Immunization with a DNA vaccine of testis-specific sodium-hydrogen exchanger by oral feeding or nasal instillation reduces fertility in female mice

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Objective: To investigate the effect of immunization with a DNA vaccine of testis-specific sodium-hydrogen exchanger (*tsNHE*) via oral feeding or nasal instillation on fertility in female mice and to look at its potential mechanism.
Design: Prospective, research study.
Setting: Institution-affiliated research laboratory.
Animal(s): Sexual mature BALB/c mice.
Intervention(s): Female mice immunized orally or nasally with the DNA vaccine at 2-week' intervals.
Main Outcome Measure(s): Number of newborns and fertility rate of the vaccinated female mice were scored.
Result(s): We identified a novel testis-specific sodium-hydrogen exchanger, *tsNHE,* which is localized to the principal piece of sperm flagellum. Immunization of female mice with the *tsNHE* DNA vaccine via oral feeding or nasal instillation statistically significantly decreased fertility rate and the newborn numbers compared with the controls. The antiserum or vaginal fluid from the *tsNHE* cDNA vaccinated female mice could specifically recognize the principal piece of sperm tail and triggered sperm agglutination. The antibodies also showed a statistically significant inhibitory effect on in vitro sperm motility and fertilization.
Conclusion(s): The sodium-hydrogen exchanger might be an excellent target molecule for developing a new contraceptive. (Fertil Steril® 2010;93:1556–66. ©2010 by American Society for Reproductive Medicine.)

Key Words: Antifertility, sodium-hydrogen exchanger, sperm antigen, *tsNHE* DNA vaccine

As the world population is increasing at a high speed, a convenient, safe, and less expensive means of contraception is urgently needed (1). Immunocontraception associated with gametes is a novel contraceptive strategy that has attracted increasing attention in recent years (2, 3). Development of a vaccine to sperm antigen is a promising route for this scheme (4); however, until now only a few specific antigens involved in sperm–egg interaction have been identified (5). It is known that sodium-hydrogen exchangers (NHEs) by mediating electroneutral exchange of Na^+ for H^+ play a vital role in osmoregulation, pH control, cell energetics (6), and sperm function (4). The direction of this exchange relies on electrochemical gradients of ions across membranes. To date, 10 NHE isoforms have been identified from various species including mouse, rat, and human (7). They are all cell-integral membrane proteins residing in plasma membranes and endomembranes (8). It has been suggested that sodium-hydrogen exchanger as a channel for regulation of intracellular pH might be a crucial modulator of sperm capacitation and motility (9). Three NHEs (NHE1, NHE5, and mspermNHE) have been identified in spermatozoa (10). Of these, NHE1 is an ubiquitously expressed normal form responsible for regulation of cell volume and pH, whereas the others are more restricted (6–8). Knockout of these NHEs in mice resulted in distinct phenotypes (9, 11, 12). According to our unpublished DNA chip data and Unigene data with a UGID: 276341 at NCBI, we produced a complementary DNA (cDNA) 1813 bp fragment. The resultant full-length cDNA encodes a new gene named mtsNHE that contains a sodium-hydrogen exchanger domain and a complete open-reading frame for a protein of 565 amino acids (the GeneBank accession number for the tsNHE DNA sequence is EU846100). The sodium-hydrogen exchanger is capable of regulating sperm intracellular pH value and calcium concentration, and modulating sperm motility and acrosome reaction (unpublished data). Because of its location on the sperm surface and NHE expression inside the blood-testis barrier, we selected mtsNHE as a DNA vaccine to examine its anti-fertility action. Furthermore, nasal administration or oral feeding of antigens could alter the subsequent immune response in animals and humans. The vaccine might overcome immune tolerance of self-antigen (13).

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We used a recombinant plasmid pCR-NHE as prototype of the DNA vaccine to immunize BALB/c female mice via oral feeding or nasal instillation and to examine the induced antifertility effect by humoral and mucosal responses. We have demonstrated that the vaccine significantly decreased the fertility rate of the immunized female mice.

MATERIALS AND METHODS

The BALB/c mice, purchased from the Institute of Genetics of Chinese Academy of Sciences, were housed in a 12-hour light/12-hour dark environment and were given food and water ad libitum (5, 13). 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 the Institute of Zoology at the Chinese Academy of Sciences. Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St. Louis, MO).

Immunization

The pCR3.1 mock vector (Invitrogen, Groningen, the Netherlands) and pCR-NHE (constructed and conserved by our laboratory) were purified with an endotoxin-free plasmid mega kit (Qiagen, Venlo, the Netherlands). The pCR-NHE contained a cDNA coding the partial transmembrane region. 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'). The recombinant was confirmed by restriction mapping and sequencing.

Female BALB/c mice were immunized with the plasmid DNA purified by Qiagen Endofree Mega (Qiagen, Valencia, CA). The mice were housed as previously described elsewhere (5, 13), and each female mouse was immunized with 20 μg of mtsNHE vaccine dissolved in 30 μL of saline via oral feeding (20 mice) or nasal instillation (20 mice). The 20 mice in the control group were immunized with 20 μg of the pCR3.1 mock plasmid dissolved in 30 μL of saline. The mice subsequently were given a booster dose twice by the same method in 2-week intervals. One week after the second immunization, the sera and the vaginal fluids from each mouse were collected. They were separated and stored individually at –20°C. The preimmune sera and vaginal washes from the negative control mice were collected in a similar manner (1, 14).

In Vivo Detection by RT-PCR

Total RNA was extracted from the mice testis by the use of Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The RNA pellets were gently resuspended in 100 μL of nuclease-free water. All RNA samples were stored at –20°C until use. Amplification of cDNA fragments was performed by reverse-transcription polymerase chain reaction (RT-PCR) according to the manufacturer's instructions (Promega, Madison, WI). Reverse transcription was allowed to proceed at 48°C for 50 minutes, followed by inactivation at 95°C for 2 minutes. The primer pair for mtsNHE detection was 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').

Expression in Cultured HeLa and 3T3 Cells in Vitro

We transfected pCR-NHE and pCR3.1 into the cultured HeLa and 3T3 cells, respectively, by LipofectAMINE (GIBCO BRL, Rockville, MD). Thirty-six hours later, the transfected cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 hour at room temperature. The slides were then rinsed three times in PBS, and the cells were permeabilized with 0.1% Triton X-100 plus 0.1% sodium citrate in PBS for 2 minutes, washed with PBS, blocked with 0.5% bovine serum albumin (BSA) in PBS (pH 7.4) for 30 minutes at room temperature, and further incubated with the antisera or the vaginal fluids (diluted 1:200 with PBS containing 0.5% BSA) at 4°C overnight. These immunized sera and vaginal fluids were obtained from the mice 6 weeks after injecting the pCR-NHE or pCR3.1. After the cells were washed thoroughly, the secondary antibodies (goat anti-mouse IgG, IgA conjugated with FITC, diluted 1:100 with PBS; Sigma) were added and incubated at 37°C for 1 hour. After the slides were washed, the cells were counterstained with Hoechst for visualizing the nuclei and were analyzed for gene expression using confocal microscopy (Zeiss, Oberkochen, Germany).

Sperm Preparation for Confocal Examination

Caudal epididymal sperm was collected by placing two minced caudal epididymides into 5 mL of PBS at 37°C. The sperm was allowed to swim out for 1 hour, then was centrifuged at 500 $\times$ *g* for 10 minutes (15). After two washes, the pellet was suspended with PBS. Sperm in suspension was dropped onto poly-L-lysine–coated cover slips, smeared, air-dried, then was fixed with 4% paraformaldehyde for indirect immunofluorescence as previously described. Instead of Hoechst, propidine iodide (PI) was used as a dye for visualizing the nuclei.

Immunohistochemical Analysis

After deparaffinization and rehydration, 5-μm sections were subjected to antigen retrieval using ethylenediaminetetraacetic acid (EDTA) buffer (10 mM [pH 8.0]) at 98°C for 15 minutes then were cooled naturally to room temperature. After two washes in PBS, the sections were sequentially incubated with 10% normal blocking serum for 30 minutes to suppress nonspecific binding, first with the antisera or the vaginal fluids from the immunized mice as the primary antibodies (diluted 1:200) at 4°C overnight, then with the biotinylated secondary antibodies (1:200) at room temperature for 30 minutes, and finally with avidin-alkaline phosphatase complex and Vector Red according to the manufacturer's protocol (Vectastain ABC-AP kit, Vector Laboratories, Burlingame,