 incubator. Then 1 μl chemicals

was added to 99 µl sperm suspensions. The ability of human spermatozoa to penetrate into the artificial viscous medium was evaluated as previously described (Zou, *et al.*, 2017).

Assessment of hyperactivation

Sperm were harvested by direct swim-up in HTF and adjusted to 10×10^6 cells/ml with HTF medium. Aliquots of 6 µl noncapacitated sperm were used to detect motion characteristics with the CASA system. Additionally, 100 µl aliquots of swimming sperm were capacitated in HTF++ medium for 4 h at 37°C in a 5% CO_2 incubator. After capacitation, 6 µl sperm samples were used to assess motion characteristics via the CASA system. Hyperactivation was defined as those cells with a curvilinear velocity ≥ 150 µm/s, linearity < 50%, and lateral head displacement ≥ 7 µm, as described previously (Alasmari, *et al.*, 2013). Percentage motility was assessed in parallel with assessment of hyperactivation, and the proportion of hyperactivated sperm was expressed as a proportion of motile spermatozoa.

Evaluation of the acrosome reaction

Sperm were harvested by direct swim-up in HTF medium, adjusted to a concentration of 20×10^6 cells/ml with HTF medium and capacitated in HTF++ medium for 4 h at 37°C in a 5% CO_2 incubator. Then 1 µl chemicals was added to 99 µl sperm suspensions and incubated for 1 h at 37°C in a 5% CO_2 incubator. The acrosome reactions were detected by chlortetracycline (CTC, Sigma Chemical Co.) staining as previously described (Luo, *et al.*, 2015). Fluorescence absence from the head represents the characteristic of acrosome-reacted sperm (AR). A total of 200 sperm were counted to evaluate sperm acrosome reaction.

Statistical analysis

Data are expressed as the means ± SEM. Differences between controls and samples were assessed by Student's *t* test analysis using the statistical software GraphPad Prism (version 5.01, GraphPad Software, San Diego, CA, USA). Statistically significant differences were determined to exist at $P < 0.05$.

Results

A man diagnosed with idiopathic infertility was identified as having normal semen parameters but no CATSPER current

By using sperm patch-clamp recording, we screened 120 men from infertile couples who had normal semen parameters (Supplementary Table SI) and identified a man (LYX-IMI-B) with complete disruption of the CATSPER current (Fig. 1A–C) but a normal KSPER current and pH (Supplementary Fig. S1) in his spermatozoa. LYX-IMI-B (24 years old) and his wife (25 years old) had a 6-year history of infertility. Other men in their genealogy had no reported fertility problems. Complete andrological examinations of LYX-IMI-B were normal, and no other significant illnesses were recorded in all of his available clinical data. LYX-IMI-B had a normal karyotype (46, XY). Y chromosome microdeletions were also not detected in LYX-IMI-B (Supplementary Fig. S2). Routine semen analysis in hospital showed that LYX-IMI-B had normal semen parameters (Fig. 2A and B and Table I) and these data were confirmed in our laboratory (Fig. 2C–I). LYX-IMI-B's wife had normal hormone levels and internal genital organs (Supplementary Table SI). Since no significant reproductive abnormalities had been identified via clinical diagnostics, LYX-IMI-B represents a case of idiopathic male infertility with normal semen parameters. The couple had undergone one cycle of IVF (Supplementary Table SI). In total, 18 oocytes were retrieved. Among these oocytes, six oocytes were used for IVF and embryo transfer (ET), which resulted in total fertilization failure. ICSI for the remaining of 12 oocytes was performed, and 10 of the 12 oocytes were fertilized (83.3% fertilization rate). From these, seven high-quality embryos were obtained, and one of them was transferred, resulting in singleton pregnancy.

A novel CNV in CATSPER2 was identified in LYX-IMI-B's genome

In total, 96.9 Gb of high-quality sequences were generated on 10 paired-end lanes by whole-genome sequencing (Supplementary Table SII). The

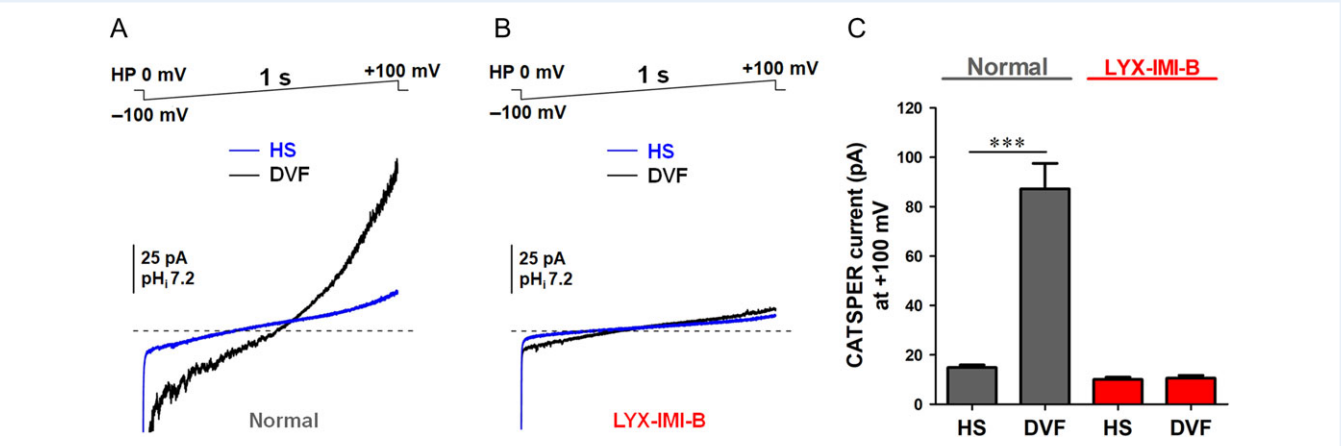

Figure 1 An infertile male individual (LYX-IMI-B) had no CATSPER current. The typical curves of CATSPER currents of the spermatozoa from normal controls (**A**) and LYX-IMI-B (**B**) were recorded via the whole-cell patch-clamp technique using a ramp protocol from −100 to +100 mV. (**C**) Statistical analysis of the mean CATSPER currents at +100 mV. Bar: mean ± SEM. ***$P < 0.001$, *t* test, 10 cells from 10 individual normal controls and six cells from LYX-IMI-B were examined.

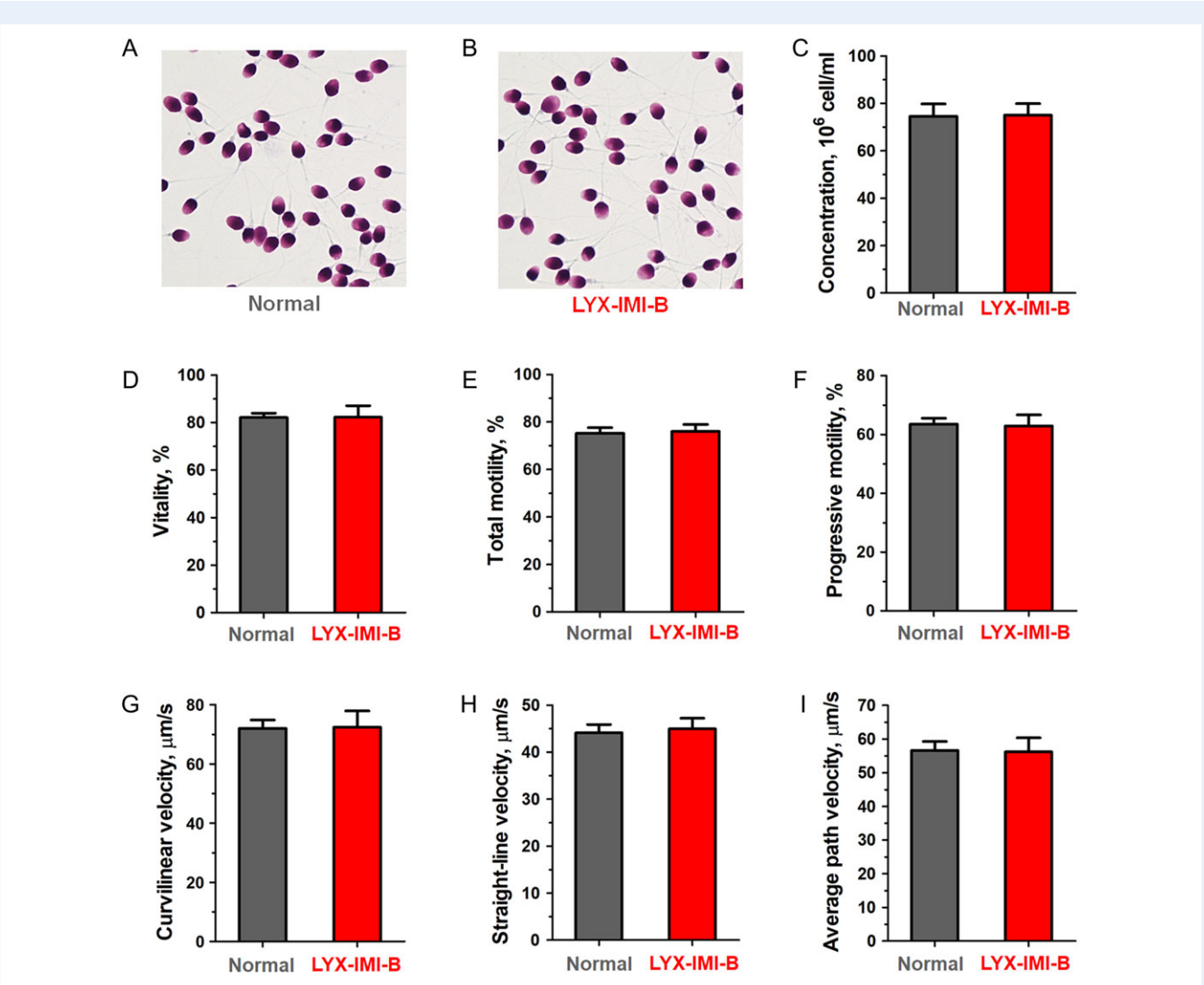

Figure 2 LYX-IMI-B exhibited normal semen parameters. Semen samples were collected by masturbation from healthy volunteers (Normal) and the infertile male individual (LYX-IMI-B) after 3–5 days of sexual abstinence. Routine semen analysis was performed to examine semen and sperm parameters. Morphology (**A** and **B**) was evaluated via Diff-Quik staining kit (Baso Diagnostics Inc., Zhuhai, China). Sperm concentration (**C**), vitality (**D**), total motility (**E**), progressive motility (**F**), curvilinear velocity (**G**), straight-line velocity (**H**) and average path velocity (**I**) were monitored by a computer-assisted sperm analysis system. Bar: mean ± SEM. *t* test, 10 replicates from 10 individual normal controls and 4 replicates from LYX-IMI-B were examined.

average sequencing depth was 32.51 (Supplementary Table SIII). The gene variations, including single nucleotide polymorphisms (SNPs), insertions and deletions (InDels), structural variations (SVs) and CNVs, were annotated according to the 1000 genomes project (http://www.1000genomes.org), dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP/), and dbVAR (https://www.ncbi.nlm.nih.gov/dbvar). There were 200 missense SNPs, 8 frameshift InDels, 53 exonic SVs and 30 exonic CNVs identified (Supplementary Table SIV). Of these mutant genes, *CATSPER2* appeared to be the most relevant candidate for the observed infertility phenotype of LYX-IMI-B, since no nonsynonymous variations in other sperm function-related genes reported previously were identified (Supplementary Table SIV). A novel CNV (one copy lost in the region of 43894500 to 43950000 in 15q15.3, GRCh37.p13 Primary Assembly, containing the entire *CATSPER2* and 80% of the DNA sequence of *stereocilin*, *STRC*) in LYX-IMI-B caused a heterozygous deletion of *CATSPER2* (labeled red in Fig. 3A and Supplementary Table SIV). Although the deletion of *CATSPER2/STRC* is the hallmark of the deafness-infertility syndrome (Zhang, *et al.*, 2007), this CNV of *CATSPER2/STRC* has not been reported previously, therefore we submitted it to dbVAR and obtained the associated identification number nsv3067119. This CNV was confirmed using a TaqMan® Copy Number Assay. This assay showed that the parents of LYX-IMI-B and a fertile male control had two copies of

CATSPER2 in their genomes, whereas LYX-IMI-B had only one copy of *CATSPER2* (Fig. 3B). Although this male has a heterozygous deletion of *STRC*, he is not hearing impaired.

Table I Semen characteristics of LYX-IMI-B.

Test	LYX-IMI-B	Reference
Age	24 years	Reproductive age
Years of infertility	6	>1 years
Semen volume (ml)	2	>1.5
Liquefaction time (min)	15	<60
Viscosity	<2 cm	<2 cm
pH	7.5	7.2–8.0
Aggregation	All free sperm	All free sperm
Concentration (10^6 cell/ml)	75	>15
Total number (10^6)	150	>39
Vitality (%)	82.3	>58
Total motility (%)	75.7	>40
Progressive motility (%)	54.9	>32
Morphology (% normal)	12	>4
Leukocytes (10^6 cell/ml)	0.5	<1

The reference limit (Reference) is according to WHO laboratory manual for the examination and processing of human semen. Semen samples were obtained from LYX-IMI-B by masturbation after 3–5 days of sexual abstinence. Routine semen analysis was carried out immediately after liquefaction.

CATSPER2 transcript and protein levels were dramatically reduced in LYX-IMI-B's spermatozoa

Only 8% of *CATSPER2* transcripts and 5% of CATSPER2 proteins were present in spermatozoa from LYX-IMI-B (Fig. 3C and D). The *CATSPER3* mRNA level in LYX-IMI-B's spermatozoa was significantly increased by 20% (Fig. 3C), while the protein level was only increased by 10% (Fig. 3D); the expression levels of *CATSPER1* and *CATSPER4* in LYX-IMI-B's spermatozoa showed no significant differences (Fig. 3C and D, $P > 0.05$). These results indicate that primarily the *CATSPER2* CNV affects the *CATSPER2* expression. The low levels of *CATSPER2* transcript and protein expression may account for the deficiency of CATSPER current in LYX-IMI-B's spermatozoa.

Progesterone exhibited no effect on LYX-IMI-B's spermatozoa in terms of increasing monovalent current and $[Ca^{2+}]_i$

Progesterone (1 μM) treatment significantly increased CATSPER current from 94.48 ± 12.81 pA to 188.6 ± 20.96 pA in normal spermatozoa ($P < 0.001$, Fig. 4A and C). However, progesterone treatment at the same concentration failed to increase any current in LYX-IMI-B's spermatozoa, even under divalent ion free conditions (Fig. 4B and C). In addition, the increase in $[Ca^{2+}]_i$ was negligible after 1 μM progesterone application, whereas a typical $[Ca^{2+}]_i$ increase was observed in normal spermatozoa (Fig. 4D–F). A Ca^{2+} increase was detectable in LYX-IMI-B's spermatozoa through the addition of 10 μM calcium

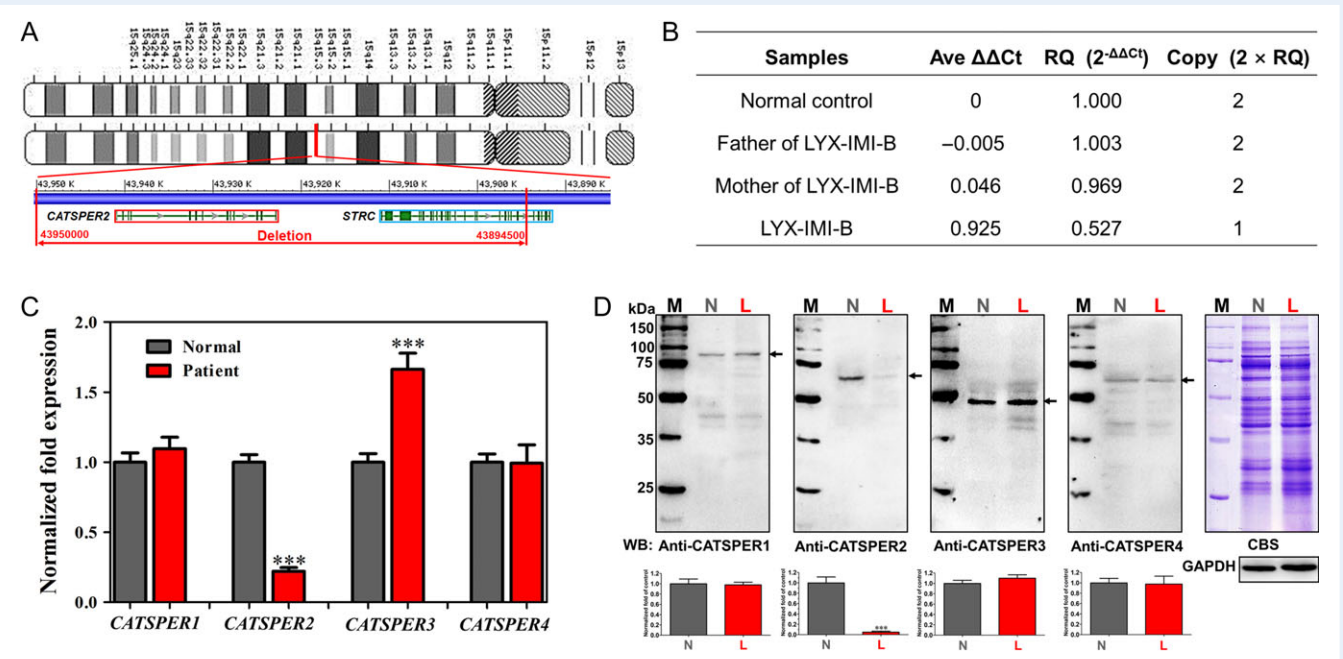

Figure 3 CATSPER2 expression was abnormal in LYX-IMI-B. (**A**) Whole-genome sequencing of LYX-IMI-B identified a novel CNV (one copy lost in region 43894500 to 43950000 in 15q15.3, GRCh37.p13 Primary Assembly, red frame, containing entire *CATSPER2* and part of *STRC*) resulting in a heterozygous deletion of *CATSPER2*. (**B**) TaqMan® CNV Assays were used to validate the *CATSPER2* CNV. (C and D) CATSPER1–4 transcript and protein expression levels were examined by quantitative real-time PCR (**C**) and Western blot (**D**). CATSPER1-4 proteins were quantified via the intensities of the bands and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using ImageJ2x software. Coomassie brilliant blue staining (CBS) showed the amount of proteins used in Western blots. Bar: mean ± SEM, ***$P < 0.001$, *t* test, three replicates from 10 individual normal controls and LYX-IMI-B were examined.

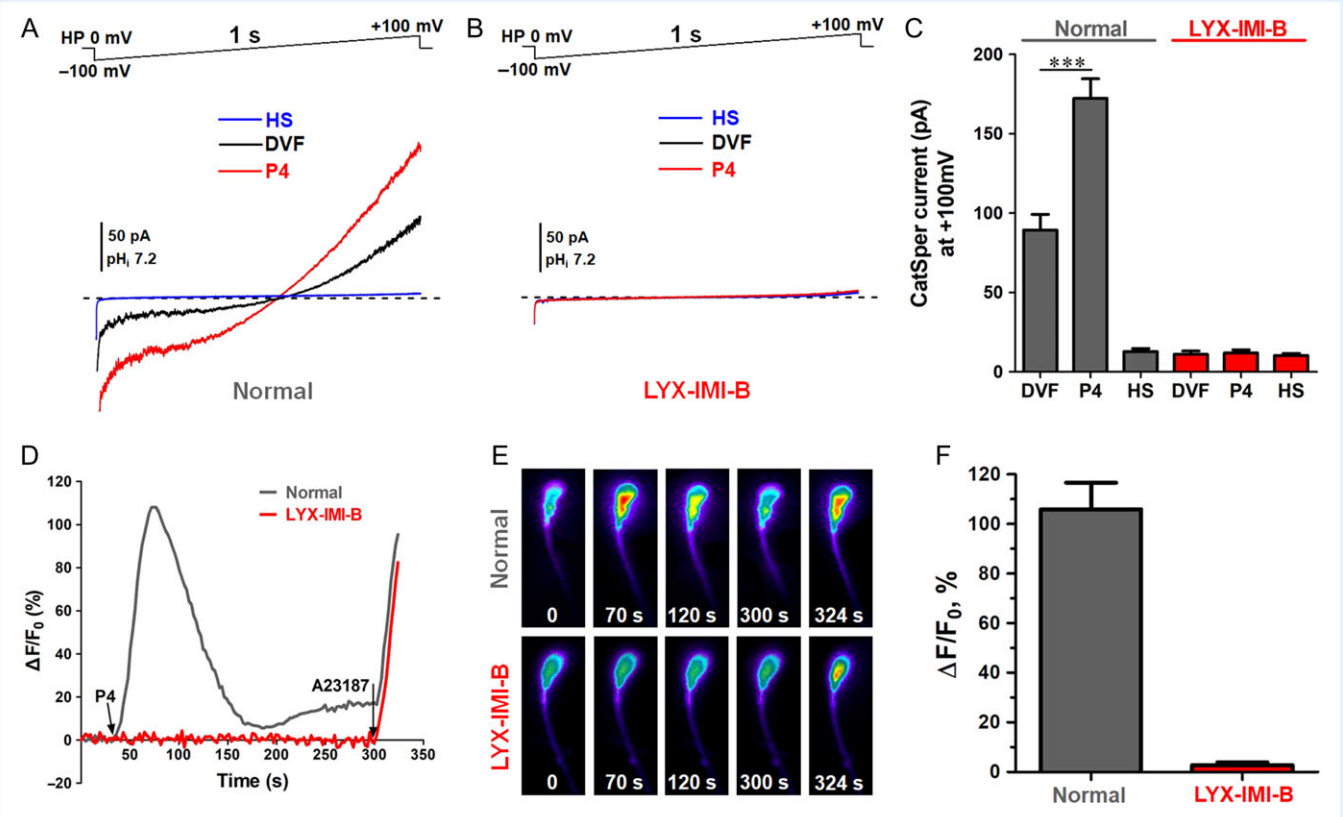

Figure 4 Progesterone-activated CATSPER current and $[Ca^{2+}]_i$ were abolished in LYX-IMI-B's spermatozoa. 1 μM progesterone (P4)-activated CATSPER currents of the spermatozoa from normal controls (**A**) and LYX-IMI-B (**B**) were recorded via the whole-cell patch-clamp technique using a ramp protocol from −100 to +100 mV. (**C**) Statistical analysis of the mean CATSPER currents at +100 mV. Bar: mean ± SEM. ***$P <$ 0.001, *t* test, eight cells from eight individual normal controls and four cells from LYX-IMI-B were examined. (**D**) Time-course changes of $\Delta F/F_0$ in response to 1 μM P4 treatment. (**E**) Examples of single cell $[Ca^{2+}]_I$ are illustrated below the graphs with each time frame. (**F**) Statistical analysis of the mean $[Ca^{2+}]_i$ at 70 s indicated in Fig. 3D (10 cells of normal controls and 6 cells of LYX-IMI-B were analyzed, bar: mean ± SEM).

ionophore (A23187, Fig. 4D and E). These results confirmed the hypothesis that CATSPER mediates the progesterone-induced calcium influx in human spermatozoa.

LYX-IMI-B's spermatozoa showed impaired penetration ability and hyperactivation, and did not respond to progesterone in terms of penetration ability enhancement and acrosome reaction inducement

The viscous medium penetration ability of LYX-IMI-B's spermatozoa was significantly reduced compared to that of normal controls (Vehicle, $P <$ 0.001, Fig. 5A and B). In addition, LYX-IMI-B's spermatozoa lost their ability to undergo hyperactivation in a noncapacitated state and showed an obviously decreased ability to undergo hyperactivation in a capacitated state (Fig. 5C). However, the spontaneous acrosome reaction in spermatozoa of both LYX-IMI-B and normal controls were below 20% and did not show any significant difference (Fig. 5D). Furthermore, the progesterone-induced penetration ability was significantly inhibited in LYX-IMI-B's spermatozoa, resembling the effects of treatment with the CATSPER inhibitor NNC 55–0396 (NNC, Sigma Chemical Co.) (Fig. 5A and B). Progesterone treatment increased the sperm acrosome reaction ratio in normal controls, from 16.70 ± 0.82% to 31.15 ± 1.78%. However, it did not induce the acrosome reaction in LYX-IMI-B's spermatozoa (Fig. 5D). Additionally, the A23187-induced acrosome reaction (a positive control) could be detected in LYX-IMI-B (Fig. 5D). These results indicate that CATSPER plays a key role in the progesterone-induced sperm functions.

Discussion

In mice, knockout of *CATSPER* genes affected sperm hyperactivation but had no effect on sperm production and morphology, indicating that murine CATSPER is important for sperm hyperactivation but not responsible for spermatogenesis (Ren, *et al.*, 2001; Quill, *et al.*, 2003; Jin, *et al.*, 2007; Qi, *et al.*, 2007; Chung, *et al.*, 2011, 2017). In humans, the findings from a CATSPER current-deficient man demonstrated the auxiliary subunit of CATSPER is not involved in spermatogenesis but is essential for sperm to respond to progesterone (Williams, *et al.*, 2015; Brown, *et al.*, 2018). However, the role of the pore-forming subunits of CATSPER played in the spermatogenesis has been obscure due to the contradictory observations from the *CATSPER2*-deleted men reported previously (Avidan, *et al.*, 2003; Zhang, *et al.*, 2007; Smith, *et al.*, 2013; Jaiswal, *et al.*, 2014). In this study, LYX-IMI-B represents

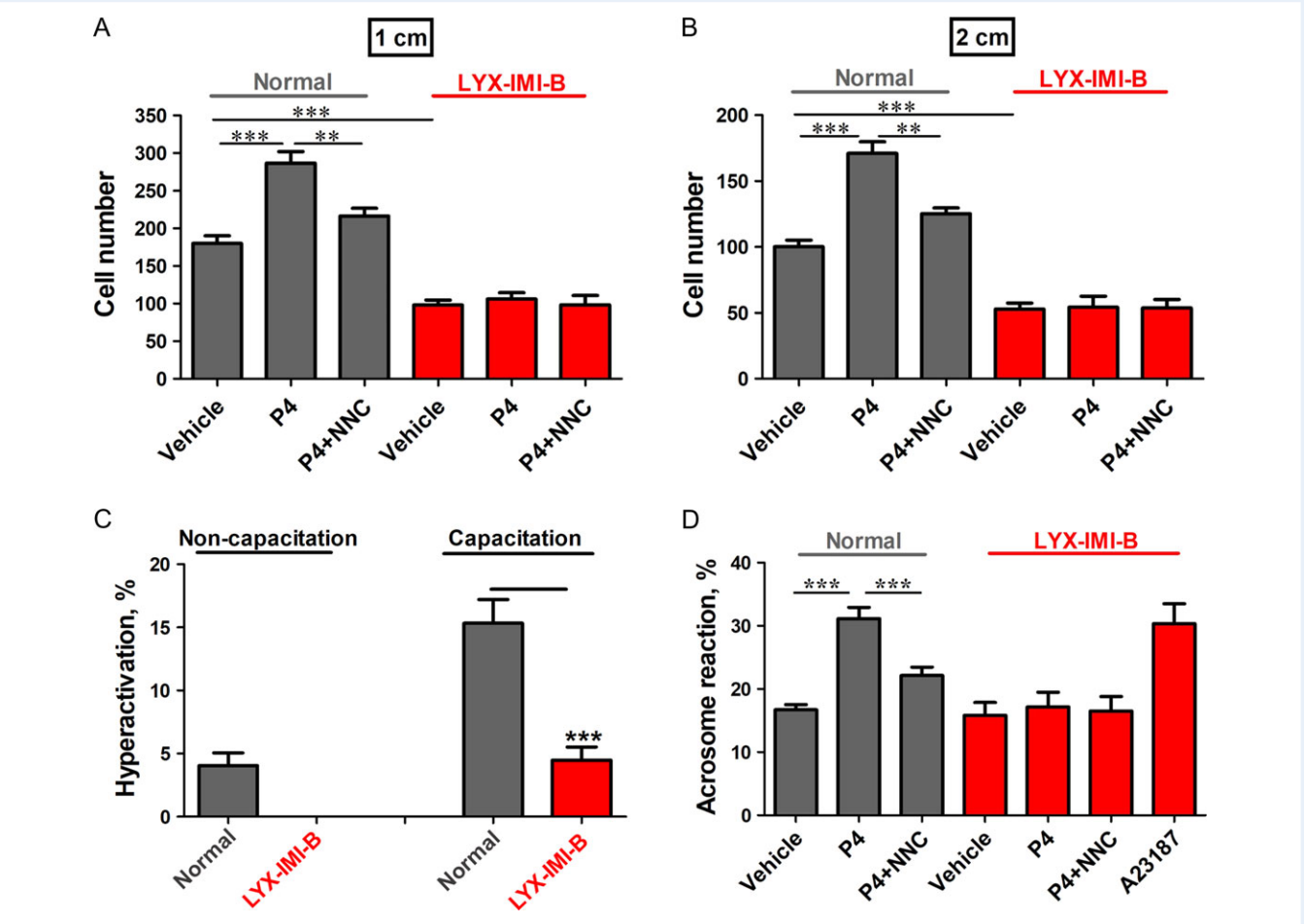

Figure 5 Sperm functions were affected in LYX-IMI-B's spermatozoa. The capacitated spermatozoa from normal controls (Normal) and LYX-IMI-B (LYX-IMI-B) were incubated with 0.1% DMSO (Vehicle, negative control), 1 μM progesterone (P4) and 1 μM P4 + 10 μM NNC 55–0396 (NNC, a CATSPER inhibitor) or A23187 for 1 h, respectively. Sperm penetration ability (**A** and **B**), hyperactivation (**C**) and the acrosome reaction (**D**) were examined. Bar: mean ± SEM. ***$P < 0.005$ and ***$P < 0.001$, *t* test, 10 replicates from 10 individual normal controls and 4 replicates from LYX-IMI-B were examined.

an ideal model to study the function of the pore-forming subunit of CATSPER in human spermatozoa because no nonsynonymous variations in commonly recognized male infertility-related genes were identified except for *CATSPER2*, and LYX-IMI-B's spermatozoa had normal KSPER current and pH_i which can affect CATSPER current indirectly. The sperm functional analyses in this case indicated that the pore-forming subunit of CATSPER is not involved in spermatogenesis but is essential for the sperm's ability to pass through the viscous female reproductive tract (via penetration ability and hyperactivation), consistent with the results previously obtained in CATSPER knockout mice. These results indicated that CATSPER plays a vital role in maintaining sperm hyperactivation and is essential for male fertility in mice and humans.

Heterozygous deletion of *CATSPER2* did not affect the expression of *CATSPER* genes in murine models (Quill, *et al.*, 2003). However, the *CATSPER2* CNV in patient LYX-IMI-B affected the expression of *CATSPER2*, resulting in a dramatically reduced level of CATSPER2 protein expression. This result suggests that the remaining *CATSPER2* copy in the LYX-IMI-B genome has particularly low transcriptional activity, which resembled genomic imprinting, an epigenetic phenomenon that causes genes to be expressed in only one of the two copies (Barlow and Bartolomei, 2014). However, *CATSPER2* is not recorded in the genomic imprinting database (http://www.geneimprint.com) and ~1% of the male population carries a heterozygous deletion of *CATSPER2* and is asymptomatic (Hoppman, *et al.*, 2013), indicating that *CATSPER2* is not an imprinted gene. Additionally, no variations were found in the predicted promoter regions of *CATSPER2* gene (43940159–43940759 and 43940923–43941575 in 15q15.3, GRCh37.p13 Primary Assembly) via WGS. Interestingly, although there was no exonic variation in *CATSPER2* on the non-deleted allele, an SNP (rs12443102) in the intron of *CATSPER2* was identified. These results imply that the low activity level of the remaining copy of CATSPER probably arises from the SNP in the intron of *CATSPER2* on the non-deleted allele. In addition, the CATSPER complex was reported to be expressed in an 'all-or-none fashion' in mice (deletion of one CATSPER subunit results in disappearance of the whole complex) (Qi, *et al.*, 2007). However, the CATSPER-deficient case reported previously cannot sufficiently confirm the existence of an 'all-

or-none' expression pattern in humans. In the present study, the nearly total absence of sperm CATSPER2 in LYX-IMI-B did not prevent the expression of the remaining subunits, challenging the concept of the 'all-or-none fashion' expression pattern in humans. Therefore, all these results suggest different expression patterns of the CATSPER complex between mice and humans.

In conclusion, this study reports a novel human *CATSPER2* 'knockout' model with complete disruption of CATSPER current. The lack of sperm CATSPER current resulted in defects in the ability of sperm to penetrate viscous media, undergo hyperactivation and respond to progesterone, which may explain the etiology of the idiopathic male infertility in LYX-IMI-B, who exhibited normal sperm parameters. Thus, this study offers the most convincing evidence to date to establish a causal relationship between CATSPER disruption and idiopathic male infertility. However, detection of CATSPER abnormalities is not currently available via clinical testing, and thus abnormalities in CATSPER may represent an underdiagnosed cause of male infertility. Fortunately, ICSI is an effective tool to allow such infertile men to obtain successful fertilization. In summary, the CATSPER channel is a promising target for the diagnosis and treatment of male infertility, although further research is required for widespread clinical applications.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

T.L., H.C. and Q.Z. conducted, analyzed and interpreted the whole-genome sequencing experiments; T.L., Q.Z. and S.W. acquired and analyzed phenotypic data; H.C. and Z.J. acquired and processed all patient samples, and analyzed data. H.C. performed and analyzed the routine semen analysis. F.W. assessed and analyzed hyperactivation. T.L. and Q.Z. conducted, analyzed and interpreted the expression analyses. T.W., Y.C., H.W. and Y.C. performed sperm patch-clamp recordings, single cell calcium imaging and intracellular pH analysis and analyzed the detailed data. S.W. and Q.Z. performed and interpreted the penetration of artificial viscous media assay and the evaluation of the acrosome reaction. T.L. collected the data and performed the statistical analyses. T.L. and X.H.Z. were responsible for primary study oversight and design, data acquisition, data analysis and interpretation, and primary writing of the article. All the authors made substantial contributions in critically revising the article. All the authors approved the final article for submission.

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Conflict of interest

The authors have no conflicts of interest to declare.

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human reproduction

SUPPLEMENTARY DATA

Supplementary Table SII **Summary of the quality of sequencing data.**

Lane	Raw reads	Raw data (G)	Effective (%)	Clean data (G)	Error (%)	Q20 (%)	Q30 (%)	GC (%)
H076LALXX_L1	22571668	97.59	99.35	96.9	0.04;0.06	93.93;91.59	85.49;82.03	41.21;41.37
H076LALXX_L4	24323979		99.31		0.03;0.05	95.70;91.46	88.98;82.88	41.37;41.17
H076LALXX_L5	24779878		99.28		0.03;0.05	95.86;92.69	89.05;84.38	41.40;41.42
H076LALXX_L6	24456040		99.28		0.03;0.05	95.72;92.64	88.74;84.23	41.34;41.38
H076LALXX_L7	24289989		99.33		0.03;0.05	95.65;92.31	88.65;83.85	41.31;41.33
H076LALXX_L8	21636953		99.31		0.03;0.05	95.54;92.15	88.41;83.71	41.34;41.38
H074BALXX_L3	34567229		99.34		0.03;0.04	96.28;93.34	90.69;86.16	41.47;41.51
H074BALXX_L5	40033659		99.26		0.03;0.03	96.33;94.50	90.41;87.55	41.45;41.53
H07PKALXX_L2	54481763		99.38		0.03;0.05	96.50;93.25	90.52;84.70	41.59;41.59
H07PKALXX_L4	54165498		99.37		0.02;0.03	96.98;95.05	92.05;88.48	41.61;41.64

Lane: the Flowcell and Lane numbers used in the HiSeq machine; Raw reads: the total number of pair read of the original sequence; Raw data (G): the total amount of the sequencing data; Clean data (G): the total amount of the clean data; Effective (%): the percentage of Clean reads in the Raw data; Error (%): the mean error of read1 and read2, respectively; Q20 (%): the percentage of the bases with accuracy rate > 99% in read1 and read2, respectively; Q30: the percentage of the bases with accuracy rate > 99.9% in read1 and read2, respectively; GC (%): the percentage of GC content in read1 and read2, respectively.

Supplementary Table SIII **The statistical data of reads and coverage.**

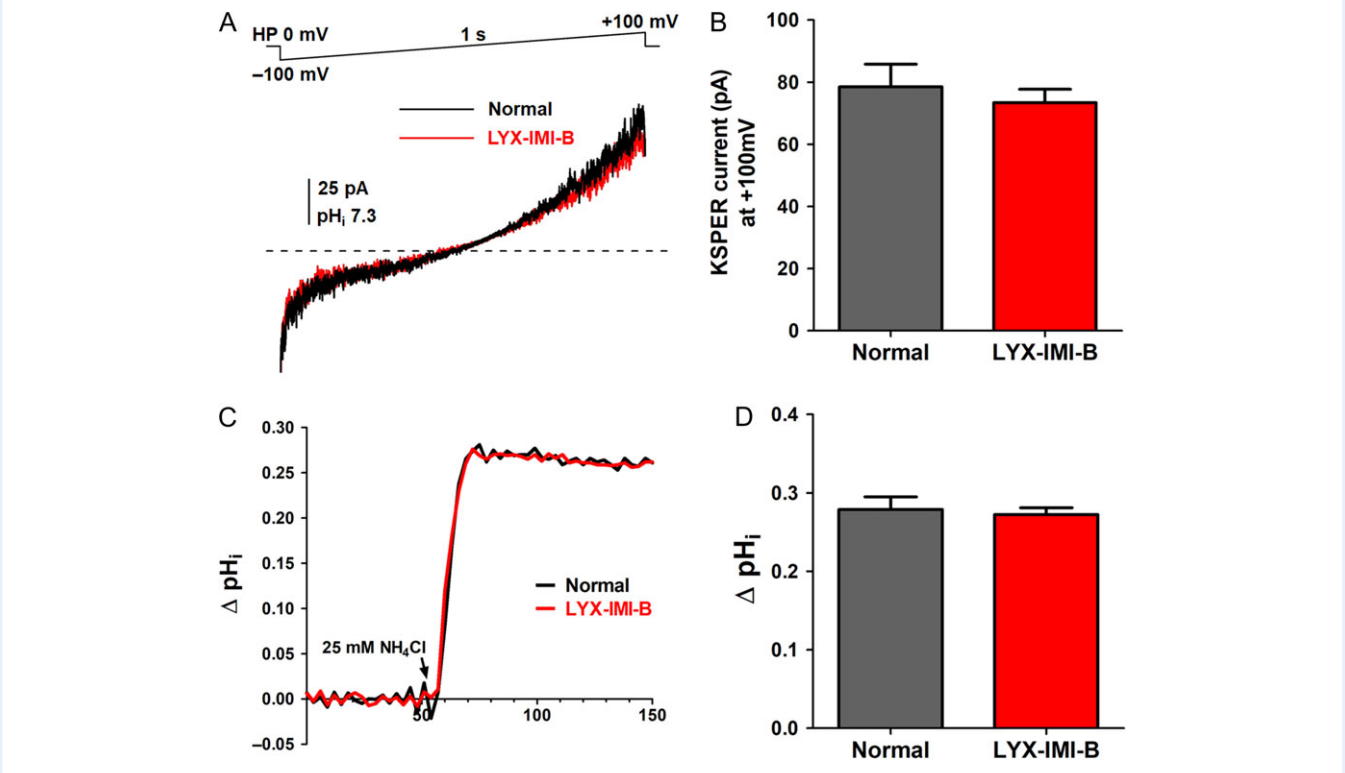
Parameters	Data
Total reads	646240862 (100%)
Duplicate reads	71997859 (11.16%)
Mapped reads	645345984 (99.86%)
Properly mapped reads	619829414 (95.91%)
Pair ends (PE) mapped reads	644607906 (99.75%)
Single end (SE) mapped reads	1476156 (0.23%)
Reads mapped to different chromosomes	8036504 (1.24%)
Average sequencing depth	32.51
Coverage	99.69%
Coverage at least 4×	99.36%
Coverage at least 10×	97.67%
Coverage at least 20×	86.44%

Supplementary Table SV **Primers used in quantitative real-time PCR.**

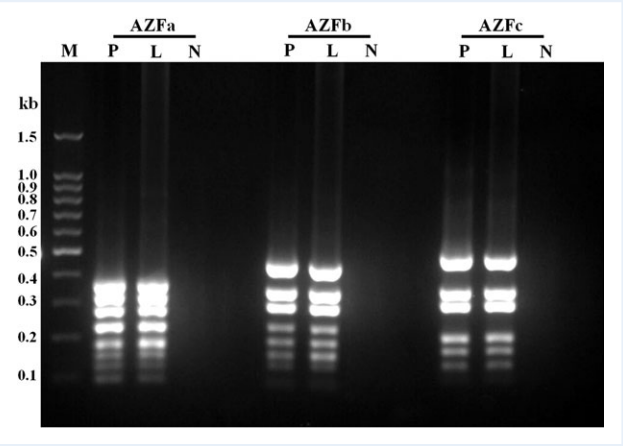
CATSPER1
5′-AGCGTCCAGGAAGTGACAGGG-3′
5′-CGTGAGCAAGGTGAAGAGGGTG-3′
CATSPER2
5′- CAGTCGCATCTTCAGCAGCATC-3′
5′-TCTGCCGCTTGAACATGTCAGC-3′
CATSPER3
5′-GCTCGCCTCTTTCATCTTCCTC-3′
5′-TCAGCCTGCTGATCTCCTCCTG-3′
CATSPER4
5′-CCACCAACCTGGAGCAAATGA-3′
5′-TCTCCTGTATTGCCTCAAGCACC-3′
GAPDH
5′-CAGGGCTGCTTTTAACTCTGGT-3′
5′-GATTTTGGAGGGATCTCGCT-3′

Supplementary Table SVI **Primary antibodies used in western blot.**

CATSPER1
1:200 dilution
Alomone Labs, Jerusalem, Israel
CATSPER2
1:250 dilution
Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA
CATSPER3
1:500 dilution
Abcam plc, Cambridge, MA, USA
CATSPER4
1:200 dilution
Alomone Labs
GAPDH
1:20 000 dilution
Proteintech Group, Inc., Wuhan, China



Supplementary Figure S1 **LYX-IMI-B's sperm had normal KSPER current and pH_i.** The typical curves of KSPER currents of the spermatozoa from normal controls and LYX-IMI-B were recorded via the whole-cell patch-clamp technique using a ramp protocol from −100 to +100 mV (**A**). (**B**) Statistical analysis of the mean KSPER currents at +100 mV. Sperm pH_i was also examined. (**C**) Time-course changes of pH_i (ΔpH_i) in response to 25 mM NH_4Cl. (**F**) Statistical analysis of the mean ΔpH_i at 80 s indicated in Fig. S1D. In total, 10 cells of normal controls and six cells of LYX-IMI-B were analyzed. Bar: mean ± SEM.



Supplementary Figure S2 Y chromosome microdeletions were not detected in the infertile male individual, LYX-IMI-B. Y chromosome microdeletion was examined by Y chromosome microdeletion detecting kit (Daan gene Co., Guangzhou, China). Genomic DNA was extracted from peripheral blood of LYX-IMI-B (L) and added to three multiplex PCR systems targeting the sequence sites in azoospermia factor (AZF) region a, b and c according to the use manual. A positive control (P, genomic DNA from fertile men) and a negative control (N, genomic DNA from female) were included in every PCR assay. All of the PCR amplification products were subjected to electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized by exposure to ultraviolet light. M, 100 bp DNA ladder (TransBionovo Co., Ltd., Beijing, China).