

A novel testis-specific Na⁺/H⁺ exchanger is involved in sperm motility and fertility

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1. ABSTRACT

Sodium-hydrogen exchanger as a channel for regulation of intracellular pH might be a crucial modulator of sperm capacitation and motility. Three members of this family have been identified in spermatozoa. A novel protein testis-specific sodium-hydrogen exchanger named mtsNHE was cloned in the present study. The mtsNHE localizing on principle piece of sperm flagellum contained 12 predicted transmembrane regions without cytoplasmic fragment at carboxyl terminus. Hydrophilic region was common in the sodium-hydrogen exchanger family members. Polyclonal antibodies to trans-membrane region significantly reduced sperm motility, acrosome reaction and ratio of *in vitro* fertilization. By in-pouring the antibodies in sperm solution, intracellular pH and calcium concentration were decreased. Muscle injection of female mice with the specific gene vaccine of mtsNHE, significantly stepped down fertility rate. Considering its specific expression and involvement in the regulation of fertility, the mtsNHE might be a potential target molecule for developing a new male contraceptive.

2. INSTRUCTION

Sodium-hydrogen exchangers (NHEs) play a vital role in osmoregulation, intracellular pH regulation and cell energetics by mediating electroneutral exchange of Na⁺ and H⁺ (1). Direction of the exchange relies on electrochemical gradients of ions across the membrane. To date, ten NHE isoforms have been identified from several species including mouse, rat and human (2). These ion antiporters are integral membrane proteins residing in plasma membranes and endomembranes of various cell types. Their tissue distribution, membrane location, sensitivities to inhibitors as well as their physiological regulations are diverse (3). Three NHEs (NHE1, NHE5 and mspermNHE) have been reported in spermatozoa (4). NHE1 is ubiquitously expressed and responsible for regulation of cell volume and intracellular pH, whereas the other NHE isoforms are more restricted (3). NHE1 knockout mice showed growth retardation, ataxia, seizures and altered intracellular pH in pancreatic acinar cells (5). However, deletion of the NHE1 failed to cause infertility,

and testis histology, sperm numbers and morphology were normal indicating that normal sperm function is preserved in NHE1-null animals. The mspermNHE has been demonstrated to be crucial for the expression and bicarbonate regulation of soluble adenylyl cyclase (6). The *mspermNHE*-null males were completely infertile with severely diminished sperm motility (6, 7).

It is well known that sperm should undergo capacitation, hyperactivity and acrosome reaction (AR) before successfully fertilizing an egg. Activation of sperm motility relies on suitable concentration of extracellular Na^+ ($(\text{Na}^+)_o$) and intracellular pH (pH_i) (8-10). Changes in extracellular pH (pH_o) linearly influence the pH_i of sperm (11). In mouse sperm, pH_i increases during capacitation mainly through Na^+ -, Cl^- -, and HCO_3^- -dependent mechanisms (12). pH_i increase observed during AR is Na^+ dependent (13). Extracellular Ca^{2+} concentration ($(\text{Ca}^{2+})_o$) is needed for zona pellucida (ZP)-induced AR in mature sperm. Essential to this process is elevation of pH_i and intracellular Ca^{2+} concentration ($(\text{Ca}^{2+})_i$) (14, 15). In many cells including sea urchin and mammalian spermatozoa, an interrelationship between $(\text{Ca}^{2+})_i$ and pH_i has been established (16-18). Mature sperm could undergo critical $(\text{Ca}^{2+})_i$ changes in response to the increase in pH_i . The NHE importing sodium and exporting proton might exert a multiple-acting effect on sperm motility, capacitation as well as AR.

The human ortholog, NHEDC1 (Na^+/H^+ exchanger domain containing 1, GenBank: BAC26494) has been reported and the preliminary data showed its testis-specific expression, encoding a putative protein of 515 amino acids, however, it was unknown whether this gene product possesses exchanger activities (19). In this report, we described cloning and functional characteristics of a testis-specific NHE from mouse germ cells. We have demonstrated that the NHE contains 12 hypothetical transmembrane segments. The transmembrane region may be an ideal target for male contraception. It has been reported that presentation of target antigen ultimately induces a full range of immunologic responses in human and various animals (20, 21), and inoculation with a testis-specific lactate dehydrogenase DNA vaccine has been proved to be effective to induce antifertility (20). Therefore we selected the testis-specific transmembrane region of mtsNHE including the extracellular domains as a DNA vaccine antigen and inserted into eukaryotic vector. Immunization of female mice with the *mtsNHE*-specific DNA vaccine significantly decreased fertility rate. It is speculated that mtsNHE might play a vital role in sperm motility and fertilization.

3. MATERIALS AND METHODS

3.1. Materials

BALB/c mice and New Zealand white rabbit were purchased from Institute of Genetics of Chinese Academy of Sciences, housed in a 12 h light: 12 h darkness and given food and water *ad libitum*. All experiments were conducted according to the guidelines of the Chinese Animal Care for Laboratory Animals, and the protocols were approved by the Ethics Committee of Institute of

Zoology, Chinese Academy of Sciences. All chemicals used in this study were purchased from Sigma (St. Louis, MO), unless otherwise noted.

3.2. cDNA amplification

Based on the data of our DNA chips and the Unigene information at NCBI, we designed the primers for the whole length cDNA of *mtsNHE*. The primer pair for cloning is: forward (5'-TTT TAA AGT TCC CTG CTG AAA CGT AAG-3') and reverse (5'-GGT GAT GAT GGC AAG CTT TTT AAT GAT-3'). Total RNA was extracted from mature testes of BALB/c mice respectively using Trizol reagent (Invitrogen, Calsad, CA, USA) following the manufacturer's protocol.

3.3. Northern blotting and *In situ* hybridization analysis

A fragment of cloned cDNA was used as probe for northern blot analysis. The primer pair for cloning is: forward (5'-TGC CTT GCT ACC TTG GGT TTG G-3') and reverse (5'-GGG TCC CAG TAT GCC GAT GAG G-3'). The procedures were performed as reported previously (22).

3.4. Recombinant protein purification in *E. coli* and polyclonal antisera production

The selected partial cDNA sequence according to the transmembrane fragment of the mtsNHE was inserted into pGEX-4T-1 (Invitrogen, Carlsbad, California, USA) to set up bacterial expression of the construct pGEX-NHE. The primer pair for cloning is: forward/BamHI (5'-TTG GAT CCC ATG GAT CTG GAG GAC T-3') and reverse/SalI (5'-AAG TCG ACG GCA CAT GCC AAT GGT T-3'). The fragment of mtsNHE protein which contained 62 amino acids, not only including the transmembrane domains, but also the extracellular domains, by hydrophobic analysis to construct recombinant protein with GST. Therefore, the polyclonal antibody produced by the fragment could be capable of accessing to the target antigen. The preparation of recombinant protein (GST plus mtsNHE fragment) and immunization of rabbits with it were performed as describes (23). Six rabbits of each group were immunized. The antisera were harvested respectively from arteriae carotis.

3.5. Immunohistochemical and Western blot analysis

The methods followed those reported before (22). Antisera or control sera were used as the primary antibodies (diluted 1:200). Secondary antibodies were biotinylated (immunohistochemical analysis) and horseradish peroxidase-conjugated (Western blot analysis).

3.6. Construction of pCR-NHE

The purified PCR product was inserted into pCR3.1 (Invitrogen, Calsad, CA, USA) according to the manufacturer's instruction. The primer pair for cloning is: forward/*HindIII* (5'-GGC GAA GCT TGT TAT GGG AGT TTT TG-3') and reverse/*EcoRI* (5'-GCG GAA TTC TTA ATG ATG GAA GTT CGA G-3'). This region contains and is larger than that used for the recombinant protein.

3.7. Immunization with DNA vaccine

The female BALB/c mice were immunized with the plasmid DNAs purified by using Qiagen Endofree Mega (Qiagen, Valencia, CA, USA). Each group of 20 female BALB/c mice were immunized i.m. with 20 µg of recombinant pCR-NHE or pCR3.1 respectively. Four groups of both styles were used. The mice were housed as previously described (24, 25), one week after the second immunization, the sera were collected respectively and stored individually at -20°C. Preimmune sera from the mice were also collected in a similar manner as the negative controls (24, 25).

3.8. Confocal analysis

pCR-NHE was transfected into cultured Hela cells by LipofectAMINE (Gibco BRL, Rockville, MD, USA). 36 h later, the transfected cells were fixed with 4% paraformaldehyde in PBS for 1 h at room temperature before test. Sperm suspension was dropped onto poly-L-lysine-coated cover slips, smeared, air-dried and then fixed with 4% paraformaldehyde (26). The confocal test of mouse sperm and transfected cells was performed as described (20). All the antisera were added at 1:200 dilutions.

3.9. Antibody detection by ELISA

Testis total protein was selected as coated antigen. The sera of the immunized mice with pCR3.1 or pCR-NHE and the sera of the immunized rabbits (inoculated or preimmune) at serial dilutions from 1:100 to 1:6000 or 1:100 to 1:4000 was added to each well. The methods followed those described previously (20).

3.10. Assessment of sperm acrosome reaction and fertilization

Mature male mice (10–12 weeks old) were used in this study. Sperm acrosome reaction was detected by CTC staining as described (7, 24). Caudal epididymal sperms were collected by placing two minced caudal epididymids into 5 ml of PBS at 37°C. Sperms were allowed to swim out for 1 h, and were then centrifuged at 500 g for 10 min (26). After two washes, the pellet was suspended with PBS. The percentage of AR form spermatozoa (AR %) at least in 200 spermatozoa per slide was scored. The method for *in vitro* fertilization followed those reported before (7). The individual antiserum used was added at 1:200 dilutions.

3.11. Sperm motility analysis

The method employed was essentially as described previously (27). Caudal epididymal spermatozoa were released into modified Krebs-Ringer bicarbonate medium (28). Sperm motility was analyzed after 40–90 min of incubation with the antiserum or the control serum (diluted 1:200) at 37°C. For percent motility experiments, at least 500 sperms were captured in a chamber remaining stable at 37°C. Four groups of both styles were used. The IVOS Sperm Analyzer (Hamilton Thorne Research, Beverly, MA, USA) was used for all motility analyses. Motile sperm percent, smoothed path velocity (VAP), mean curvilinear velocity (VCL) and progressive velocity (VSL) of spermatozoa under the same conditions were measured. At least, four times repeat of each group were performed.

3.12. Immunocontraceptive test

The test was carried out as reported previously (20). One week after last booster, the treated and the control mice were placed by pairing each immunized female mouse with one normal male respectively for 2 weeks. They were checked daily for mating as evidenced by a vaginal plug. The number of female mice which gave birth was recorded.

3.13. Measurement of pH_i and (Ca²⁺)_i in spermatozoa

The procedure was performed as describes previously (24). The coverslips were immersed in 50 µg/ml polyD-lysine overnight and air dried before they were laid on the chamber. The sperm squeezed from the caudal epididymis were prepared on the coverslips and loaded with 20 µM SNARF-4F (Molecular Probes) or 10 µM Fluo3/AM (Molecular Probes) respectively for intracellular pH or Ca²⁺ measurement. After 30 min incubation at 37°C in the dark, the sperms were washed with HS medium containing (in mM) 135 NaCl, 5 KCl, 2 CaCl₂, 1 MgSO₄, 20 HEPES, 5 glucose, 10 lactic acid and 1 Sodium pyruvate, pH 7.4 (NaOH). Fluorescence changes were monitored and recorded by a Laser Scanning Confocal Imaging System (TCS SP2; Leica microsystem, Germany). In the intracellular pH measurement, the excitation wavelength was set at 488 nm, and the ratio of fluorescence intensities of emission wavelengths 580/ 640 nm was obtained. Calibration was performed by using the nigericin/high K⁺ method (24). The same was the excitation wavelength of intracellular Ca²⁺ measurement with that of intracellular pH measurement, and an emission of 525 nm was performed. The change of fluorescence intensity after the antibody treatments at 1:50 dilutions was normalized with the initial intensity (F₁/F₀).

3.14 Statistic analysis

All experiments were repeated at least three times. All data were analyzed by ANOVA. Differences among groups were calculated by Turkey's multiple-comparison test, and a difference was considered to be significant at $P < 0.05$.

4. RESULTS

4.1. Cloning and sequence analysis of mtsNHE cDNA and its protein

According to the DNA chip data which decreased on day 1, 4, 7, 14, and 28 after experimental cryptorchidism (EC) using Affymetrix MOE430A microarray in our group and the Unigene data with a UGID: 276341 at NCBI, we performed RT-PCR and obtained a cDNA fragment of 1776 bps, containing a complete open-reading frame for a protein of 565 amino acids (Figure 1). By mouse genome search, this gene was located in the G3 region of chromosome 3. Bioinformatics analysis of the conserved protein domain showed existence of sodium-hydrogen exchanger conserved domain in the NHE protein (Figure 2). Using DNASTAR to produce Kyte-Doolittle plot, we found 12 predicted transmembrane domains (Figure 3A).

A sequence alignment of mtsNHE with 19 other NHE protein sequences was carried out with GeneBee, comparing the predicted open reading frames of each NHE.

mtsNHE is involved in sperm fertility

The tree generated with topological algorithm showed that the mtsNHE seemed closely related to the mspermNHE.

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1      TTTTAAAGTTCCTGCTGAAAACGTAAAGATATCTTAAGACTGCAGACAAACTCCTGAAAAGA
      Forward primer
61     TGAGTGAGCAGCAGCTAGAATCAAAACAAGGATGATGGTTTCCAATCATCTGTAACGTG
      M S E H D V E S N K K D D G F Q S S V T
121    TTGAGATGTCCAAAGATCCTGATAGTTTTCATGAAGAAACTGTGGAACCAAAACCAGAAC
      V E M S K D P D S F H E E T V E P K P E
181    TGAAGGAACCGGAACCAAGGAACCAAGAACCGAAGGAACCAAGGAAGGAACCAAGAAA
      L K E P E P K E P E P K E P E R K E P E
241    GGAAGGAACCAAGGAAGGAACCAAGGAAGGAACCAAGGAAGGAAGGTACCAGGAA
      R K E P E R K E P E R K E P E R K V P G
301    GAAAGGAACCAAGGAAGGAACCAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGA
      R R E T Q T K E T Q T T E I E R K E T K
361    AGAAGAGAGGGACAAATAGTTACTGTCTCCACAAGGAACAATAAATAAACAATTACAG
      K K R G T N S Y C P P Q G T I N K T I T
421    ATGGAGCTGCACCTATTGCACCTATGGACTCTACTCTGGGCCCTTATAGGTCAAGAGTTC
      D G A A L I A L W T L L W A L I G Q E V
481    TCCCTGGTGGAAATTTGTTGGACTTGTAGTTATTTTACAGTCTTTCCTGGGGGTA
      L P G G N L F G L V V I F Y S A F L G G
541    AAAATTTAGAAATTCATTAATAACCTGTAGTGCCTCCCTCCACCTCTCATTGGGATGT
      K I L E F I K I P V V P P L P P L I G M
601    TACTGGCTGGTTTCACCATCAGGAATGTTCCGATTATTTATGAATTTGTCCATATTCCTA
      L L A G F T I R N V P I I Y E F V H I P
661    CCACATGGTCTTCAGCTTTAAGAAACACTGCCCTTACTATTATCCTAGTACGAGCTGGGC
      T T W S S A L R N T A L T I I L V R A G
721    TTGGACTGGATCCACAGGCCTTGAAGCATCTGAAGGGAGTCTGTCTGCGCTTGTCAATTG
      L G L D P Q A L K H L K G V C L R L S F
781    GTCCATGCTTCCTAGAACCTGCTCAGCCGCTCTCTCTCCCACTTCATTATGAACTTTC
      G P C F L E A C S A A L F S H F I M N F
841    CTTGGCAATGGGGTTCTACTAGGTTTTGTCTAGGCGCTGTCTCTCTGCTGTTGTGCG
      P W Q W G F L L G F V L G A V S P A V V
901    TCCCAACATGCTAATGTTGCAAGGAATGGGTATGGGTGTTGAGAAAGGCATCCCGACCT
      V P N M L M L Q E N G Y G V E K G I P T
961    TGCTGGTGGCAGCTAGCAGCATGGACGACATTGTGGCCATCACTGGATTCAACACATTCT
      L L V A A S S M D D I V A I T G F N T F
1021   TGAGCATAGTCTTTCTCGGGTAGTGTGATTAGCAACATCCTATCGTCCTTGGCGGACG
      L S I V F S S G S V I S N I L S S L R D
1081   TTCTTATTGGGGTCTGGTAGGAATGTTATGGGAGTTTTTGTTCAGTATTTTCCAAGTG
      V L I G V L V G I V M G V F V Q Y F P S
1141   GAGATCAGGAGAGACTTACGCAGAGCGAGCCTTCCTAGTTCTGAGTATGTGTATTCTG
      G D Q E R L T Q R R A F L V L S M C I S
1201   CTGTCTTAGGCTGCCAGCAGTATGGCTTACATGGATCTGGAGGACTGGTCACACTAGTAT
      A V L G C Q H I G L H G S G G L V T L V
1261   TGTCGTTTCATGGCAGCAAAAGATGGGCTGAAGAGAAGGTTGGAATCCAAAAAATTGTTG
      L S F M A A K R W A E E K V G I Q K I V
1321   CAAACACATGGAATGTTTTCAGCCGCTTCTTTTGGCTTAGTTGGAACAGAAGTATCTG
      A N T W N V F Q P L L F G L V G T E V S
1381   TTGAATCTCTGAATCAAAACCATTTGGCATGTGCCCTTGCTACCTTGGGTTTGGCACTAT
      V E S L E S K T I G M C L A T L G L A L
1441   CTGTTGCAATTTATCCACTTTTGTACTGATGTCTTTTGCTAACTTCCGTTTAAAGGAGA
      S V R I L S T F V L M S F A N F R F K E
1501   AAGTATTTATTGCTTTATCATGGATCCCTAAAGCTACAGTCCAGGCTGTCTTAGGACCTC
      K V F I A L S W I P K A T V Q A V L G P
1561   TGGCTCTAGAAACAGCAAGGGTCAATGGCACCCCACTTGAAGGATACGCGAAGGCTGTGA
      L A L E T A R V M A P H L E G Y A K A V
1621   TGACAGTGGCTTCTAGCATCTGATTACAGCTCCAAATGGAGCCCTCCTCATCGGCA
      M T V A F L A I L I T A P N G A L L I G
1681   TACTGGGACCCAAATCCTTGAACAGAGTGAGGTGACATTCCCTTTGAAAGTTGAGCTCT
      I L G P K I L E Q S E V T F P L K V E L
1741   CGAACTTCCATCATTAAAGGCTTGGCATCATCACC
      Reverse primer
S N F H H *

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Figure 1. Full length mtsNHE cloning and deduced protein sequence. ATG: initiator condon; TGA: terminator condon. The primer pair for cloning mtsNHE cDNA was lined out with grounding. The amino acids of antigen region to produce polyclonal serum was signed italic with grounding.

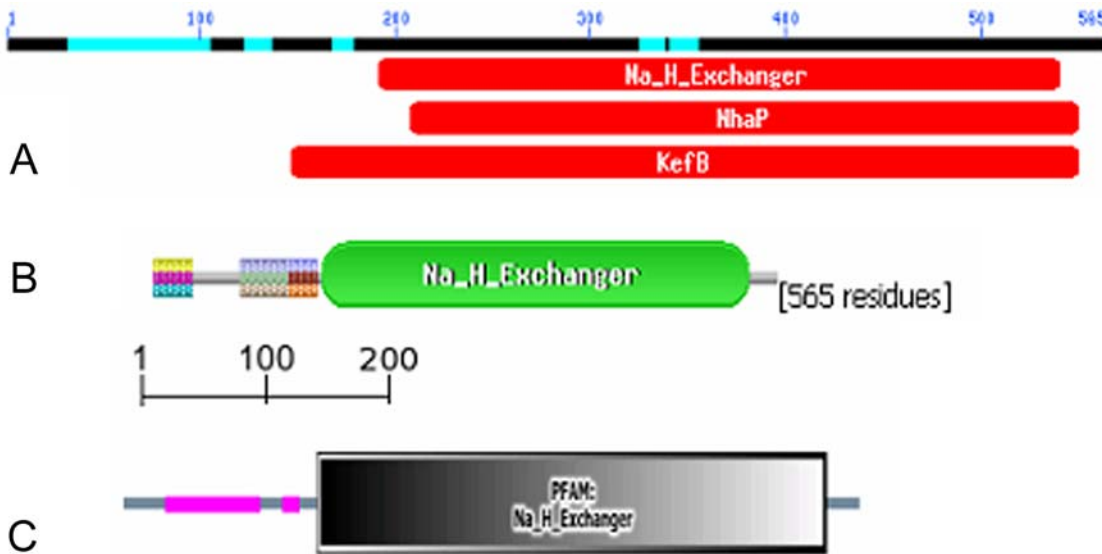


Figure 2. Bioinformatics analysis of conserved domain of mtsNHE protein. Sodium-hydrogen exchanger conserved domain was shown. A. by NCBI Conserved Domain Search at NCBI (<http://www.ncbi.nlm.nih.gov>), B. by Pfam server (<http://www.sanger.ac.uk/Software/Pfam>), C. by SMART sever (Simple Modular Architecture Research Tool, <http://smart.embl-heidelberg.de/>).

and mNHE6 (Figure 4).

4.2. Expression pattern of mtsNHE and its protein in testis and sperm

The Northern blot analysis (Figure 3B) and RT-PCR (data not shown) both confirmed that transcript of the *mtsNHE* was only expressed within the testis. *In situ* hybridization confirmed the specific expression of the NHE in spermatocytes and spermatids (Figure 5A).

The individual anti-mtsNHE serum obtained from the immunized rabbits using the purified mtsNHE recombinant protein was utilized to examine distribution of the mtsNHE protein in adult mouse testis by immunohistochemistry. At higher magnification, the mtsNHE signal was observed in spermatids and spermatozoa (Figure 5B). Furthermore, indirect immunofluorescence of mouse caudal epididymal sperm demonstrated that the NHE was localized to the principle piece of sperm flagellum (Figure 6A). Western blot analysis using the antisera was accomplished with the proteins extracted from testes or spermatozoa. In both of them, a protein with a relative molecular mass (M_r) of about 68 kDa was detected (Figure 3C1). Seems like mspNHE, construct of mtsNHE in frame with GFP was transiently transfected into 3T3 cells and spermatocyte cell line GC2. In both cases, the NHE failed to be transported to the plasma membrane (data not shown).

4.3. Construction of DNA vaccine and confirmation of its specificity.

We selected the partial fragment of *mtsNHE* gene as the target, which was inserted into the eukaryotic expression vector pCR3.1 to construct pCR-NHE as the prototype gene vaccine. An intense fluorescence along the

principle piece of flagella was observed when sperms were reacted with the antisera from the mice inoculated with the pCR-NHE (Figure 6A). In order to confirm whether the mtsNHE fragment could be expressed in eukaryotic cells, the pCR-NHE plasmid was transfected into Hela cells. Same with the antisera from the rabbits inoculated with the recombinant protein, the antisera from the mice immunized with the pCR-NHE could bind to the transfected Hela cells, while no positive signal was observed in the control (Figure 6B).

The specificity of the antibodies binding to the mtsNHE was also determined by Western blotting. Only the testis protein and the purified recombinant mtsNHE protein probed with the antisera from the pCR-NHE immunized mice showed a specific protein band (Figures. 3C2&3C3).

4.4. ELISA

As shown in Figure 7, the mtsNHE-specific antibodies of the immunized mice by the pCR-NHE and the antisera of the immunized rabbits with the recombinant protein were elicited significantly as compared with that of the pCR3.1 or the preimmunized sera.

4.5. Immunocontraceptive effects of pCR-NHE

To determine the effect of pCR-NHE DNA vaccine on contraception, birth rate of the immunized female mice was scored. The results clearly showed that the pCR-NHE vaccine significantly reduced birth rate; only 40.0% of the immunized females were able to give birth (Figure 8A).

4.6. Inhibitory effect of antisera on *in vitro* fertilization

As shown in Fig.8B, the mean percentage of two-cell embryos was approximately 29.4% after

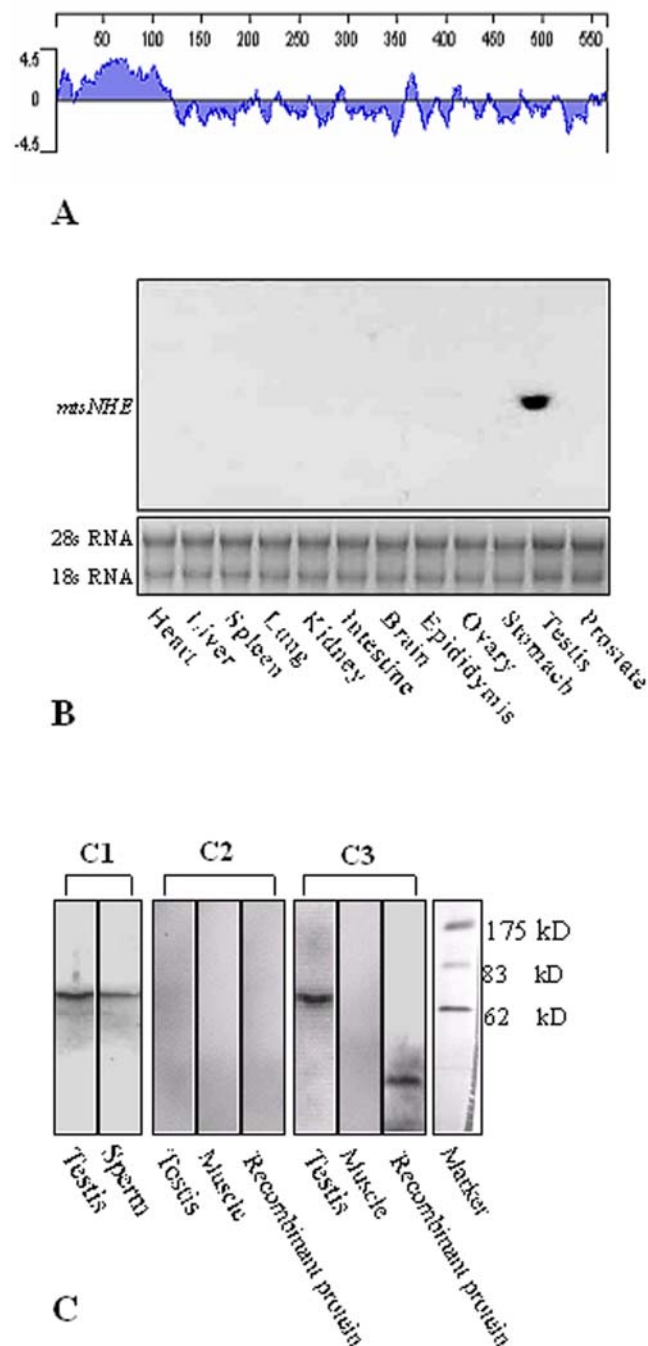


Figure 3. A novel testis-specific sodium-hydrogen exchanger. A. Transmembrane and hydropathic analysis of mtsNHE. DNASTAR was used to produce the Kyte-Doolittle plot with a window size of 11. The plot predicts 12 transmembrane domains. B. Northern blot analysis of mouse multi-tissue total RNA (25µg each). Antisense DIG-cRNA probe was used for Northern blotting of multi-tissue total RNA according to the method detailed in Materials and Methods. A signal was detected only in testis. C. Detection of mtsNHE expression in mouse testis, sperm, muscle and recombinant (GST plus mtsNHE fragment) protein by Western blot. C1. The antisera from rabbits immunized with the recombinant protein used as primary antibody for western blot of testis and sperm total protein. Specific band was detected in both of them which were about 68 kDa. C2, C2 & C3. Analysis of specification of antibodies via DNA vaccine immunization by Western blot. The antisera from mice immunized with pCR-NHE and pCR3.1 via muscle injection were used as primary antibodies. Specific band was detected only in testicular protein and fusion protein by antisera from mice immunized with pCR-NHE via muscle injection.

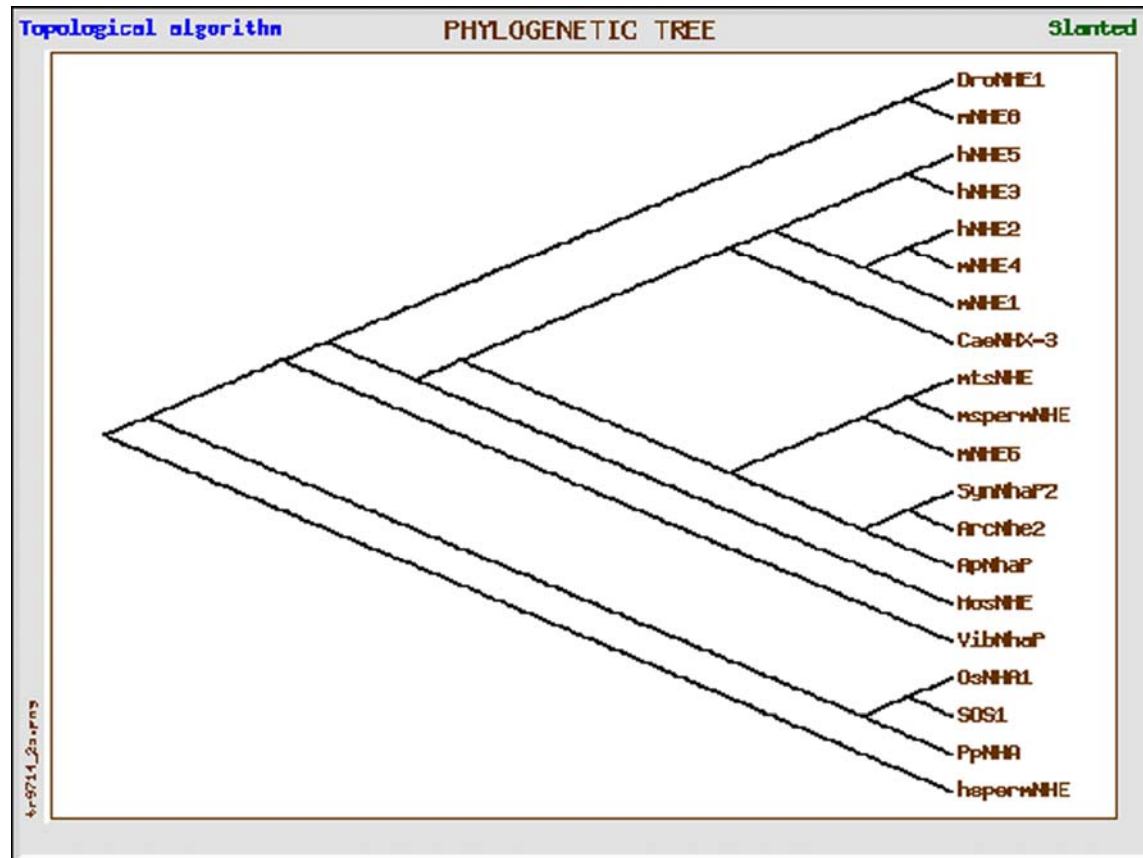


Figure 4. Phylogenetic tree displays the relationship between the mtsNHE and other NHEs. The tree was generated with GeneBee <http://www.genebee.msu.su/genebee.html> aligning the predicted open reading frames of each NHE with topological algorithm. (The Genbank accession numbers are listed below. DroNHE1: AAD32689, mNHE8: NP_683731, hNHE5: NP_004585, hNHE3: P48764, hNHE2: A57644, mNHE4: BAC39504, mNHE1: Q61165, CaeNHX-3: AAG24015, mspermNHE: AY368685, mNHE6: BAC27816, SynNhaP2: NP_441812, ArcNHE2: NP_069680, ApNhaP: BAB69459, MosNHE: AAM63432, VibNhaP: NP_761650, OsNHA1: AAP93587, SOS1: AAF76139, PpNHA: CAD91921, hspemNHE: BAC 87265).

insemination by the sperms treated with the antisera of the rabbits inoculated with the recombinant protein compared with that of the preimmunized control sera (83.1%). Similarly, sperms treated with mouse antisera produced by muscle injection of the pCR-NHE also significantly reduced the ability to fertilize oocyte (44.3%) as compared to the control (84.0%).

4.7 Inhibitory effect of antisera on spermatozoa acrosome reaction

We performed CTC fluorescence assay to assess the influence of the antisera of inoculated mice and rabbits on acrosome reaction. The antisera from rabbits immunized with recombinant protein significantly suppressed the frequency of AR pattern. The AR rate was decreased from 56.9% to 33.3% (Figure 8C1). The antisera from the mice inoculated with pCR-NHE also showed an obvious inhibitory effect on AR compared with the antisera from pCR3.1 immunized mice. The percentage of acrosome-reacted sperms was reduced from 53.6% to 39.4% (Figure 8C2).

4.8. Alterant spermatozoa motility by antisera

To evaluate whether the antisera from rabbits or mice affect spermatozoa viability, spermatozoa motility was scored respectively. The mean percentage of motile spermatozoa among sperm populations mixed with the antisera from the rabbits was significantly less than that with the preimmune sera. The percentage was brought down from 76.1% to 43.9% (Figure 9A1). The antisera from immunized rabbits also significantly decreased the relative VAP, VCL and VSL, as compared with the control group (Figures. 9A2-9A4). Similar results were also observed in the experiments using antisera from immunized mice.

4.9. Effect of antisera on sperm intracellular pH and calcium concentration

We probed changes in spermatozoa intracellular pH and calcium concentration after treatment with the antisera from rabbits immunized with the recombinant

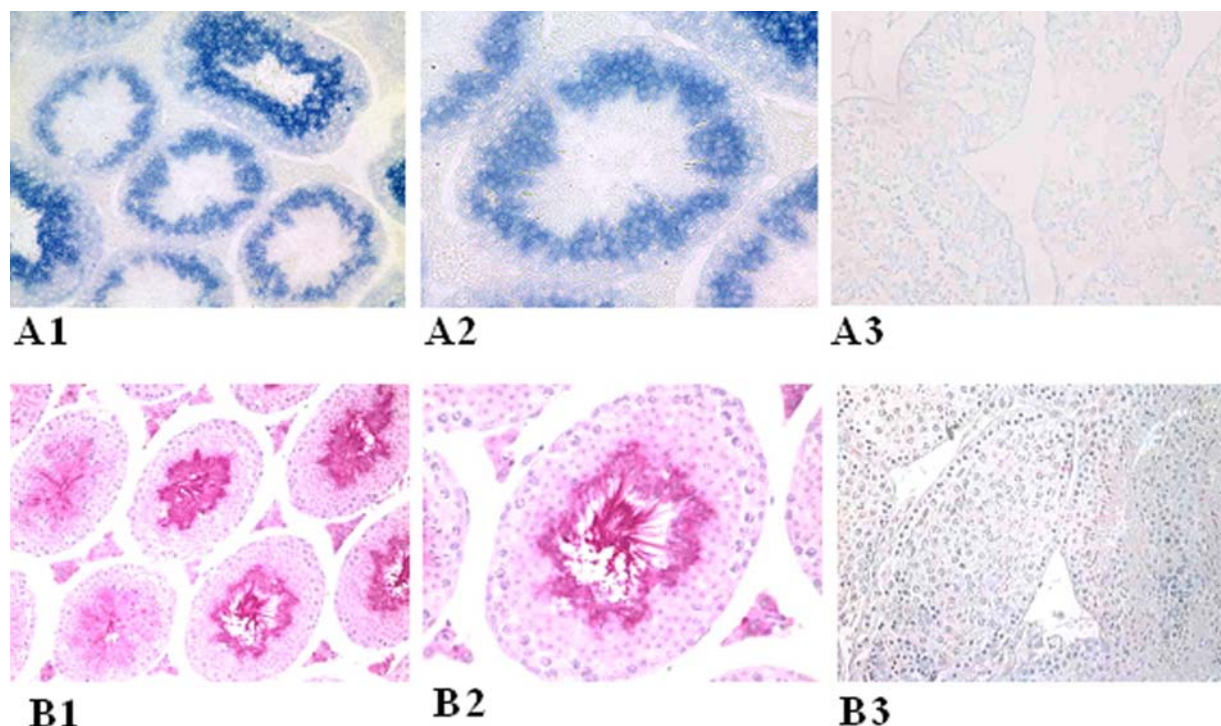


Figure 5. The restricted expression pattern of mtsNHE. *A.* *In situ* hybridization of testicular tissue. Antisense and sense DIG-cRNA probes were used for *in situ* hybridization of paraformaldehyde-fixed adult mouse testis section. The mtsNHE mRNA is specifically expressed in spermatocytes. *A1* Mouse testicular section was probed by antisense DIG-cRNA ($\times 200$ magnification); *A2* a higher magnification of *A1* ($\times 400$); *A3* Mouse testicular section was probed by sense DIG-cRNA ($\times 200$ magnification). *B.* Immunohistochemical localization of the mtsNHE antigen in adult mouse testis. Mouse testicular section was reacted with antiserum and preimmune serum from rabbit immunized with recombinant protein as primary antibodies. The mtsNHE protein was specifically expressed in spermatids and spermatozoa. *B1* Mouse testicular section was reacted with antiserum from immunized rabbit ($\times 200$ magnification); *B2* a higher magnification of *B1* ($\times 400$); *B3* Mouse testicular section was reacted with preimmune serum ($\times 200$ magnification).

protein. The antisera were diluted and fluxed into the chamber, as shown in Figure 10A, the sperm pH_i descended in a time-dependent manner, then the plot returned stable at a lower level; On the contrary, in pouring of the diluted preimmune sera had no obvious effect. The pharmacological experiment with the Na⁺/H⁺ exchanger inhibitor amiloride showed the same effect of the antisera on the sperm intracellular pH (data not shown).

Similarly, as shown in Fig 10B, by treatment of the sperms with the diluted antisera, the fluorescence intensity of the sperms showed that (Ca²⁺)_i reduced gradually to half of the control. At 170 sec, the plot became stable again. Therefore, it is suggested that the mtsNHE antisera might have inhibitory effect on both pH_i and (Ca²⁺)_i.

5. DISCUSSION

A protein of testis-specific sodium-hydrogen exchanger, mtsNHE, has been cloned in the present study (22, 29-31). The mtsNHE mRNA was detected in spermatocytes and spermatids, the NHE protein was expressed in the spermatids and the spermatozoa, such

expression pattern of the mtsNHE mRNA and its protein in spermatogenesis cells has been also reported in other genes (29, 32), and suggested that such genes might be on account of preparation of mRNAs in the early stage, and for proteins expressed in the later stage of spermatids and spermatozoa (33).

Protein sequence analysis proved existence of NHE conserved domain in the protein. Equivalent results were also observed in Kyte-Doolittle plot by DNASTAR, showing 12 transmembrane domains might exist. So far, the number of NHE family has reached to ten (2). By comparing predicted open reading frames of the mtsNHE with other NHEs, the resulted phylogenetic tree with topological algorithm showed that the mtsNHE seemed to be most closely related to mspermNHE. It is therefore suggested that the novel protein detected in the present study might be a new member of NHE family. Three members of the family, NHE1, NHE5 and mspermNHE, have been identified and expressed in spermatozoa (4, 7). The mtsNHE was localized to principle piece of sperm flagellum, which was proved to be critical for spermatozoa movement (34, 35). The predicted M_r of the NHE was about 62.1 kDa, but both in testis and spermatozoa, a

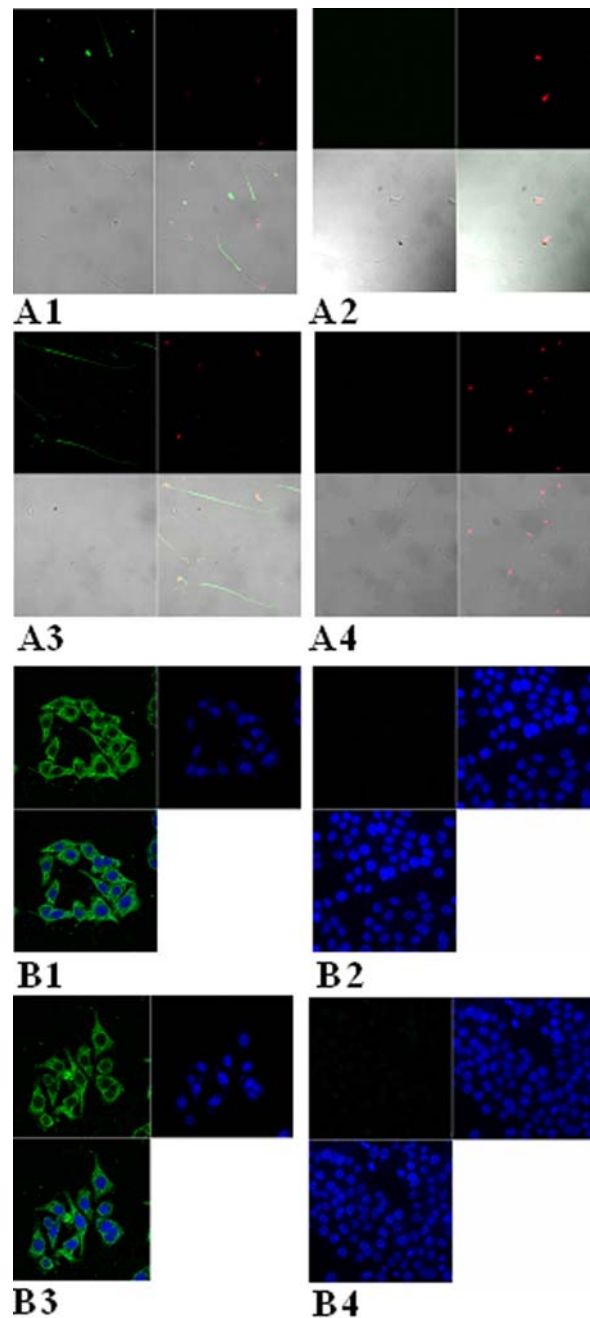


Figure 6. Indirect immunofluorescence of mtsNHE protein. *A* sperms obtained from mouse caput epididymis. The formaldehyde-fixed spermatozoa were probed with antiserum from rabbit immunized with recombinant mtsNHE protein or immunized mouse via DNA vaccine as primary antibodies and secondary antibody conjugated with FITC (panel one, green) according to the method detailed in Materials and Methods. The cells were counterstained with propidium iodide (panel two, red) for visualizing nuclei. Light field was used as panel three. The fourth part of each picture was the combination of all the three panels. The mtsNHE protein specifically localized at principle piece of sperm flagellum. *A1* Antiserum of rabbit immunized with recombinant protein; *A2* Serum from preimmunized rabbit; *A3* Antiserum of immunized mouse via DNA vaccine; *A4* Serum from inoculated mouse via mock plasmid. *B*. Transfected HeLa cells. pCR-NHE transfected cells were reacted with the antisera as primary antibodies and the secondary antibodies as described in *A*. The cells were counterstained with Hoechst for visualizing nuclei. The green fluorescence emitted by activated FITC represents the expression of mtsNHE protein or its fragment. *B1* Antiserum of rabbit immunized with recombinant protein; *B2* Serum from preimmunized rabbit; *B3* Antiserum of immunized mouse via DNA vaccine; *B4* Serum from inoculated mouse via mock plasmid. Magnification is $\times 400$.

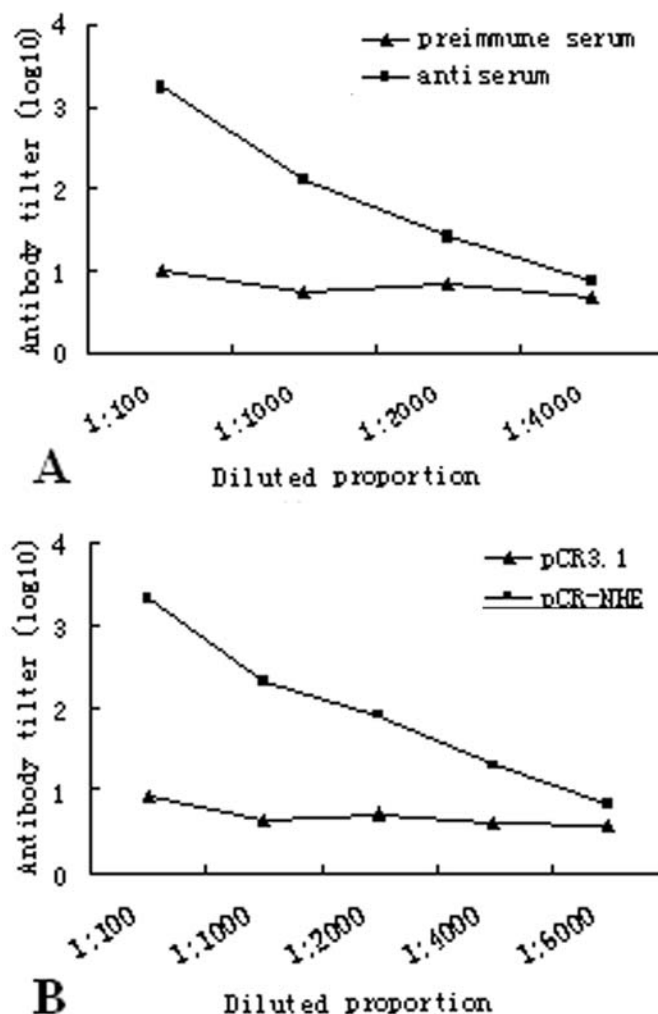


Figure 7. Analysis of serum antibody titers specific to mtsNHE by ELISA. Testicular total protein was chosen as coated antigen. *A.* Antiserum and preimmune serum of the rabbit immunized with recombinant protein were serially diluted from 1:100 to 1:4000. *B.* Antisera from the pCR-NHE and pCR3.1 immunized mice via muscle injection were serially diluted from 1:100 to 1:6000. The antibody titers were pooled and expressed as log₁₀ titers according to the method detailed in the above part of this literature.

protein with a relative M_r of about 68 kDa was detected in the present study. Considering the predicted protein subcellular transmembrane location, we proposed the augmented M_r might be due to glycosylation of this type of protein (36). In the transferred 3T3 or GC2 cells (a spermatocyte cell line) with the mtsNHE, the NHE failed to be transported to the plasma membrane. This phenomenon was also observed in mspermNHE (7), indicating a cell-specific transport mechanism present in the spermatid, in which NHE might be responsible.

Kyte-Doolittle plot analysis by DNASTAR showed there was no cytoplasmic region at the carboxyl terminus of the mtsNHE, suggesting that the region might be important in modulating NHE domain by phosphorylation (37).

During capacitation, sperm (Ca^{2+})_i increased, and then remained at a stable level (10). Alkaline pH_i was

speculated to play an important role during that period (12). Evidence has shown that pH_i increases during capacitation and AR via a Na^+ -dependent mechanism (12, 13). It is suggested that elevation of pH_i is essential for (Ca^{2+})_o dependent AR (14, 15), therefore, the new "housekeeping" NHE might play a role as a basic exchanger for hydrogen and sodium, and then indirectly modulate the AR.

It has long been accepted that pH_i is a vital regulator of both invertebrate and vertebrate spermatozoa motility (38, 39). Activation of motility depends on concentration of external Na^+ (Na^+)_o and internal pH (pH_i) (8-10). Motility initiation in sperm was regulated by reverse sodium-calcium exchange as calcium influx and sodium efflux (35). The mtsNHE-specific antisera which could modulate sperm concentration of both pH_i, (Na^+)_i and (Ca^{2+})_i, also depress the sperm motility. It is therefore proposed that physiological function of the mtsNHE might

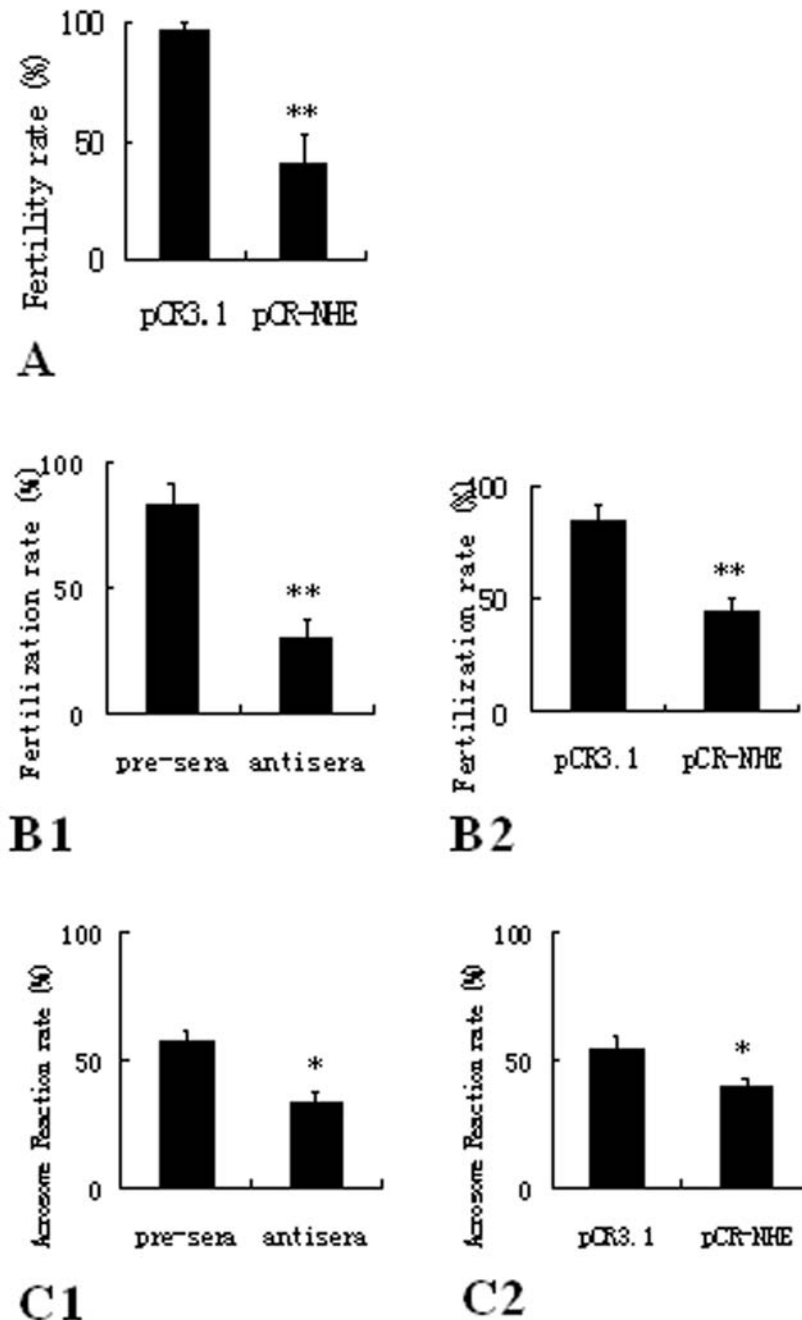


Figure 8. Effect of pCR-NHE vaccination on female mouse fertility. *A.* Female mice were inoculated with pCR3.1 and pCR-NHE respectively via muscle injection. Analysis of fertility rate was described in Materials and Methods. The fertility rate (mean \pm SD) of vaccinated female mice with pCR-NHE was decreased from $96.7\% \pm 2.9\%$ (inoculated with pCR3.1) to $40.0\% \pm 13.2\%$ (with pCR-NHE). *B.* *In vitro* fertilization. Two-cell embryos were scored at 24-27 h after insemination. *B1* The antiserum from immunized rabbit significantly decreased fertilization rate (mean \pm SD) from $83.1\% \pm 8.5\%$ (preimmune serum from immunized rabbit) to $29.4\% \pm 7.5\%$ (antiserum from immunized rabbit). *B2* Antiserum from mouse inoculated with pCR-NHE significantly decreased fertilization rate from $84.0\% \pm 7.9\%$ (antiserum from mouse inoculated with mock plasmid) to $44.3\% \pm 6.3\%$ (with pCR-NHE). *C.* Effect of antisera on spermatozoa AR. Percentage of acrosome-reacted spermatozoa (AR %) at least in 200 spermatozoa was scored. *C1* Antiserum from immunized rabbit significantly decreased the AR rate (mean \pm SD) from $56.9\% \pm 5.1\%$ (preimmune serum from the immunized rabbit) to $33.3\% \pm 4.2\%$ (Antiserum). *C2* Antiserum from mouse inoculated with pCR-NHE significantly decreased the AR rate (mean \pm SD) from $53.6\% \pm 6.2\%$ (antiserum from mouse inoculated with mock plasmid) to $39.4\% \pm 3.7\%$ (with pCR-NHE) ** significant difference ($P < 0.01$) and * significant difference ($P < 0.05$). Data are expressed as mean \pm SD from 4 replicate of experiments.

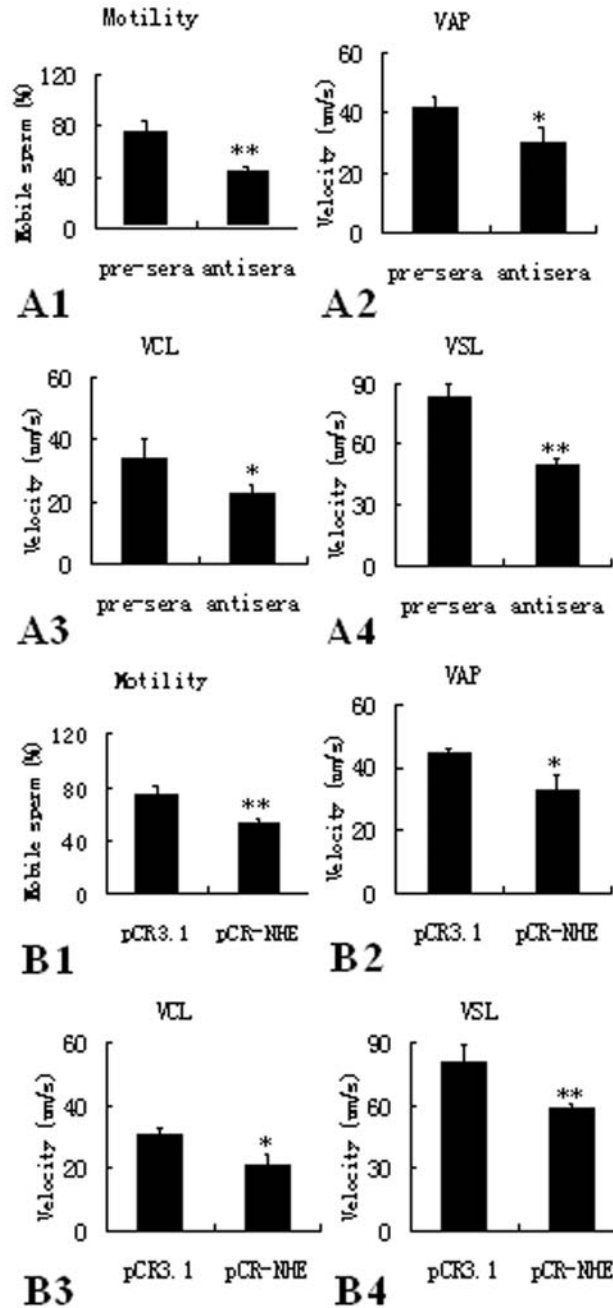


Figure 9. Effect of NHE antisera on spermatozoa motility. *A.* Antiserum and preimmune serum of rabbit immunized against the recombinant protein. *A1* The antiserum decreased the percentage of motile spermatozoa (mean \pm SD) from $76.1\% \pm 8.3\%$ (pre-serum) to $43.9\% \pm 3.2\%$. *A2* The antiserum significantly decreased the smoothed path velocity (VAP, mean \pm SD) from $42.1 \pm 3.2 \mu\text{m/s}$ to $30.3 \pm 4.4 \mu\text{m/s}$. *A3* Antisera significantly decreased the relative track velocity (VCL, mean \pm SD) from $33.6 \pm 6.6 \mu\text{m/s}$ to $22.8 \pm 2.5 \mu\text{m/s}$. *A4* Antisera significantly decreased the relative straight-line velocity (VSL, mean \pm SD) from $83.2 \pm 6.9 \mu\text{m/s}$ to $50.5 \pm 2.4 \mu\text{m/s}$. *B.* Antisera from inoculated mice with pCR-NHE and pCR3.1 (control mock plasmid) via muscle injection were used for motility test of mice sperm. *B2-B4* showed the value of velocities of sperms respect to those which were incubated with sera from pCR-3.1 inoculated mice. *B1* Antisera from pCR-NHE immunized mice apparently decreased the mobile sperm percent (mean \pm SD) from $73.4\% \pm 7.6\%$ (pCR3.1) to $52.2\% \pm 4.2\%$. *B2* Antisera from pCR-NHE immunized mice significantly decreased the VAP from $44.3 \pm 1.7 \mu\text{m/s}$ to $32.5 \pm 5.1 \mu\text{m/s}$. *B3* Antisera from pCR-NHE immunized mice significantly decreased the VCL from $30.2 \pm 2.8 \mu\text{m/s}$ to $20.9 \pm 3.3 \mu\text{m/s}$. *B4* Antisera from pCR-NHE immunized mice significantly decreased the VSL from $80.6 \pm 8.3 \mu\text{m/s}$ to $58.7 \pm 1.5 \mu\text{m/s}$. ** indicates significant differences ($P < 0.01$) and * indicates significant differences ($P < 0.05$).

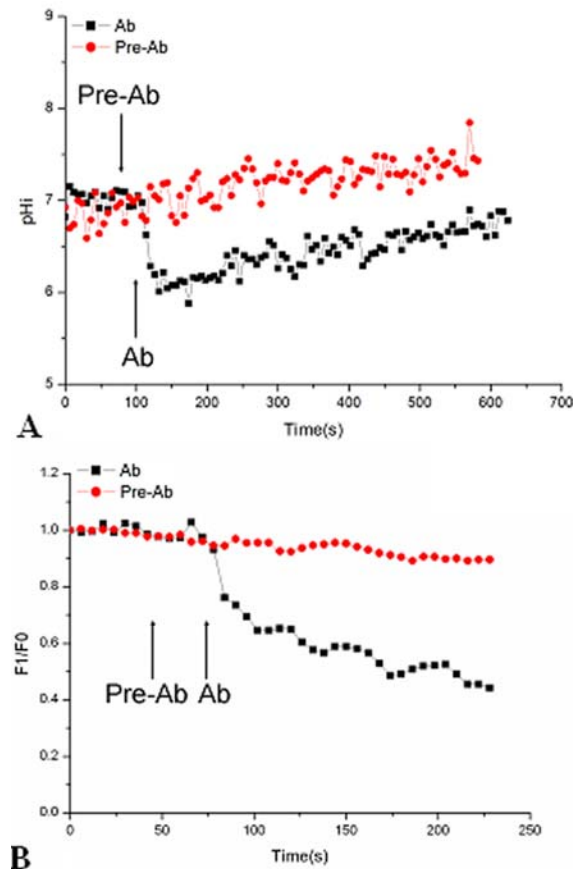


Figure 10. Effect of NHE antiserum from rabbit immunized with recombinant protein on mouse sperm pH_i and (Ca²⁺)_i. The antiserum and preimmune serum were used for measurement of mouse sperm pH_i and (Ca²⁺)_i according to the method detailed in this article. *A*. Treatment of sperms with the antiserum decreased the pH_i. The vertical line showed the inner pH values (ratio of fluorescence intensities at 580/ 640 nm). *B*. After treatment of sperms with the antiserum at 40 sec, the fluorescence intensity of (Ca²⁺)_i decreased gradually, at 170 sec only half of the fluorescence intensity could be detected. The vertical line showed the inner calcium concentrations by calculating the emission of 525 nm.

be involved in modulation of intracellular pH by importing Na⁺ and exporting H⁺, as suggested by Wakabayashi *et al* (3). This transportation can be driven by electrochemical gradient of H⁺ or Na⁺ between outside and inside of the cells (40). To examine inhibition of Na⁺/H⁺ exchanger activity by the mtsNHE-specific antisera, we designed experiment to look at possible effect of the antisera on sperm intracellular pH value. Because of the combination of extrusion and influx Na⁺, the decrease in pH value observed might be result of both extrusion of H⁺ and influx of Na⁺ in the sperm. Calcium homeostasis of spermatozoa is maintained by several transporting proteins among which Na⁺/Ca²⁺ exchanger may play a major role (38-41). Evidence has demonstrated that regulation of sperm capacitation, motility and AR may be more sensitive to changes in (Ca²⁺)_i by modulating protein phosphorylation

(41, 42). Na⁺/Ca²⁺ exchanger might pose certain effect during AR (43). An alkaline pH_i may be necessary for capacitation and AR (10). Influence of Na⁺/K⁺-ATPase which contributes to pH_i regulation by keeping low (Na⁺)_i can not be ruled out. Na⁺/K⁺-ATPase via (Na⁺)_i modulates NHE, and then regulates pH_i (44). A subtle balance must be preserved between conditions that promote premature AR and those that counterbalance environmental changes so that only the tightest and fittest sperm survive excursion through epididymis and female reproductive tract achieve fertilization (10). Therefore, a relationship may exists among mtsNHE, Na⁺/Ca²⁺ exchanger and Na⁺/K⁺-ATPase, and the cationic function might be independent on direct interaction between proteins. Changes in (H⁺), (Na⁺) and (Ca²⁺) may modulate activity of these three exchangers. Considering the testis-specific expression of the mtsNHE mRNA, the transmembrane region of mtsNHE located in principle piece of sperm flagellum was selected as an antigen containing domain and inserted into eukaryotic vector. The inhibitory effect of IVF with the antiserum from the immunized rabbit and the remarkable decrease in the female mice fertility rate with the pCR-NHE vaccine via muscle injection confirmed the requirement of mtsNHE *in vivo* and *in vitro* for sperm fertility.

The inoculated pCR-NHE vaccine plasmid contains the DNA fragment coding the transmembrane region of mtsNHE, which might be small enough to avoid cytotoxic responses (25). The generated antibodies by the vaccination could not only identify the purified recombinant mtsNHE protein, but also the native mtsNHE on both sperm surface and in testicular protein. Both *in vitro* and *in vivo* experiments have demonstrated the anti-fertility potential of the pCR-NHE DNA vaccine. Because the selected fragment of mtsNHE protein which contained 62 amino acids, not only including the transmembrane domains, but also extracellular domains, therefore the polyclonal antibodies produced by the fragment may not only specifically inhibit Na⁺/H⁺ exchanger activity, but also other sperm activities. Our *in vitro* experiment showed that the normal sperm suspension mixed only with the antisera from the pCR-NHE immunized mice, but not from that of the black pCR immunized mice was observed with sperm aggregation, therefore, the antibody-induced sperm aggregation should be specifically. It is therefore suggested that the antibodies *in vivo* may not only specifically affect sperm Na⁺/H⁺ exchanger activity, but also capable of decreasing sperm fertility by inhibiting sperm capacitation, AR and inducing sperm aggregation in the reproductive tract. These might be how the vaccine works and reduces the total female fertility.

In conclusion, we have cloned a testis-specific sodium-hydrogen exchanger protein, mtsNHE, the NHE was expressed in spermatozoa. Treatment of sperm with the antibodies decreased intracellular pH and calcium concentration. Muscle injection of female mice with the specific mtsNHE gene vaccine significantly stepped down fertility rate. Considering the specific expression and involvement in regulation of fertility, the mtsNHE might be

a candidate molecule for developing a new male contraceptive.

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Key Words: Mouse, Sodium-Hydrogen Exchanger, Testis, Sperm Specific Antigen, Sperm Motility, Fertility

mtsNHE is involved in sperm fertility

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