Induction of specific immune response and suppression of fertility by B-cell-epitope-based mimovirus vaccine

Zi-gang Shen¹, Wei He², Ji Zhang¹, Hai-yang He¹, Xia Yang¹, Zheng-qiong Chen², Ping Yang², Jian Li¹, Zhi-qing Liang², Yu-zhang Wu¹ and Jin-tao Li¹

¹Institute of Immunology, PLA and ²Department of Obstetrics and Gynecology, Southwest Hospital, Third Military Medical University, 30 Gaotanyan Street, Shapingba District, Chongqing 400038, People's Republic of China

Correspondence should be addressed to Y Wu; Email: wuyuzhang20006@sohu.com; J Li; Email: ljtgms@yahoo.com.cn

Z Shen and W He contributed equally to this work

Abstract

SPINLW1 (previously known as eppin (epididymal protease inhibitor)) is a target under intense scrutiny in the study of male contraceptive vaccines. B-cell-dominant epitopes are now recognized as key parts of the induction of humoral immune responses against target antigens. The generation of robust humoral responses *in vivo* has become a crucial problem in the development of modern vaccines. In this study, we developed a completely novel B-cell-dominant-epitope-based mimovirus vaccine, which is a kind of virus-size particulate antigen delivery system. The mimovirus successfully self-assembled from a cationic peptide containing a cell-penetrating peptide of TAT_{49–57} and a plasmid DNA encoding both three SPINLW1 (103–115) copies and adjuvant C3d3. The male mice were immunized with the epitope-based mimovirus vaccine, which resulted in a gradual elevation of specific serum IgG antibody levels. These reached a peak at week 4. Mating for the fertility assay showed that the mimovirus vaccine had accomplished a moderate fertility inhibition effect and investigation into the mechanism of action showed that it did so by interfering with the reproductive function of the sperm but that it did not damage the structures of the testes or cause serum testosterone to decline. Our results suggest an ideal protocol for suppressing fertility in mice by an engineered mimovirus vaccine.

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Introduction

In the present day, birth control and family planning are of major concern (Speidel *et al.* 2009). However, the contraceptive options available to men are limited to vasectomy, condoms, and early withdrawal, all of which present certain problems. The former is considered to be a permanent method of birth control, considering that the surgery to reverse infertility has only a limited success rate (Silber & Grotjan 2004, Patel & Sigman 2008). The latter two methods have failure rates even under perfect use. They are between 80 and 90% successful under actual use (Fu *et al.* 1999). Therefore, it is necessary to develop new male contraceptives that are safe, reversible, and more effective than current offerings. Contraceptive vaccines may be an attractive addition to the currently available range of family planning methods, offering several potential advantages. Currently, efforts toward developing a steroidal-based male contraceptive vaccine have had few successes. In the past two decades, there have been many studies focusing on sperm-specific molecules, such as the

fertilization antigen (FA)-1, PH-20, PH-30, SP-10, SP-17, testis-specific antigen-1, protein A-kinase anchoring protein (AKAP), and sperm-associated antigen 9 (SPAG9; Primakoff *et al.* 1988, Herr *et al.* 1990, Hardy *et al.* 1997, Lea *et al.* 1998, Naz 1999, Santhanam & Naz 2001, Miki *et al.* 2002, Shankar *et al.* 2004). None have been proven to be successful contraceptive agents.

SPINLW1 (previously known as eppin (epididymal protease inhibitor)) is one of several serine protease or serine protease-like inhibitors that have been recently identified and characterized as a 'male-only' molecule from the testes and epididymis. SPINLW1 is a $\approx$ 133 amino acid protein secreted by the epididymis/testis in an androgen-dependent manner. It is found on the surface of spermatozoa and seems to be important for sperm maturation (Richardson *et al.* 2001). It has been shown that, in immunized male monkeys, SPINLW1 may be neutralized by antibodies that are believed to affect fertilization by interfering with sperm maturation (O'Rand *et al.* 2004).

However, a previous study has shown that the full-length, mature protein is generally not suitable as

a vaccine immunogen for reasons of safety (Agadjanyan *et al.* 2005). Because activation of the CD4⁺T cell is dependent on recognition of specific peptides (epitopes) (Hernandez *et al.* 1997), it is necessary to search these dominant B-cell epitopes of SPINLW1 to produce safe effective immunogens. Previously, a B-cell epitope, MFVYGGCQGNNNN, from SPINLW1 amino acids 103–115 (GenBank Accession No: QBDA01) was predicted using GUATIF, TEPITOPE, and ANTHIWHIN software. Previous studies have also shown that this B-cell epitope can induce a SPINLW1-dominant B-cell-epitope-specific immune response and suppress fertility (Chen *et al.* 2009). For this reason, a mimovirus vaccine strategy was developed and carried out to improve upon the immune potential of this B-cell epitope as a vaccine immunogen (Wu *et al.* 2002). This nanometer-sized particle antigen delivery system was constructed by combination of the epitope from SPINLW1 and the plasmid encoding the same epitope. Complement (C) component C3d has previously been proven to enhance antibody responses to several antigens when three copies of C3d (3C3d) are combined with the antigen as a fusion protein (Wang *et al.* 2006, Movsesyan *et al.* 2008). Herein, we engineered a plasmid to include 3C3d as the molecular adjuvant. We packed it into a B-cell-epitope-based mimovirus, which we used to immunize mice. We found that the engineered mimovirus robustly induced an immune response that may enhance the anti-SPINLW1 antibody response and effectively suppress fertility in mice.

Results

Plasmid construction and western blotting

We constructed two plasmids as shown in Fig. 1A. The 3SPINLW1_{103–115}PADRE fragment was cloned into the eukaryotic expression vector pSG.SS.YL with or without three copies of C3d, resulting in combinational DNA plasmids P1 and P2 respectively. After the HEK293 cells were transiently transfected with P1, P2, or pSG.SS.YL plasmids, the targeted proteins were expressed *in vitro*

and confirmed by western blotting using anti-SPINLW1 antibody. As shown in Fig. 1B, the ≈ 7 kDa band appeared in plasmid P1 transfection (lane 1), and a ≈ 118 kDa band appeared in plasmid P2 transfection (lane 3).

Formation of the mimovirus

The mimovirus was prepared with r ranging from 0 to 16 and formed by the self-assembly of the cationic peptide (rkrrrrMFVYGGCQGNNNN) with the plasmid through electrostatic interactions. Peptide–DNA complexes were examined by electrophoresis mobility in the DNA retardation assay. In Fig. 2A, no migration of the plasmid DNA band was observed when $r \geq 4$, indicating that the negative charge of the plasmid was fully neutralized by the cationic peptide.

When a mimovirus is formed, plasmid DNA is expected to be protected from DNaseI. So, we evaluated the ability of each complex via a DNaseI digestion assay. As shown in Fig. 2B, the protection of plasmid DNA was first examined at $r=4$ (lane 5). At this point, the DNA was fully retarded. The formation of the mimovirus was also confirmed by transmission electron microscopy with negative staining to observe the structure of the mimovirus. We prepared the mimovirus at a ratio of 4.0, and particle size analysis showed that the diameter of most particles ranged between 30 and 50 nm, with a peak value of 37.84 nm (Fig. 2C and D) and average diameters of 40 nm. All these data indicated that the virus complexes had been constructed successfully. $r=4$ was used for all the experiments.

Antibody response

Next, we tested the IgG-specific response to rhSPINLW1 in the vaccinated mice by standard ELISA. The mean reciprocal of endpoint titers showed the details of the four groups with different inoculation regimens at different times (Fig. 3). The anti-SPINLW1 antibody levels could be detected after the first immunization

Figure 1 Schematic representation and *in vitro* expression of plasmids. (A) 3SPINLW1_{103–115}-PADRE and 3SPINLW1_{103–115}-PADRE-3C3d genes were cloned into the pSG.SS.YL and pSG.SS.C3d.YL expression vectors, respectively, which contained the SS signal peptide and YL for termination of transcription. (B) Both 3SPINLW1_{103–115}-PADRE and 3SPINLW1_{103–115}-PADRE-3C3d proteins were expressed in HEK293 cells transfected with appropriate plasmids. Lane 1: HEK293 cells transfected with plasmids P1 (≈ 7 kDa). Lane 2: HEK293 cells transfected with pSG.SS.YL. Lane 3: HEK293 cells transfected with plasmids P2 (≈ 118 kDa).

Figure 2 The formation of mimovirus. (A) DNA retardation assay. Mimovirus was prepared at the input molar ratio of peptide to DNA (r), $r=0, 0.25, 0.5, 1, 2, 4, 8$, and 16 and then analyzed by electrophoresis on a 1% agarose gel stained with goldview (lanes 1–7). Mimovirus1 solutions were produced and analyzed in the same way with similar results (data not shown). (B) DNaseI digestion assay. Mimovirus samples at $r=0, 0.25, 0.5, 1, 2, 4, 8$, and 16 were subsequently digested with DNaseI and trypsin. The remaining DNA was extracted and analyzed on a 1% agarose gel stained with goldview (lanes 1–8). (C) The size of the mimovirus particles was measured by transmission electron microscopy with negative staining. Mimovirus particles were condensed at $r=4$ and were examined using transmission electron microscopy at a magnification of $80\,000\times$. The size of particles was ~ 30 nm. Scale bar: 200 nm. (D) Particle size analysis of mimovirus.

and reached a peak at week 4. The PBS group was considered as the background. The antibody responses in the mice inoculated with mimovirus2 appeared significantly earlier than in mice inoculated with mimovirus1. At every point in time, the mean reciprocal of the endpoint titers in the former was higher than that of the latter. Furthermore, the mean reciprocal of the endpoint titers of groups inoculated with the mimovirus was higher than that observed for the peptide A group. These data indicated that the mimovirus effectively triggered an immune response in the vaccinated mice.

Fertility assay

Then we tested the fertility of mimovirus immunized male mice. At weeks 5 and 20, immunized male mice were mated with female mice, and litter sizes were counted. The mimovirus2 group had the smallest litter size (3.07 ± 1.10), the mimovirus1 group had an average litter size of 5.07 ± 0.18 , the peptide A group had an average litter size of 8 ± 0.7 , and the PBS group had an average litter size of 8.4 ± 0.50 (Fig. 4A). Furthermore,

the fertility rates of the males from the mimovirus1 and mimovirus2 groups were $60\% \pm 0.035$ and $28\% \pm 0.053$, respectively, both significantly lower than those of the peptide A ($92\% \pm 0.051$) and PBS groups ($98\% \pm 0.019$). To test their ability to recover fertility, we carried out a fertility recovery assay at 20 weeks. The fertility rates of the females mating with males from the mimovirus1 and mimovirus2 groups were 94.44 and 92.59%, respectively, and the mean litter size among the conceiving female mice showed no difference among the four groups (Fig. 4C and D), suggesting that male mice recovered fertility after the immunization process had ceased.

Characterization of sperm cells from vaccinated mice

Sperm cells were obtained from the cauda epididymides from the immunized male mice and several factors were detected. In Table 1, there was no correlation among the four groups with regard to the weight of the testes. Furthermore, the sperm count was $63 \pm 0.0126\%$ in the mimovirus2 and $58 \pm 0.0289\%$ in the mimovirus1 groups, both lower than in the PBS ($82\% \pm 0.0289$) and peptide A groups ($77 \pm 0.0577\%$). Importantly, hyperosmotic swelling (HOS) test results were presented as % HOS, together with total sperm motility and viability, which were all greatly inhibited in the mimovirus1 and mimovirus2 groups relative to the control PBS group, as determined by independent t -test.

Detection of serum testosterone concentration

Serum testosterone concentrations were detected by RIA. The levels of serum testosterone in the mimovirus1 and mimovirus2 groups were 2.41 ± 0.25 and 2.35 ± 0.15 respectively. Serum testosterone levels were 2.47 ± 0.17 in the peptide A group and 2.7 ± 0.19 in the PBS group. These results showed no significant difference between the test groups and the control PBS group.

Figure 3 Specific immune response in specific pathogen-free BALB/c mice injected with mimovirus vaccine. Each animal received a total of four injections of the same immunogen at 3-week intervals. Serum IgG titers of mice were detected by the standard ELISA method.

Figure 4 Fertility assay of immunized mice. (A) The fertility rate of female mice mated with immunized males at 5 weeks after the first immunization. (B) The fertility rate of female mice mated with immunized males at 5 weeks after the first immunization. Asterisk indicates significant difference from group PBS ($P < 0.05$). (C) The fertility rate of female mice mated with immunized males 20 weeks after immunization had been stopped. (D) The fertility rate of female mice mated with immunized males 20 weeks after immunization had been stopped.

Histological analysis

We also performed a duct size histological examination of the testes and epididymides of mice from each group to exclude the possibility that the reduction in fertility in immunized male mice was due to any deleterious effect. The testes and epididymides were stained with hematoxylin–eosin (HE) and observed under phase contrast microscopy. Very finely coiled seminiferous tubules and germ cells were observed in the testis sections (Fig. 5). Mature sperm cells were present in the lumen. These results showed no difference between the testes of test group mice and control-PBS group mice.

Inhibition of antisera to human sperm motility

Computer-assisted sperm analysis (CASA) has been shown in a variety of species to be an accurate and

practical means of assessing male reproductive quality and the impact of various treatments on sperm motility (Owen & Katz 1993). The effect of antisera obtained from immunized male mice on human sperm motility was monitored by CASA. As shown in [Supplementary Figure 1](#) (see section on [supplementary data](#) given at the end of this article), serum from the mimovirus1 and mimovirus2 groups was found to significantly inhibit the viability and progressive motility of human sperm relative to the control PBS groups in three independent experiments ($P < 0.05$).

Discussion

The world of male contraception has been limited to condoms, vasectomies, and behavioral methods. However, these choices have their shortcomings. Hence, it is of great importance to develop a safe, effective, convenient, and reversible method of male contraception. In the last decade, scientists have developed contraceptives based on hormones. These were designed to suppress spermatogenesis. However, none of them has been proven fit for human use until now. SPINLW1 (eppin) is specifically expressed in the testes and is an epididymis protein. It is a potential target for hormonal methods of contraception and it may be relevant to the development of safe, effective, and reversible contraceptive vaccines (O'Rand *et al.* 2004). When monkeys were injected with SPINLW1, seven out of nine males tested generated high anti-SPINLW1 antibody levels and showed suppressed fertility. Of these, five out of seven recovered their fertility once the treatments were stopped. However, many studies indicate that full-length SPINLW1 proteins are not fit for human vaccines for safety reasons. Recently, we screened a fragment of SPINLW1 (MFVYGGCQGNNNN), residues 103–115, using a protein prime–peptide boost strategy. It showed high serum antibody levels with considerable anti-fertility efficacy (Chen *et al.* 2009). This strategy has a disadvantage in that it uses a full-protein immunogen. However, epitope-based vaccines offer the prospect of directional immunity leading to safer and more effective antigen-specific immune responses

Table 1 Effects of mimovirus vaccine on the seminal parameters of immunized mice.

	PBS group (control)	Treated groups		
		Peptide A	Mimovirus1	Mimovirus2
Testis weight (mg)	1216.1 ± 60.45	1156 ± 89.65	1109.67 ± 124.52	1174 ± 55.51
Sperm density (10 ⁶ /ml)	82 ± 2.89	77 ± 5.77	63 ± 1.26*	58 ± 2.89*
% Motility	52 ± 7.64	47 ± 5.77	38 ± 7.63	37 ± 2.87*
% Viability	90 ± 5.00	85 ± 5.00	68 ± 7.64*	57 ± 7.64†
% HOS	81 ± 5.13	79 ± 4.04	69 ± 3.60*	64 ± 5.29*

Data represents the mean ± s.d. * $P < 0.05$ and † $P < 0.01$.

Figure 5 Histological analysis of testes of mice immunized with mimovirus vaccines and PBS. Histological sections of the testes of mimovirus-immunized and PBS-control mice were compared. Panels depict the testes of (A) PBS-control mice; (B) peptide-A-immunized mice; (C) mimovirus1-immunized mice; and (D) mimovirus2-immunized mice.

(Chiarella *et al.* 2009). In this study, we further investigated the anti-fertility potential of the SPINLW1 B-cell epitope in BALB/c mice.

However, epitope-based peptides alone cannot elicit competent immune responses (Naz *et al.* 2005). Research has reported that mimovirus vaccines with

sizes similar to viruses can induce cellular immunity (Wu *et al.* 2002, Yang *et al.* 2008). Other studies also have indicated that mimovirus vaccines induce cellular and humoral immune responses (Zhang *et al.* 2008). These studies demonstrate that mimovirus vaccines could be developed as an ideal contraceptive vaccine to induce immune responses and suppress fertility. Herein, we formed a mimovirus vaccine containing DNA plasmid and cation peptide. We developed a DNA plasmid composed of a strong foreign T helper epitope fused with three copies of the B-cell epitope of SPINLW1_{103–115}. Many reports have demonstrated that the efficacy of DNA vaccines encoding antigens from different pathogens dramatically induce humoral immune responses after fusion of these antigens with 3C3d molecular adjuvant (Wang *et al.* 2006, Movsesyan *et al.* 2008). Thus, to take advantage of the capacity of 3C3d to act as a molecular adjuvant inducing humoral immune responses to SPINLW1_{103–115}, a DNA plasmid encoding the 3SPINLW1_{103–115}-PADRE epitope fused with 3C3d was constructed (Fig. 1A). Meanwhile, we tested the expression of both plasmids in HEK293 cells. Results demonstrated that the theoretical molecular weights of 3SPINLW1_{103–115}-PADRE epitope and 3SPINLW1_{103–115}-PADRE-3C3d fusion protein corresponded (Fig. 1B).

A cell-penetrating peptide such as TAT_{49–57} can be bound to plasmid DNA through electrostatic attractions and mediate the entry of heterologous proteins into the cells (Torchilin *et al.* 2003). During mimovirus development, the peptide–DNA plasmid may condense into a spherical particle upon electrostatic interactions. When the charge was fully neutralized, migration of the plasmid DNA was completely restrained (Fig. 2A). Mimoviruses have a favorable stability that can alter the accessibility of the DNA plasmid to DNaseI and protect it from being degradation by the enzyme. In our experiment, total protection from DNaseI degradation was only observed at $r \geq 4$ (Fig. 2B). Furthermore, in our study, when $r = 4$, the mimovirus successfully condensed into a group of dense particles with diameters of 40 nm (Fig. 2C and D); they were able to enter cells easily.

Research has suggested that the targeting of the antigen to CD21 by C3d tagging can increase antigen processing and presentation by all B-cells regardless of their specificity (Thornton *et al.* 1994, Cherukuri *et al.* 2001). To augment mimovirus-induced antibody production, 3C3d was chosen as an adjuvant and incorporated into the mimovirus design. Mice immunized with the mimovirus vaccine fused with 3C3d showed significantly higher anti-SPINLW1 antibody levels than those exposed to the mimovirus vaccine without 3C3d or to PBS alone. In our study, the mimovirus vaccine fused with 3C3d induced higher levels of anti-SPINLW1 antibody titer than the mimovirus vaccine without 3C3d, and much higher levels than PBS. We observed that the levels of antibody could be maintained for a long

time. Moreover, in the fertility assay, the former group showed a higher rate of fertility inhibition than the latter or PBS groups (Fig. 3). In this way, it was confirmed that 3C3d augments and maintains the levels of antibody, resulting in long-term fertility inhibition in male mice. Importantly, most of the male BALB/c mice regained their fertility when the immunizations were stopped for 20 weeks (Fig. 4D). Our results suggest that this method is a good strategy for the development of safe, effective, and reversible male contraceptives.

Although the mechanism of action of SPINLW1 is not completely understood at the molecular level, there is a hypothesis that anti-SPINLW1 antibodies neutralize SPINLW1 on surface of the sperm cells, interfering with the process of sperm maturation and eventually suppressing fertility (O'Rand *et al.* 2004). In this study, human SPINLW1 B-cell epitope SPINLW1_{105–113} (MFVYGGCQGNNNN) shares 84.6% homology with mouse SPINLW1_{105–113} (VFIYGGCQGNNNN), differing by only two amino acids, at positions 105 and 107. We then wondered if the antisera of each immunized mouse could also inhibit human sperm motility. Our results showed that the antisera of immunized mice could indeed inhibit human sperm motility, indicating the mimovirus vaccine's potential for limiting fertility. We observed that total sperm motility and viability in immunized male mice was more inhibited than those of mice exposed only to PBS. In the fertility assay, male mice immunized with the mimovirus developed high antibody levels and displayed suppressed fertility. Thus, we believe that the mimovirus works by impairing sperm motility, preventing the sperm from accessing the uterus and oviducts to fertilize the egg. There was no effect on serum testosterone levels in the immunized males and no effect on their testes or epididymic structures. From our observations, the mimovirus vaccine did not induce any visible side effects.

In conclusion, a B-cell-epitope-based mimovirus vaccine produced high levels of antibody and showed a moderately inhibitive effect on fertility by interfering with sperm function but not by impairing the structure of the testes or reducing levels of serum testosterone. For this reason, we believe that a mimovirus vaccine containing multiple sperm antigens and effective molecular adjuvant may be an effective form of contraceptive. These promising results indicate that further evaluation of B-cell-epitope-based mimovirus vaccines is warranted.

Materials and Methods

Animals

For experiments, 8- to 10-week-old specific pathogen-free BALB/c mice were obtained from the Animal Research Center of the Third Military Medical University (Chongqing, China). Animals were raised in a temperature- and light-cycle-controlled animal facility at the Institute of Immunology, PLA,

Third Military Medical University. The numbered mice were randomly divided into four groups of ten mice and ear-coded. All experiments were conducted according to the guidelines of the Chinese Animal Care for Laboratory Animals, and the protocols were approved by the Animal Care and Use Committee at the Third Military Medical University.

Peptide synthesis and western blotting

The 23-mer cationic peptide rkkrrqrrrMFVYGGCQGNNNN (TAT_{49–57}SPINLW1_{103–115}) was synthesized and purified (Invitrogen), the purity of the peptides was over 95% as determined by mass spectrometry.

Plasmids pSG.SS.YL and pSG.SS.C3d3.YL were provided by Dr Fearson (Wellcome Trust Immunology Unit, Department of Medicine, School of Clinical Medicine, University of Cambridge, UK). The sequences of 3SPINLW1_{103–115}PADRE (BglII/BamHI) were cloned into eukaryotic expression vectors pSG.SS.YL and pSG.SS.C3d3.YL to obtain plasmids pSG.SS.3SPINLW1_{103–115}PADRE.YL(P1) and pSG.SS.3SPINLW1_{103–115}PADRE.C3d3.YL(P2) respectively. A GGG linker was used between each SPINLW1_{103–115} repeat. The plasmids were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection, cells were cultured at 37 °C for 36 h, and protein expression was examined by western blotting.

Construction and identification of mimovirus

Construction of mimovirus took place as described previously (Wu *et al.* 2002). Briefly, the mimovirus was prepared in microcentrifuge tubes by adding plasmids P1 and P2 to TAT_{49–57}SPINLW1_{103–115} with different molar ratios (0, 0.5, 1, 2, 4, 8, and 16) in HEPES-buffered saline containing 10 mM HEPES and 150 mM NaCl. The final concentration of the DNA plasmid in all of the samples was identical (100 µg/ml). The input molar ratio (*r*) of peptide to DNA plasmid was the ratio of moles of lysine to nucleotide (NH₄⁺/PO₃). The mimovirus was identified by a DNA retardation assay, DNaseI digestion assay, transmission electron microscopy, and particle size analysis as described previously (Wu *et al.* 2002). Mimovirus1 was produced by a combination of cationic peptide TAT_{49–57}SPINLW1_{103–115} and plasmid P1. Mimovirus2 was composed of peptide TAT_{49–57}SPINLW1_{103–115} and plasmid P2.

Mouse immunization

Sexually mature Balb/C male mice were divided into three test groups and one control group (PBS group) comprising ten mice each. These four groups of Balb/C male mice were immunized with mimovirus1 (containing 100 µg peptide and 20 µg plasmid P1), mimovirus2 (containing 100 µg peptide and 20 µg plasmid P2), peptide (containing 100 µg peptide and complete Freund's adjuvant), or PBS (pH 7.2). Each mouse received 200 µl in total of the emulsion, subcutaneously. Three independent experiments were carried out, and for each experiment, the immunization was repeated four times with a 21-day interval.

Antibody detection in the sera of immunized mice

Animals were fixed and tail serum samples were collected at certain times during the study. Anti-SPINLW1 antibody levels were analyzed by indirect ELISA as below: the plates were coated with optimized concentrations of recombinant human SPINLW1 (rhSPINLW1) (100 ng in 100 μ l PBS each well) and allowed to sit overnight at 4 °C in a humidified atmosphere. Then the rhSPINLW1 solution was removed and BSA (30 mg/ml) in PBS was added. The plates were allowed to sit for 1 h at 37 °C. Then they were washed with PBS containing 0.05% Tween-20. Hundred microliter serums from each of the various dilutions by PBS were added into the wells. The diluent PBS was the negative control. After incubation for 1 h at 37 °C and a thorough wash, the plates were incubated with 100 μ l per well of HRP-conjugated goat anti-mouse IgG (dilution 1:5000) for 1 h at 37 °C. After washing, bound antibody was detected using the TMB detection system. The chromogenic reaction was stopped by addition of 2 M H₂SO₄ and the absorbance was measured at 405 nm (Bio-Rad). If the ratio between the OD of a well and the negative control exceeded 2.1, the sample in the well was defined as positive. Among the serial and positive serum samples, the maximum dilution ratio was the antibody titer.

Mating for in vivo fertility assay

Each immunized male mouse was caged for 1 week with three female 8-week-old Balb/C mice. Female mice were examined for vaginal plugs each morning and litter sizes were recorded. Two successive mating trials were carried out at 1 and 16 weeks after the final immunization respectively.

Sperm analysis of vaccinated mice

After the ultimate fertility assay, three male mice from each group were randomly chosen and killed. The cauda epididymides was carefully excised and used to collect epididymal sperm as has been described previously (Tayama *et al.* 2006). Briefly, the cauda epididymides was cut into small pieces and flushed using pre-warmed (37 °C) Ham's F-10 medium. The solution was centrifuged at 225 g for 10 min to collect the sperm cells, which were then resuspended in 1.0 ml Ham's F-10 medium. Sperm cell concentration was expressed as 10⁶/ml. Motility of epididymis sperm was evaluated via the method described by Reddy & Pushpalatha (2006). Sperm motility was expressed as the percentage of motile sperm out of the total sperm counted. Live and dead spermatozoa were determined using 1% trypan blue (Talbot & Chacon 1981). Simultaneously, hypoosmotic swelling test (which has been proven to be a better predictor of fertilizing capacity than motility) was employed to evaluate the functional activity of the sperm membrane (Jeyendran *et al.* 1984). In brief, 0.1 ml of the sperm suspension was mixed with 1 ml of the hypoosmotic solution and incubated at 37 °C for 30 min. A minimum of 200 cells in 10 different fields was assessed under phase contrast microscopy. Sperm with typical tail curling were considered to be hypoosmotic reactive cells.

Inhibition of antisera to human sperm motility

Normal human semen was collected from three volunteers whose routine semen examinations had been shown to be within normal parameters. First, active motile spermatozoa were recovered by the 'swim-up' procedure (Alvarez *et al.* 1993) and were adjusted to 20 $\times$ 10⁶ cell/ml in F-10 medium at 37 °C. Then the recovered spermatozoa were assessed by CASA to evaluate the effect of the procedure upon sperm motility. Last, incubation with pooled antisera of each immunized sample was loaded in Makler's chamber and a range of 5–10 fields was acquired for motility analysis (Owen & Katz 1993).

Measurement of serum testosterone concentration

Serum testosterone levels were measured using an RIA kit obtained from the North Biological Technology Research Institute (Beijing, China). Three male mice were anesthetized and serum was collected by cardiac puncture. The intra-coefficients of variation were <10% and the sensitivity reached 0.02 ng/ml. The antibody used in this study had 0.011% cross-reactivity with 5 α -dihydrotestosterone, 1.2 $\times$ 10⁵% with androstenedione, 0.032% with progesterone, and no cross-reactivity with rostanediol. All of the samples were run at the same time to avoid inter-assay variation.

Histological examination

Testes epididymides of the immunized mice were dissected, fixed in 4% (w/v) paraformaldehyde and embedded in paraffin. The slices (4–5 μ m) were cut and deparaffinized by being immersed three times in xylene for 5 min each, three times in 95% ethanol for 5 min each, and four times in 70% ethanol for 5 min. The slices were stained with HE and observed under phase contrast microscopy.

Statistical analysis

Data are expressed as mean $\pm$ s.d., except as noted. Statistical analysis was performed by the ANOVA (Statistical Package for Social Sciences (SPSS) Version 11.5; Armonk, NY, USA). Statistical significance was defined as $P < 0.05$.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0161>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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