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Original Article

The antibody against a nuclear autoantigenic sperm protein can result in reproductive failure

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Abstract

To study whether the antibody against the testis form of the nuclear autoantigenic sperm protein (tNASP) could result in reproductive failure, we successfully cloned and expressed a 339-bp cDNA fragment of mouse tNASP (mtNASP). Using mouse as a model, recombinant mtNASP (rmtNASP) and a synthetic peptide, human $tNASP_{393\text{-}408}$ ($htNASP_{393\text{–}408}$), were investigated for their antifertility effect. Active immunization with rmtNASP or the synthesized peptide raised high antibody titers in the immunized mice. Sperm-egg binding and fusion assay were carried out in 8–10-week-old BALB/c mice. Sperm-egg binding and *in vitro* fertilization of mouse oocytes were inhibited by co-incubation of zona-free mouse oocytes with capacitated mouse spermatozoa in the presence of varying concentrations of the antisera against rmtNASP. There was a significant antifertility effect in animals immunized with rmtNASP or the synthesized peptide. The effect on fertility in the mice immunized with the synthesized peptide was reversible. Our data indicate that active immunization with rmtNASP antigen may induce a strong antibody response that causes an inhibition of fertility.

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1 Introduction

NASP, a nuclear autoantigenic sperm protein (GenBank locus ID: 4678; Accession number: P49321), initially described as a highly autoimmunogenic testis- and sperm-specific protein, is present in the nucleus of spermatozoa and spermatogenic cells [1], hence its name. Previous studies have shown that NASP is a histone-binding protein that binds H1 linker histones *in vivo* and has been proposed to transport them into the nucleus of dividing cells [2]. Two major forms are encoded by transcript variants of this gene, a testis form NASP (tNASP) and a somatic form NASP (sNASP). The sNASP, expressed in all mitotic cells, is localized to the nucleus, and is coupled to the cell cycle. The tNASP is expressed in embryonic tissues, tumor cells, and testis. In male germ cells, this protein is localized to the cytoplasm of primary spermatocytes, the nucleus of spermatids, and the periacrosomal region of mature spermatozoa. Mouse NASP (mNASP) somatic protein (Mr 45 751) is identical in amino acid sequence to the testis form (Mr 83 934), except that a region coded by exon 6 has been deleted [2]. Human NASP (hNASP) was first described in the testis [3], but also occurs in a somatic form with identical deletion to that found in mouse.

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The blood-testis barrier provides an immunological privileged status to the testis, preventing late spermatogenetic cell components from encountering the immune system. Not surprisingly, many testicular isoenzymes and other proteins are autoantigenic during immunological challenges occurring from testicular injury, infection, or vasectomy [4]. In an earlier study [5], it was found that 86% of the vasectomized patients with anti-sperm antibodies had anti-tNASP autoantibodies.

Early epidemiological studies indicated that some infertile men who were infected by *Ureaplasma urealyticum* [6] had positive antisperm antibodies (ASAs) in their serum and/or semen [7]. Recently, we found a pentapeptide identity (IERLT) both in the urease complex component UreG, one of the proteins in *Ureaplasma urealyticum*, and in hNASP [8]. The pentapeptide, present in tNASP, is located in the region excluded in sNASP. It may explain why infertile men infected with *Ureaplasma urealyticum* displayed a higher titer of serum and/or semen ASA, but had no symptoms. It has also been shown that the presence of antinuclear antibody (ANA) significantly reduces pregnancy rates [9].

The purpose of this study is to analyze whether the anti-tNASP antibodies affect fertility or not. Mouse tNASP (mtNASP) was cloned and expressed. *In vitro* fertilization (IVF) assays were performed in the presence of anti-tNASP antibodies. In addition, we examined the effect of active immunization with recombinant mtNASP (rmtNASP) antigen or a synthesized peptide (containing the pentapeptide IERLT) on the fertile female mice *in vivo*.

2 Materials and methods

2.1 Subjects and animals

This study was approved by the Ethical Review Board at the Shanghai Jiao Tong University School of Medicine. All subjects signed their informed consent before participation in the study. Healthy donors and infertile patients who had ASAs were recruited from Ren Ji Hospital, affiliated to the Shanghai Jiao Tong University School of Medicine (Shanghai, China). BALB/c mice and New Zealand white rabbits were obtained from the Animal Center of the Chinese Academy of Sciences. Animals were housed in specific pathogen-free conditions at the Shanghai Jiao Tong University School of Medicine. All animal work was conducted in accordance with the Shanghai Jiao Tong University School of Medicine Animal Studies Committee.

2.2 Cloning and expression of exon 6 of mtNASP

The 16 565–17 539-bp cDNA of mNASP was proved to contain exon 6 of mtNASP; therefore, two specific primers were designed by Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA), which would amplify a 339-bp fragment. The forward primer used was 5'-GCGGATCC ATG GAA CTG CTA GGG CAA GA-3' (containing a *BamH* I site) and the reverse primer was 5'-GCAAGCTT TTT GTC TTC AGG TGC TTT-3' (containing a *Hind* III site). Total RNA was extracted from mouse testis according to the manual of Qiagen RNAeasy Kit (Qiagen, Hilden, Germany) and quantified with UV absorbance at 260 nm. Reverse transcription was carried out according to the manual of the TaKaRa AMV reverse transcription-polymerase chain reaction (RT-PCR) kit (TaKaRa, Da Lian, China). PCR was carried out [10] in a final volume of 25 μL on a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). The cycling parameters employed were 94ºC, 2 min; 94ºC, 30 s; 51ºC, 1 min; and 72ºC, 1.5 min, for 35 cycles. PCR reaction products were separated on agarose gels, and a band of 339 bp was isolated and subcloned in pMD-18 vector (TaKaRa) to transform *Escherichia coli* (strain DH16B) [11]. Multiple cDNA clones were sequenced (Sangon, Shanghai, China). Recombinant pET-28a (+) plasmid was propagated in *E. coli* BL21 (DE3) host cells and the encoded proteins were expressed as IPTG-induced 6 × Histidine-tagged fusion proteins [12]. The molecular size of expressed proteins was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The rmtNASP was purified [13] on a His-binding Ni^{2+} chelation affinity resin column by a modification of the manufacturer's procedures (Pierce, Rockford, IL, USA). Protein concentrations were determined by Coomassie Plus-200 using bovine serum albumin (BSA) as a standard.

2.3 Polyclonal antibody production and Western blot analysis

Three healthy male New Zealand white rabbits aged 6 months (body weight about 2.5 kg) were housed in the animal facility for at least 3–4 days to acclimate them to the new surroundings. The rabbits were immunized with purified rmtNASP as described [14]. At the first and third day, a mixture of antigen and the complete Freund's adjuvant (Sigma, St. Louis, MO, USA) was

injected subcutaneously on the back and proximal limbs of the rabbits. On the 28th day, a mixture of antigen and the incomplete Freund's adjuvant (Sigma) was injected in the same way. The target antigen (200–300 μg) was injected into each rabbit at each time. On the 35th day, the titer of the antiserum was checked with enzyme-linked immunosorbent assay (ELISA). The control sera were obtained from animals immunized with only the adjuvant. The blood was collected and the serum was removed and purified with Protein A affinity chromatography according to the manual (Millipore, Billerica, MA, USA). The specificity of the antiserum was judged by (a) the reactivity of the antiserum to rmtNASP, (b) the inability of the antiserum to cross-react with any other protein from mouse total sperm extracts on Western blot, (c) the ability of antigen pre-adsorption to abolish immunorecognition, and (d) reproducibility of the results with antiserum from different animals. rmtNASP, mouse sperm, testis, and epididymis extracts were subjected to 1D SDS-PAGE [15]. Proteins were then blotted to nitrocellulose. All blots were blocked with 5% BSA in TBS with 0.05% Tween 20 (TBS-T) for 30 min at room temperature. Immunoblotting was tested using different antisera as primary antibodies [16]. The blots were then incubated with 1:4 000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody for 1 h. The blots were then developed with ECL reagent (Amersham Corp., Buckinghamshire, UK).

2.4 Sperm-egg binding and fusion assays

Female 8-week-old BALB/c mice were primed with 10 IU of pregnant mare's serum gonadotropin (PMSG, Sigma) and then induced to ovulate with 10 IU of hCG (Sigma) 48–56 h later [17]. Cumulus-enclosed egg complexes were collected from the oviductal ampullae 15 h after hCG administration, and treated with 0.1% hyaluronidase (Sigma) in M16 medium (Sigma) containing 10% fetal calf serum to disperse the cumulus cells. The ZP was removed with acidic (pH 2.5) Tyrode's solution (Sigma) [18]. The eggs were washed thrice with M16 medium. Spermatozoa were obtained from male BALB/c mice (8–10 weeks old) by placing the cauda epididymides and vasa deferentia in 900 μL Tyrode's solution (containing 3 mg mL^{-1} BSA). Spermatozoa were allowed to swim out for 10 min, and then the tissue was removed from the solution and 5 μmol L^{-1} calcium ionophore A23187 (Sigma) was added for another 60 min for the acrosome reaction [19].

Eggs were placed in 50-μL drops of M16 medium. Capacitated spermatozoa were pretreated with different concentrations of affinity-purified IgG from immune serum against rmtNASP or IgG from adjuvant control serum for 1 h [20]. The treated spermatozoa were added to a final concentration of 1.0×10^6 per mL and incubated for 30 min in a 5% CO_2, 37ºC incubator [21]. After three washes, eggs were stained with 10 μg mL^{-1} Hoechst 33342 for 15 min, and then washed three times in fresh medium [22]. About 10–20 eggs were analyzed for each treatment, and the average number of spermatozoa-bound per egg was determined.

The treated spermatozoa were added to a final concentration of 1.0×10^6 mL^{-1} and incubated with eggs for 3 h in a 5% CO_2, 37ºC incubator [23]. Eggs were then washed and mounted on glass slides for analysis for evidence of sperm penetration under phase-contrast microscopy. Eggs were considered to be penetrated if a decondensing sperm head or two pronuclei and at least a sperm tail were present in the ooplasma [24]. The fusion rate (percentage of eggs fertilized) was determined.

2.5 Synthesized peptide and competitive ELISA

The peptide human tNASP (htNASP) 393–408 (EPQTSIERLTETKDGS), corresponding to amino acids 393–408 of htNASP, was synthesized. The peptide was coupled to keyhole limpet hemocyanin (KLH) [25] by the manufacturer (Sangon). The polyclonal antiserum against the peptide was made as described [14].

Antiserum against rmtNASP at a nonsaturating dilution was incubated with various concentrations (0.1–1 mmol L^{-1}) of competing synthesized peptides at room temperature for 2 h [26]. This mixture of peptide and antiserum was then transferred to a plate coated with rmtNASP and further incubated for 2 h at room temperature to measure antibody unabsorbed by the synthesized peptide with ELISA [27].

2.6 Sperm motility analysis

Spermatozoa were obtained from male BALB/c mice (8–10 weeks old) by placing the cauda epididymides and vasa deferentia in 900 μL Tyrode's solution (containing 3 mg mL^{-1} BSA). The cells were incubated with 100 μg mL^{-1} of purified adjuvant control IgG or purified anti-rmtNASP IgG or anti-$NASP_{393–408}$ IgG at 37ºC for 1 h. Computer-assisted sperm motility

analysis was performed by using a semen autoanalyser (Hamilton Thorne, Beverly, MA, USA). Motile percentage, curvilinear velocity (VCL), and straight-line velocity (VSL) were measured.

2.7 Binding of ASAs with rmtNASP or htNASP$_{393–408}$

Sera were obtained from 22 infertile men who had ASAs and 25 men with normal fertility. The study groups were matched by age (31.0 ± 3.5 years and 29.4 ± 3.4 years, respectively). Each infertile man's wife was found to be healthy, lacking any detectable reproductive-system anomalies or other known clinical disorders. Additionally, the wives had not been pregnant for at least 1 year. Polystyrene 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with rmtNASP or htNASP$_{393–408}$ KLH at a volume of 100 μL per well (0.5 μg per well). Using sera from the infertile men as primary antibodies, ELISA was performed as described previously [28]. Sera from the fertile men were used as control.

2.8 Fertility tests

Female BALB/c mice of proven fertility were immunized against purified rmtNASP or synthesized peptides htNASP$_{393–408}$ by i.m. and s.c. routes as described previously. Each animal received a total of three injections. Each injection consisted of 100 μL of phosphate buffered saline (PBS) containing 50 μg rmtNASP protein or the synthesized peptide htNASP$_{393–408}$ emulsified with 100 μL Freund's adjuvant. Control animals were injected with KLH in Freund's adjuvant. One week after the last immunization, male animals, proven to be fertile, were individually and continuously caged with experimental and control female mice at a ratio of 1:2 (eight animals per group) [29]. After 3 weeks, pregnant females were counted. The animals were kept for a longer time, up to 240 days, to examine the effect of disappearance of antibody titers on the regain of fertility.

2.9 Statistical analysis

Experimental and control group averages were reported as mean ± SD (Table 1) or mean ± SEM (Table 2, Figure 1). The results were analyzed by upaired *t*-test. Correlation between the antibody titer and fertility was analyzed by linear regression. Results were considered statistically significant at $P < 0.05$.

3 Results

3.1 Characteristics of the testicular-specific exon 6 of NASP

The exon 6 of the *NASP* gene is selectively transcribed in the testis. Some infertile men displayed high titers of serum anti-NASP antibody, but they did not show any

Table 1. Effect of the antibody against rmtNASP and anti-NASP393–408 antibody on sperm motility. BALB/c mouse spermatozoa were incubated with 100 mg mL^{-1} of purified adjuvant control IgG or the same concentration of purified anti-rmtNASP IgG or anti-NASP$_{393–408}$ IgG at 37ºC for 1 h. Motile percentage, VCL and VSL were measured. All results were expressed as mean ± SD.

Group	Motile (%)	VCL (mm/s)	VSL (mm/s)
Control group	67 ± 12	272.5 ± 68.0	145.2 ± 76.0
Anti-rtNASP IgG group	58 ± 15	262.0 ± 72.0	127.3 ± 62.0
Anti-tNASP$_{393–408}$ IgG group	62 ± 17	255.0 ± 66.0	146.6 ± 75.0

Abbreviations: VCL, curvilinear velocity; VSL, straight line velocity.

Table 2. Effects of rmtNASP and the synthesized peptide immunization on fertility of female mice. Female BALB/c mice of proven fertility were immunized with purified rmtNASP or the synthesized peptide htNASP393–408 coupled to KLH. The control animals were immunized with keyhole limpet hemocyanin (KLH) emulsified in phosphate buffered saline (PBS) and Freund's adjuvant. The animals were then housed with males of proven fertility. The number of babies delivered by each mated female was counted.

Group	No. of animals	No. of pregnancies		Pups born (mean ± SEM)	
		Housed for 21–30 days with male	Housed for 150–180 days with male	Housed for 21–30 days with male	Housed for 150–180 days with male
Control	8	7	7	8.63 ± 0.26	7.43 ± 0.35
rtNASP immunized	8	3	3	2.50 ± 2.12**	2.75 ± 0.27**
Synthesized peptide immunized	8	4	7	1.75 ± 1.34**	8.72 ± 0.93

$^{**}P < 0.01$, compared with the control.

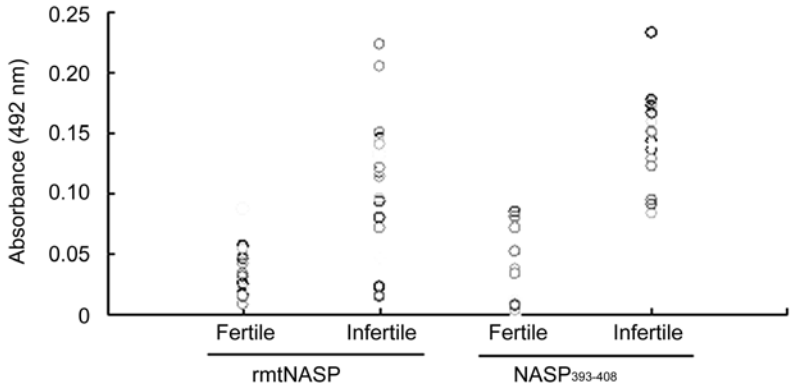

Figure 1. Cross-reactivity of patients' antisperm antibodies with rmtNASP or $NASP_{393–408}$. Microtiter plates were coated with rmtNASP or $htNASP_{393–408}$-KLH and ELISA was performed using sera from the infertile men as primary antibodies. Sera from fertile men were used as control. The absorbance was measured at 492 nm.

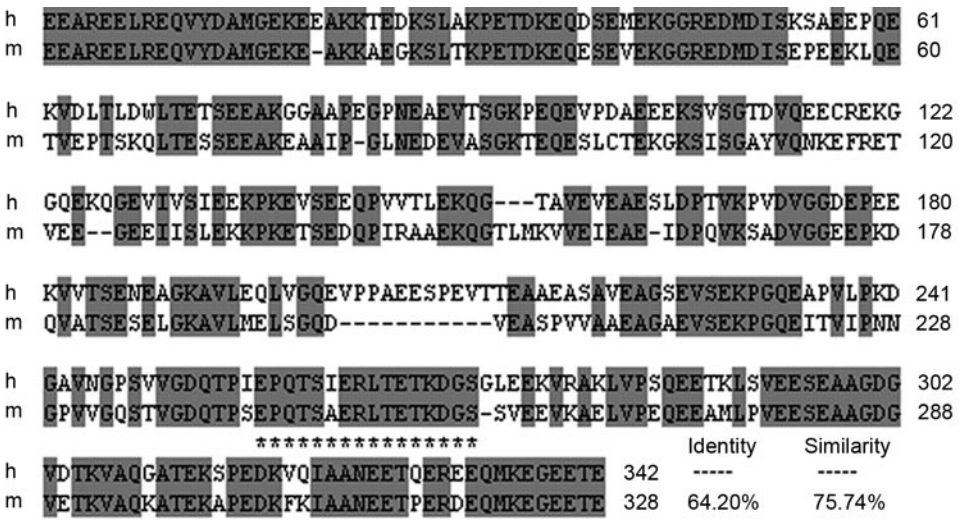

Figure 2. Alignment of the amino-acid sequence encoded by exon 6 of human (h) and mouse (m) tNASP. Identical residues are shaded. The asterisk indicates the amino-acid sequence of the synthesized peptide.

other symptoms. The phenomenon implied that exon 6 of tNASP may play a key role in producing anti-NASP antibody, which results in infertility. Comparison of human and mouse exon 6 of tNASP using DNAssist software shows that the region coded by exon 6 in mouse tNASP is 75.74% similar and 64.20% identical to that of humans (Figure 2), which suggests that NASP is conserved. Further analysis of the amino-acid sequence coded by mouse tNASP exon 6 revealed that it had strong hydrophilicity and antigenicity using DNAStar software according to reported procedures [30, 31]. Thus, a highly conserved and antigenic region, corresponding to amino acids 393–408 of the tNASP (EPQTSIERLTETKDGS), was selected and synthesized for antibody production.

3.2 Expression of mouse tNASP exon 6 fragment and specificity of the antibody

A cDNA sequence encoding mouse tNASP exon 6, containing the pentapeptide (AERLT) identical between *Ureaplasma urealyticum* and tNASP (Figure 2), was expressed as His_6-tagged recombinant protein running at approximately 18×10^3 Mr. After Ni^{2+}-affinity purification, an aliquot of the protein was separated on

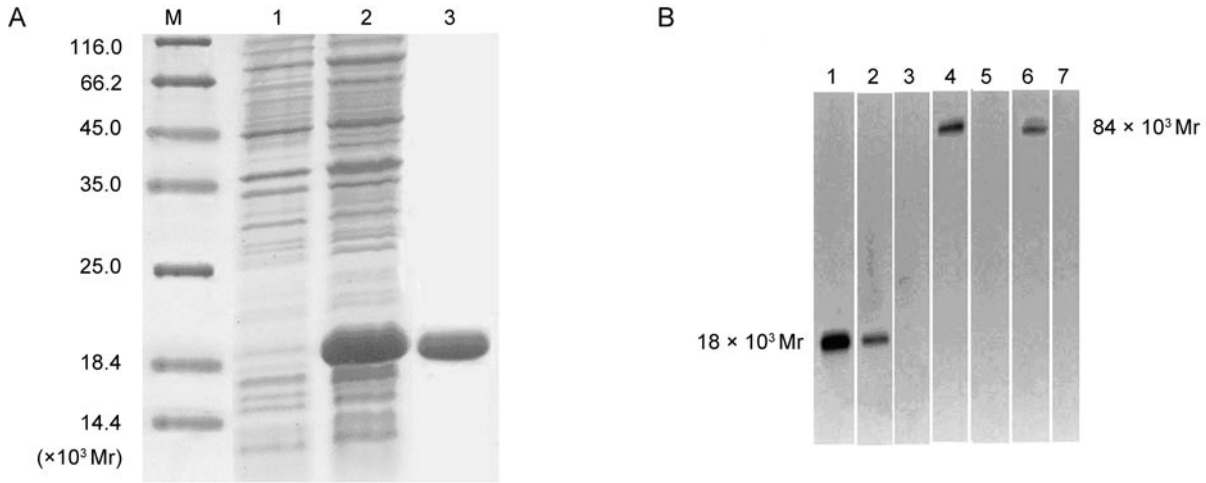

Figure 3. Expression and purification of rmtNASP and specificity of its antisera. (A): Expression and purification of His-tagged rmtNASP (~18 × 10^3 Mr) from BL21-DE3 cell lysate stained with Coomassie Blue. M: molecular weight marker; lane 1: uninduced cells; lane 2: cells induced with IPTG for 4 h; lane 3: purified rmtNASP. (B): Western blot analysis showing specificity of the antisera. Purified rmtNASP was probed with anti-polyhistidine (1:3 000, lane 1) and with anti-rmtNASP antisera (1:2 000, lane 2). Cauda epididymal mouse sperm extract was probed with adjuvant control sera (1:1 000, lane 3) and anti-rmtNASP antisera (1:1 000, lane 4). Mouse testis extract was probed with adjuvant control sera (1:1 000, lane 5) and anti-rmtNASP antisera (1:1 000, lane 6). Sperm-voided epididymis extract was probed with anti-rmtNASP antisera (1:1 000, lane 7).

SDS-PAGE, stained by Coomassie Blue and blotted to probe with anti-histidine antibody (Figure 3). A prominent band at 18 × 10^3 Mr was noted, indicating that rmtNASP-exon 6 used for this study was highly purified. A rabbit polyclonal antibody against the exon 6 fragment was subsequently generated, and its specificity examined by Western blot. The antiserum recognized the 18 × 10^3 Mr antigens (Figure 3B), whereas the adjuvant control serum did not. The antiserum also reacted only with an 84 × 10^3 Mr band in mouse sperm and testis extracts, whereas the adjuvant control serum showed no reactivity (Figure 3B). Somatic NASP protein was not detectable in testicular or sperm-voided epididymal extracts. These results indicated that a specific antibody had been generated against the testicular-specific exon 6 of mouse NASP, which detected tNASP as a single band in sperm and in testis.

3.3 Involvement of tNASP in sperm-egg binding and fusion

To determine the role of tNASP during fertilization, capacitated spermatozoa were pretreated with different concentrations of affinity-purified IgG from rmtNASP antiserum or IgG from adjuvant control serum prior to insemination. Sperm-egg binding and fusion were conducted in the presence of the antibody. Treatment with 50 or 25 μg mL^{-1} of the rmtNASP IgG significantly reduced binding (67.69% and 45.89% inhibition, respectively, Figure 4A and 4B) and fusion (74.24% and 48.57% inhibition, respectively, Figure 4C) as compared with the respective control values. However, a significant effect on fertilization was not observed at the 10 μg mL^{-1} level. Importantly, no difference was observed in the percentages of motile spermatozoa compared to the control (data not shown), suggesting that anti-rtNASP did not affect sperm motility but oolemma binding and subsequent fusion. The effect of anti-$htNASP_{393–408}$ on the interaction between sperm and eggs is the same as that of anti-rmtNASP (data not shown). Taken together, these results support the participation of tNASP in the binding and fusion between mouse sperm and egg.

3.4 Effect of anti-rmtNASP and anti-$NASP_{393–408}$ antibodies on sperm motility

To assess whether the antibody against rmtNASP and anti-$NASP_{393–408}$ antibody inhibit sperm motility, we performed computer-assisted sperm motility analysis. In the experimental group, the motile percentage, VCL,

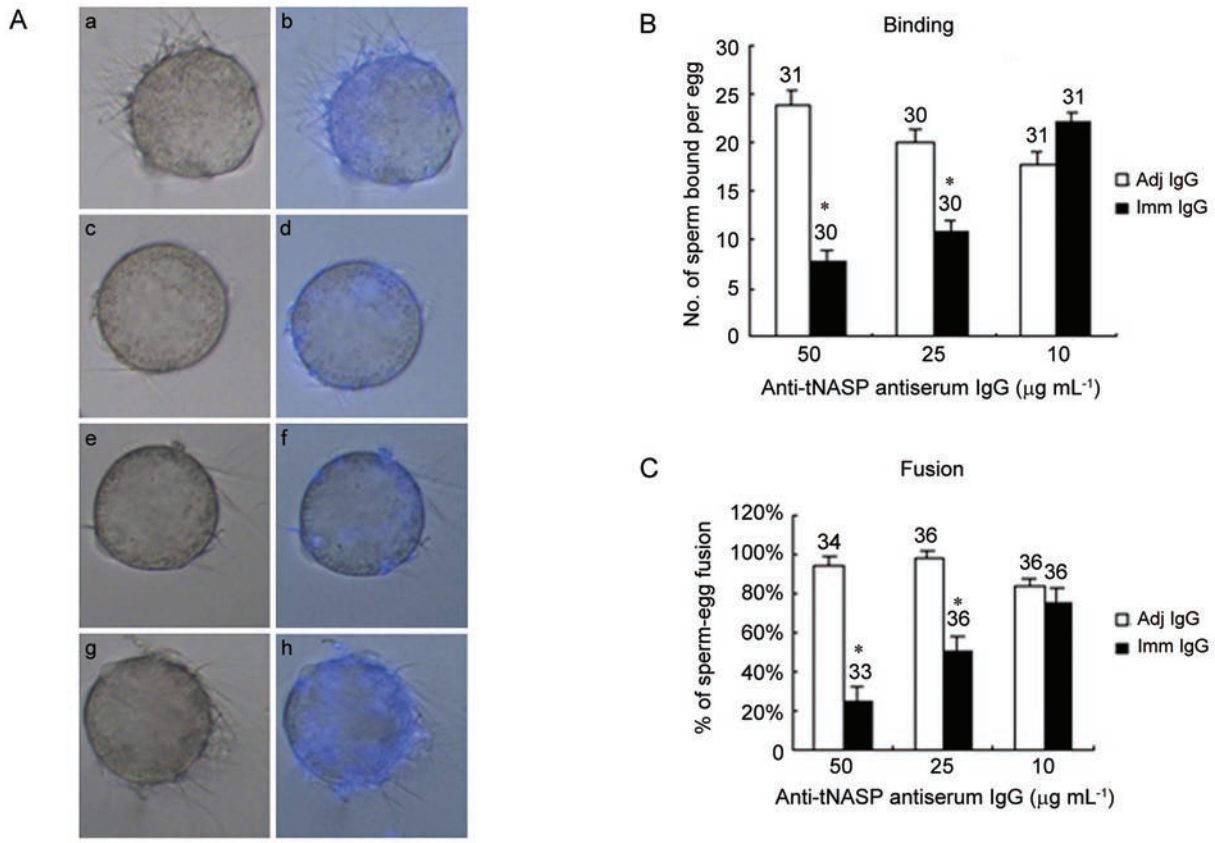

Figure 4. Involvement of tNASP in sperm-egg binding and fusion. Capacitated mouse sperm, preincubated with different concentrations of IgG from immune (Imm) serum against rmtNASP or from adjuvant control (Adj) serum, were co-incubated with zona-free mouse eggs. (A): Phase contrast (a, c, e, g) and Hoechst staining (b, d, f, h) of IgG-treated sperm 0.5 h after insemination. a, b: 50 μg mL^{-1} Adj IgG; c, d: 50 μg mL^{-1} Imm IgG; e, f: 25 μg mL^{-1} Imm IgG; and g, h: 10 μg mL^{-1} Imm IgG (Bar = 20 μm). (B) and (C): The number of sperm bound per egg was counted and the fusion rates were determined under a laser-scanning confocal microscope. Histograms represent mean ± SEM. The numbers above the columns indicate the total number of eggs per group. $^{*}P < 0.05$, comparison between the experimental groups and the adjuvant control groups.

and VSL were not significantly changed (Table 1). Thus, sperm motility appeared to be unaffected by the antibody against rmtNASP and anti-NASP$_{393–408}$ IgG at concentrations of 100 μg mL^{-1}.

3.5 Antisperm antisera from infertile men reacted with rmtNASP or htNASP$_{393–408}$

To confirm that ASAs contained immunoreactivities against the testicular-specific portion of NASP, which may lead to infertility, we collected sera from infertile men with ASAs and assessed their reactivity against rmtNASP and htNASP$_{393–408}$ by ELISA assay. The absorbances against the rmtNASP and htNASP$_{393–408}$ antigens for sera from the infertile men (mean ± SD, 0.11 ± 0.06 and 0.16 ± 0.06, respectively) were significantly higher ($P < 0.01$) than those for sera from the fertile men (0.034 ± 0.020 and 0.029 ± 0.027, respectively, Figure 1). The results showed that sera containing ASAs from infertile men could react with rmtNASP and the synthesized peptide htNASP$_{393–408}$.

3.6 In vivo immune responses to tNASP

Fertile female BALB/c mice were immunized with two injections of rmtNASP or the synthetic peptide-KLH conjugate on days 1 and 3. Serum antibodies to rmtNASP or the peptide-KLH conjugate became measurable by day 12 (Figure 5A). After a booster immunization on day 28, the antibody titer rose to a plateau of 10^5–10^6 in animals immunized with rmtNASP and 10^3–10^4 in animals immunized with the peptide-

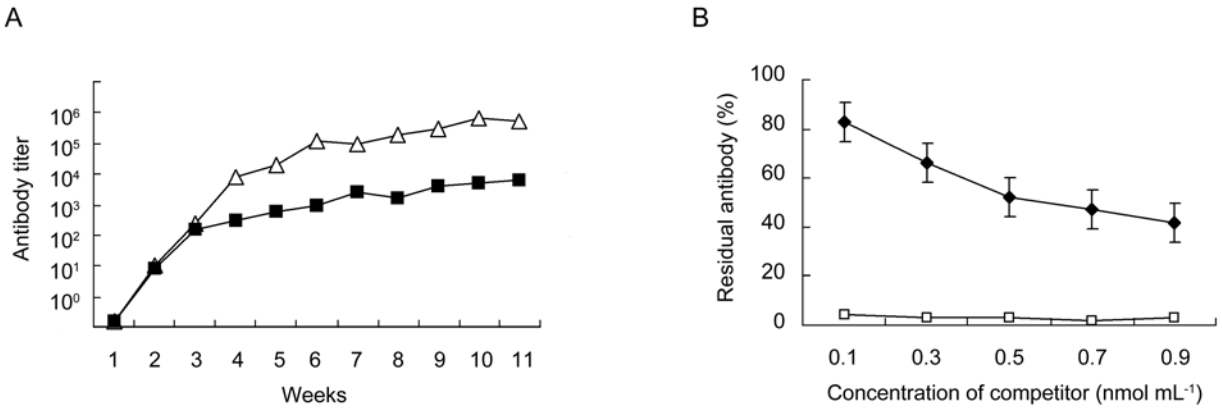

Figure 5. (A): Immune response to tNASP in BALB/c mouse immunized with rmtNASP or the peptide-keyhole limpet hemocyanin (KLH) conjugate. Antibody titers to tNASP were measured by ELISA (triangles, immunized with rmtNASP; squares, immunized with synthesized peptide–KLH conjugate). (B): Residual antibody is expressed as the percentage ratio between the peptide-preabsorbed antisera (diamonds) and the unabsorbed antisera. rmtNASP (squares) is used as a positive control.

KLH conjugate. Animals immunized with adjuvant plus KLH had no detectable serum antibodies. The serum antibody against rmtNASP could be completely absorbed by rmtNASP and partly absorbed by the peptide in competitive ELISA experiments (Figure 5B).

3.7 Fertility study of BALB/c female mice immunized with rmtNASP or the peptide

As shown in Figure 5A, injections of the peptide immunogens led to a persistently high antibody response over a 3-month period in the female mice. When these immunized mice were mated with normal males, the pregnancy rate was significantly reduced as compared with control female mice (Table 2), and the reduction in litter size correlated with antibody titer ($r = -0.6721$, $P < 0.01$, Figure 6) in animals immunized with the peptide–KLH conjugate. Collectively, 87.5% of the control mice became pregnant and delivered litters compared with 37.5% of the rmtNASP-immunized mice and 50% of the peptide-immunized mice. After the antibody titer had declined, the peptide-immunized mice regained fertility at rates that matched those of the control mice (Table 1). However, animals immunized with rmtNASP remained infertile, although some of them also showed a decrease in antibody titer by the end of the study.

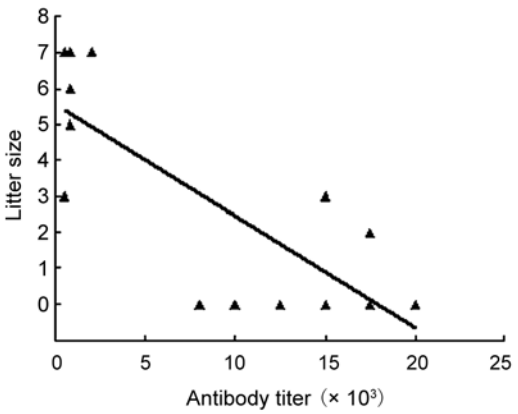

Figure 6. Correlation between litter size borne by mice immunized with the peptide-keyhole limpet hemocyanin conjugate and the antibody titers against the peptide as detected by ELISA.

4 Discussion

ASAs are postulated to interfere with the fecundity process through various mechanisms, such as interference with sperm transport within the female genital tract, alteration of sperm capacitation or acrosomal reaction, interference with fertilization, or inhibition of implantation of the early embryo.

Active immunization with rmtNASP and the $htNASP_{393–408}$ peptide antigen sharply raised the antibody titers, which were enough to cause a significant reduction in fertility compared with the controls. The anti-fertility effect was transient in mice immunized with the $htNASP_{393–408}$ peptide in that they delivered healthy babies without any effect on the litter size when

the antibody titer dropped to normal levels. However, although all animals immunized with rmtNASP showed a decrease in antibody titer by the end of the study, some remained infertile, suggesting that other immune reactions were likely to be involved. There was a significant linear correlation between antibody titer and reduction in fertility in animals immunized with the synthetic peptide, so that animals with a higher circulating antibody titer bore a lower number of babies. However, the correlation was not perfectly linear. There were animals that, despite having high titers, showed a lesser reduction in fertility compared with other animals that had relatively lower antibody titers. The data indicate that there are additional factors besides circulating antibodies, probably local immune response, that are involved in affecting fertility after active immunization with the sperm antigens [29].

We also examined the mechanism by which anti-NASP antibodies inhibited fertility *in vivo* by testing their effect on zona-free sperm-egg binding and IVF. The results demonstrated that the anti-rmtNASP antibody significantly inhibited binding and fusion of sperm with zona-free mouse eggs. Furthermore, in the presence of either antibody, sperm binding and fusion to the oolemma was inhibited in a concentration-dependent manner, indicating a role for tNASP in fertilization. The antibodies neither agglutinated nor immobilized sperm; therefore, there was no apparent effect of the antibodies on sperm motility. Thus, the antibodies may inhibit or block sperm-egg binding, resulting in fertilization failure.

It is believed that the antibody effects such as inhibiting sperm-egg binding and fusion must then be associated with the cell surfaces and not with the cytoplasm or nucleus. Lee and O'Rand [32] reported the ultrastructural localization of NASP; in later spermatids, testicular spermatozoa, and ejaculated spermatozoa, NASP is concentrated over the nucleus, although some is still present in the acrosome. It is likely that some part of the tNASP may be exposed for a short time when it is carried into the ovum with the sperm nucleus at fertilization. It is perhaps the only opportunity for the specific antibodies to bind the tNASP antigen to affect the reproductive process.

It has been shown that the presence of ANA might reduce pregnancy rates after IVF-embryo transfer. However, the mechanism of implantation failure by ANA has not yet been clarified [33]. NASP is also involved in human involuntary immunoinfertility [5]. Our fertility study of BALB/c female mice *in vivo* indicates that anti-NASP antibodies can result in reproductive failure. We will carry out intracytoplasmic sperm injection (ICSI) in our further study to investigate the direct effects of the anti-NASP antibodies on embryo development and implantation.

It seems that no single sperm antigen has been shown to cause a 100% reduction in fertility. It is hypothesized that the immunization with multiple fertility-related antigens will increase the efficacy of a vaccine by an additive effect. Therefore, with administration of multiple antigens including tNASP and other sperm surface antigens, there is a greater possibility of activating the host individual's immune system to produce a range of antibodies to provide more effective protection.

In conclusion, our data showed that tNASP is an evolutionarily conserved and immunogenic antigen, and anti-tNASP antibodies can result in reproductive failure.

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