DNA Immunization Against Proacrosin Impairs Fertility in Male Mice

Lucila García[1], María F. Veiga[2], Livia Lustig[3], Mónica H. Vazquez-Levin[2], Carolina Veaute[1]

[1]Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, Santa Fe, Argentina;
[2]Instituto de Biología y Medicina Experimental (IBYME), National Research Council of Argentina (CONICET), Buenos Aires, Argentina;
[3]Instituto de Investigaciones en Reproducción, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

Keywords
Acrosomal exocytosis, fertilization, genetic immunization, infertility, proacrosin/acrosin, sperm antigen, *zona pellucida*

Correspondence
Mónica H. Vazquez-Levin, Instituto de Biología y Medicina Experimental (IBYME), National Research Council of Argentina (CONICET), Vuelta de Obligado 2490, Zip Code C1428ADN, Buenos Aires, Argentina.
E-mail: mhvazl@gmail.com

Mónica H. Vazquez-Levin and Carolina Veaute equally contributed to the work.

Submission January 3, 2012;
accepted February 19, 2012.

Citation
García L, Veiga MF, Lustig L, Vazquez-Levin MH, Veaute C. DNA immunization against proacrosin impairs fertility in male mice. Am J Reprod Immunol 2012

doi:10.1111/j.1600-0897.2012.01127.x

Problem
Evaluation of proacrosin/acrosin ability to induce an immune response in male mice after genetic immunization and assessment of animal fertility.

Method of study
Mice received 50 μg per animal of a plasmid containing the human proacrosin cDNA (pSF2-Acro) (control: empty plasmid, pSF2). The humoral response was evaluated by ELISA and immunocytochemistry. *In vivo* fertility was assessed by mating immunized males with control females. The effect of antibodies upon Ca^{+2}-ionophore-induced acrosomal exocytosis (AE) and *in vitro* sperm–*zona pellucida* (ZP) binding was also studied.

Results
pSF2-Acro-immunized mice developed high levels of specific antibodies ($P < 0.05$) that recognized the sperm acrosomal cap. The number of fertile mice was lower ($P = 0.027$) in pSF2-Acro-immunized animals than in controls. Litter size was smaller ($P < 0.05$) in the pSF2-Acro group compared with controls. A negative correlation ($P < 0.05$) between antibody levels and litter size was found. Antiproacrosin/acrosin antibodies inhibited sperm–ZP binding ($P < 0.0001$) and Ca^{+2}-ionophore-induced AE ($P < 0.05$).

Conclusion
DNA immunization against proacrosin elicits an immune response in male mice associated with abnormal sperm functions and reduced fertility.

Introduction

Several immunologic factors, both of humoral and cellular origin, have been found to influence human reproductive failure.[1] Although the relative contribution of individual immune components to infertility remains controversial, presence of antisperm antibodies (ASA) has been associated with 2–30% of human infertility cases.[2,3] As the testis is an immunoprivileged organ and sperm cells appear late after the establishment of self-tolerance, sperm proteins could act as immunogens when they are exposed to the immune system.[4,5] ASA may impair some steps of fertilization, among them sperm transport within the female genital tract, sperm capacitation, acrosomal exocytosis (AE), sperm–oocyte interaction, implantation, and early embryonic development.[6]

Animal immunization with sperm proteins has been broadly described in several species and has been reported as a useful approach to study the molecular bases of fertilization as well as ASA-related infertility. In addition, information from

these studies has contributed to the design of strategies for fertility regulation.[7,8] With regard to sperm antigens, immunization with testis homogenates has been found to induce autoimmune orchitis, and some sperm antigens that elicit strong inflammation and damage the tissue have been identified.[9] On the other hand, other purified sperm antigens, mostly surface proteins, have been utilized as immunogens with slight or no adverse effects on the testis; however, the ability to induce fertility inhibition varies in different models.[10] Besides sperm proteins, other molecules have been tested as immunocontraceptive candidates; among them are *zona pellucida* (ZP) proteins[8] and hormones, such as human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone (GnRH). Presently, an anti-hCG has reached a phase two clinical trial,[11] and a commercial vaccine is available for a population control of elk and white-tailed deer.[12]

Acrosin (EC 3.4.21.10) is a trypsin-like protease localized to the sperm acrosome as an inactive zymogen (proacrosin) that is converted to the active enzyme and released during the AE.[13,14] Human and mouse acrosin proteins have been detected in pachytene spermatocytes.[15,16] Activation of proacrosin (53 kDa) leads to the 34 kDa enzymatically active form, β-acrosin, through proteolytic cleavage at both the N- and C-protein termini.[17,18] Several functions have been attributed to the proacrosin/acrosin system, mainly binding to ZP glycoproteins, participation in the release of acrosomal content, and limited proteolysis of the ZP.[19–22] Recently, a role for proacrosin in binding to the egg envelope in birds has been reported.[23]

Immunity to proacrosin/acrosin has been found to inhibit functions of this protein system and to block fertility in several models.[24–29] In particular, our group has described an immune response to proacrosin/acrosin that impaired *in vivo* fertility, with the production of antibodies that inhibited *in vitro* fertilization and interfered with functions of the protease system, in a DNA immunization model of female mice injected with a plasmid encoding human proacrosin.[29] Moreover, our group has described the detection of antibodies toward proacrosin in 19% of women consulting for infertility.[30] Despite its potential relevance, the effect of immunity toward proacrosin in a male model has yet not been tested. As proacrosin is exclusively expressed during spermatogenesis, an immune response toward this specific target in male mice represents an attractive model to study the molecular basis of male immunologic infertility.

In this study, the ability of sperm proacrosin to induce an immune response in male mice was investigated using a DNA immunization approach. DNA vaccines are easy to construct and to develop, and they have been demonstrated to be efficient in inducing strong immune responses to a variety of immunogens.[31] The effect(s) of proacrosin immunization upon animal fertility and sperm functions was also investigated.

Materials and methods

Chemicals

Unless specified, chemicals were of culture and analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA). Electrophoresis reagents were products of Bio-Rad (Richmond, CA, USA), or specifically indicated throughout the article. Molecular biology reagents were of highest quality and purchased from Qiagen (Hilden, Germany) and Invitrogen-Life Technologies (Carlsbad, CA, USA), unless indicated.

Plasmids and Proteins

A plasmid containing the human proacrosin (h-proacrosin) coding sequence was obtained as previously described[29] and subcloned in an expression vector for DNA immunization. Briefly, the proacrosin cDNA (kindly donated by Prof. Dr. W. Engel, Institut für Humangenetik, Göttingen, Germany) was subcloned in the pSF2-cytomegalovirus (CMV) eukaryotic expression vector (generously provided by Dr. A.S. Johnston, Texas University, Southwestern Medical Center, Dallas, TX, USA), under the control of the CMV promoter and downstream of the α1-antitrypsin leader sequence; this construction was named pSF2-Acro. Plasmid DNA was then obtained from bacterial cultures and purified by anion-exchange chromatography using a commercial kit (Jetstar, Genomed, Löhne, Germany) following manufacturer's instructions. Quality and quantity of purified DNA were determined by performing agarose gel electrophoresis and by assessing optical density (OD) readings at 260 and 280 nm, respectively.

Recombinant h-proacrosin was also expressed in bacteria using the pET22b expression vector as previously reported.[32] Briefly, subcloning of the same

h-proacrosin cDNA was carried out in two steps, combining PCR amplification and restriction enzyme digestion, and a selected clone (pET22b-525) was subjected to nucleotide sequence analysis. *Escherichia coli* B21 (DE3) cells were transformed with the plasmid pET22b-525, and bacteria were cultured in Terrific broth medium (1.2% bactotryptone, 2.4% yeast extract, 0.17 M KH_2PO_4, 0.072 M K_2HPO_4, 0.4% glycerol) containing 50 μg/mL of carbenicilin at 37°C until OD_{600nm} = 0.6–1. Protein expression was induced by the addition of 1 mM of isopropyl-thio-β-D-galactoside to bacterial cultures. Bacterial cells were harvested 2 hrs after induction, recovered by centrifugation, and cell pellets were kept at −70°C until further analysis. Under the conditions assayed, an expression product corresponding to the h-proacrosin full-length protein (Rec-40: 42–44 kDa) as well as N-terminal truncated fragments (Rec-30: 32–34-kDa, Rec-20: 21-kDa, and Rec-10: 18-kDa) were obtained. Recombinant proacrosin and N-terminal products were purified by preparative SDS–PAGE as previously described.[32]

Animals and Immunization Protocol

For the immunization protocol, six-eight-week-old Balb/c male mice were used. Animals were obtained from the Instituto de Biología y Medicina Experimental animal facility and transferred to the Facultad de Bioquímica y Ciencias Biológicas. All experimental procedures were performed according to the recommendations of the Guide for the Care and Use of Laboratory Animals.[33] Adult male mice were inoculated in the quadriceps muscle with 50 μg of pSF2-Acro or pSF2 (plasmid without insert, control) in 50 μL of sterile phosphate-buffered saline (PBS). Injections were repeated four times, every 3 weeks. Blood samples were taken by tail puncture 1 week before each inoculation and, 1 week after the last dose, animals were bled by cardiac puncture and killed. Animal serum was obtained after centrifugation of blood at 2000× ***g*** for 10 min and was stored at −20°C until further analysis. Two different trials were performed including ten mice in each group in the first trial and eight mice in each group in the second trial.

Assessment of Immune Response

The immune response of mice to proacrosin was evaluated by an indirect ELISA (ELISA-Acro), as previously reported.[29] Briefly, 96-well polystyrene plates (Costar, Corning, NY, USA) were coated with 0.8 μg per well of recombinant h-proacrosin (Rec-40) diluted in PBS containing 8 M urea. After blocking non-specific binding sites with PBS supplemented with 3% skimmed milk, 1:200 dilutions of sera in PBS-1%-skimmed milk were tested. To assess sera reactivity toward different proacrosin protein regions, 1:200 sera dilutions were tested using N-terminal truncated acrosin proteins Rec-30, Rec-20, and Rec-10. Binding of the antibody was evaluated by incubation with anti-mouse IgG conjugated to peroxidase (Jackson, Baltimore, MD, USA) followed by incubation with hydrogen peroxide and tetramethylbenzidine (Zymed, San Francisco, CA, USA). OD was measured at 450 nm using a microplate reader (Emax Microplate Reader; Molecular Devices, Sunnyvale, CA, USA). Results are expressed as the ratio between the OD of the sample and the OD of the pre-immune sera (OD/ODpre-immune).

Assessment of Male Mice Fertility

Immunized male mice were mated with non-treated 14–16-week-old female mice of proven fertility in a proportion 1:1, starting at week 15 of the immunization protocol. Animals were monitored daily for the presence of vaginal plugs; when detected, it was considered day 0 of pregnancy, and male mice were killed to analyze sperm parameters and to obtain tissue and blood samples. At term, female mice were monitored, and numbers of born pups were registered.

Evaluation of Sperm Parameters

Immunized male mice were killed by cervical dislocation, and *caudae* epididymides were immediately excised, diced in small pieces, and placed into 0.5 mL Whittingham's media based on Tyrode solution containing 0.5 mM pyruvate and 3 mg/mL BSA at 37°C. Sperm cells were allowed to swim out into the medium for 10 min. Sperm concentration was estimated in 20 μL aliquots of the sperm suspension using a hemocytometer. Sperm motility was assessed by counting progressively motile cells and expressed as a percentage of the total sperm count.

To assess sperm ability to undergo AE, cell concentration was adjusted to 1×10^6 sperm/mL and gametes were capacitated by incubating in Whittingham's medium, for 1 hr at 37°C, in an atmosphere of 5% CO_2 in humidified air. Aliquots of each sam-

ple (25 μL) were added to 25 μL 10 μM calcium (Ca^{+2})-ionophore A23187 (Sigma) dissolved in dimethylsulfoxide (DMSO); control assays were performed in parallel adding sperm cells to 25 μL of DMSO (Ca^{+2} -ionophore vehicle). At incubation times zero, 30, 60, and 90 min, spermatozoa were fixed in 2% paraformaldehyde for 10 min at room temperature, concentrated by centrifugation, and resuspended in PBS (200 μL). Aliquots (10 μL) were loaded onto microscope slides, air–dried, and stained with Coomassie staining solution (0.22% Coomassie Brilliant Blue G-250 in 50% methanol, 10% glacial acetic acid, and 40% water).[34] Acrosome-reacted (AR) spermatozoa were identified by the loss of intense blue staining on the anterior region of the sperm head and expressed as a percentage of the total scored cells. To evaluate the effect of antibodies on the ability of spermatozoa to undergo AE, mouse sera containing antiproacrosin/acrosin antibodies were added to the sperm suspension after 30 min of capacitation at 1:500 and 1:5000 dilutions, and further incubated during 30 min at 37°C in an atmosphere of 5% CO_2 in air. After spermatozoa were washed, induction and evaluation of AE was performed as previously described.

Histopathologic Analysis of the Male Gonad

Testes from immunized mice were removed after fertility testing (weeks 15–16 of immunization protocol), fixed in Bouin's solution, and embedded in paraffin. Transversal sections (5 μm thick) obtained from the poles and equatorial areas of testes were stained with haematoxylin and eosin and analyzed.

Immunocytochemistry

Cauda epididymal spermatozoa were recovered from untreated animals as described earlier and fixed in 2% formaldehyde in PBS during 10 min at room temperature. At the end of the fixation procedure, sperm cells were washed twice with PBS–Dulbecco (PBS-D; PBS containing 1% $MgCl_2$ and 1% $CaCl_2$), loaded on sylanized microscope glass slides (50,000 spermatozoa/slide), and allowed to dry at 37°C. Spermatozoa were washed in PBS-D for 5 min and permeabilized with 100% methanol during 10 min at 4°C. Non-specific binding sites were blocked by incubating cells with PBS supplemented with 4% BSA. Sera from immunized mice with pSF2-Acro or pSF2 were added at a 1:25 dilution in PBS-1% BSA and incubated overnight at 4°C. Unbound antibodies were washed with PBS, and Cy3-conjugated anti-mouse IgG (Sigma) in PBS with 1% BSA was added as secondary antibody for 1 hr at room temperature in darkness. Samples were mounted with Vectashield and observed under epifluorescence microscopy at ×400 magnification using a Nikon epifluorescence microscope (Nikon Instruments Inc., Melville, NY, USA) (excitation at 510 nm).

Sperm–ZP Binding

CF1 (45–60 d of age) female mice were superovulated by performing subcutaneous injections of 7.5 IU of pregnant mare serum gonadotropin (Sigma); 54–56 hrs later, animals were injected with 7.5 IU of hCG (Sigma). Animals were killed 12–14 hrs after hCG injection, and *cumulus* masses containing the unfertilized oocytes were harvested, freed of cumulus cells after treatment with 0.3 mg/mL of hyaluronidase for 5 min at 37°C, and washed in Whittingham's culture medium to remove the hyaluronidase excess. Spermatozoa were recovered as indicated earlier and capacitated in the same medium for 1.5 hr at 37°C in an atmosphere of 5% CO_2 in air. After that, pooled sera (from pSF2-Acro- or pSF2-immunized mice) were added to the sperm suspension to a final dilution of 1:1000 or 1:5000, and incubated for an additional 30 min. Oocytes (randomly distributed in groups of 10–15 in 50-μL drops of culture medium) were incubated with 100,000 spermatozoa/mL for 30 min at 37°C in an atmosphere of 5% CO_2 in air. At the end of the incubation, oocytes were washed to dislodge loosely attached spermatozoa, fixed with 2% formaldehyde in PBS during 10 min, and observed at an optical microscope. The number of attached spermatozoa per ZP was registered.

Statistical Analysis

Results are reported as mean ± standard error of the mean (S.E.M.) for each group. Statistical differences between groups were analyzed by Student's *t* test or ANOVA, when normal distribution of data was verified, or by Fisher's exact test, Mann–Whitney test, or Kruskal–Wallis test, followed by post hoc comparisons, when appropriate. *P* values <0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

Results

DNA Immunization Against Proacrosin Induces Humoral Response in Male Mice

In a first set of experiments, Balb/c male mice were immunized with a plasmid encoding human proacrosin (pSF2-Acro, $n = 10$); controls were inoculated in parallel with the empty plasmid (pSF2, $n = 10$). The humoral response was assessed in all animals during the immunization protocol and until week 15 from the first injection, by means of an indirect ELISA using recombinant h-proacrosin as antigen. Two immunization trials were performed giving similar findings; results are shown for one of them. Antibody levels increased at week 11 compared with weeks 3, 5, and 7 ($P < 0.05$) (Fig. 1A). At week 15, antibody levels, expressed as the ratio between the OD of the sample and the OD of the pre-immune sera (OD/ODpre-immune), were significantly higher in pSF2-Acro group than in control group (31.8 ± 5.8 versus 1.8 ± 0.2; mean ± S.E.M; $P < 0.05$) (Fig. 1B).

Five immunized mice were followed up to week 44 to perform long-term evaluation of the humoral immune response. At week 24, levels of anti-acrosin antibodies were similar to those registered at week 15 in four animals. At week 32, antibody levels were between 39 and 94% of the maximum values. Finally, at week 44, three mice were evaluated, finding high levels of anti-acrosin antibodies in two animals (Table I). Control mice did not show detectable levels of anti-acrosin antibodies throughout the study.

The ability of anti-acrosin antibodies to bind to different regions of the protein was also evaluated in the indirect ELISA-Acro test. Evaluations were performed using as antigens the recombinant proenzyme (Rec-40) and C-terminal-truncated products expressed in *E. coli* (Rec-40: amino acids (aa) 1-402, Rec-30: aa 1-300, Rec-20: aa 1-190, and Rec-10: aa 1-160). All immune sera recognized the recombinant products; Rec-40 was the antigen showing the highest response. Rec-30, Rec-20, and Rec-10 exhibited 85, 41, and 30% of the signal observed with Rec-40, respectively (Fig. 2).

Table I Long-term Response of Antiproacrosin/Acrosin Antibodies in pSF2-Acro-immunized Mice

	$OD/OD_{pre\text{-}immune}$ (% of maximum)			
Animal	Week 15	Week 24	Week 32	Week 44
1	35 (100)	35 (100)	14 (39)	6 (16)
2	34 (100)	32 (95)	32 (94)	25 (74)
3	15 (100)	8 (53)	10 (66)	9 (60)
4	18 (100)	15 (83)	9 (50)	N/A
5	21 (100)	21 (100)	11 (53)	N/A

N/A, not assessed; OD, optical density.

Antibodies Toward h-proacrosin Bind to Mouse Spermatozoa

To assess the ability of antibodies generated after immunization to h-proacrosin of recognizing the mouse protease, an immunocytochemistry assay with mouse spermatozoa was performed. Sperm cells from non-immunized mice incubated with the immune sera (from pSF2-Acro injected animals) and with a Cy3-labeled anti-mouse IgG depicted a fluorescent signal on the anterior portion of the sperm head over the acrosomal cap (Fig. 3A). Staining was specific because no label was observed in spermatozoa incubated with sera from animals inoculated with the pSF2 control plasmid (Fig. 3C).

Fertility is Impaired in Male Mice Immunized with h-proacrosin

Immunized male mice were mated with females of proven fertility at week 15 of the immunization protocol. Animals were separated after the first evidence

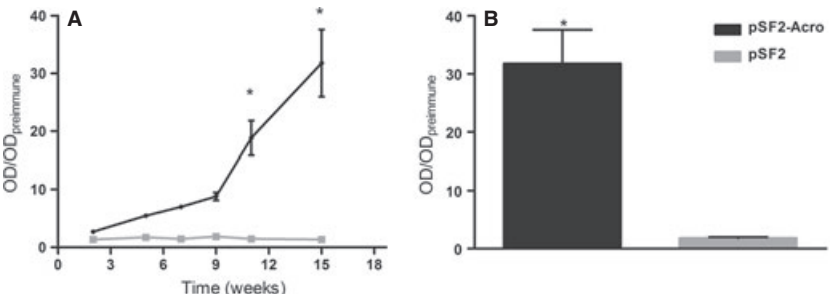

Fig. 1 Humoral immune response of mice inoculated with pSF2-Acro or pSF2. Antibody levels were assessed by an indirect ELISA. Results are expressed as optical density (OD)/ODpre-immune ratio (mean ± S.E.M.). (*) indicates statistical significance. (A) Time course of antibody levels until week 15. (B) Antibody levels at week 15 in pSF2-Acro group and pSF2 groups.

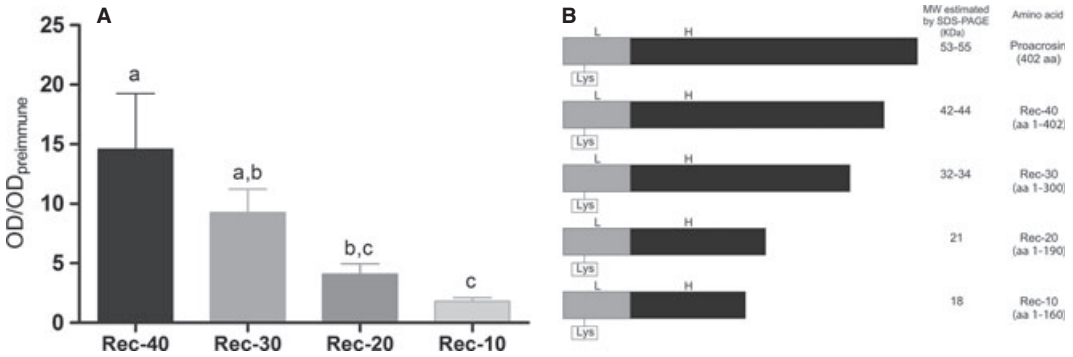

Fig. 2 Humoral immune response of mice to recombinant h-proacrosin and N-terminal protein fragments. (A) Antibody levels against different regions of h-proacrosin assessed by indirect ELISA at week 15 using each recombinant fragment as antigen. Results are expressed as optical density (OD)/ODpre-immune ratio (mean ± S.E.M.). Different superscripts denote significant differences. (B) Schematic representation of recombinant protein products of h-proacrosin expression in bacteria. L: light chain, H: heavy chain. Lys: lysine residue.

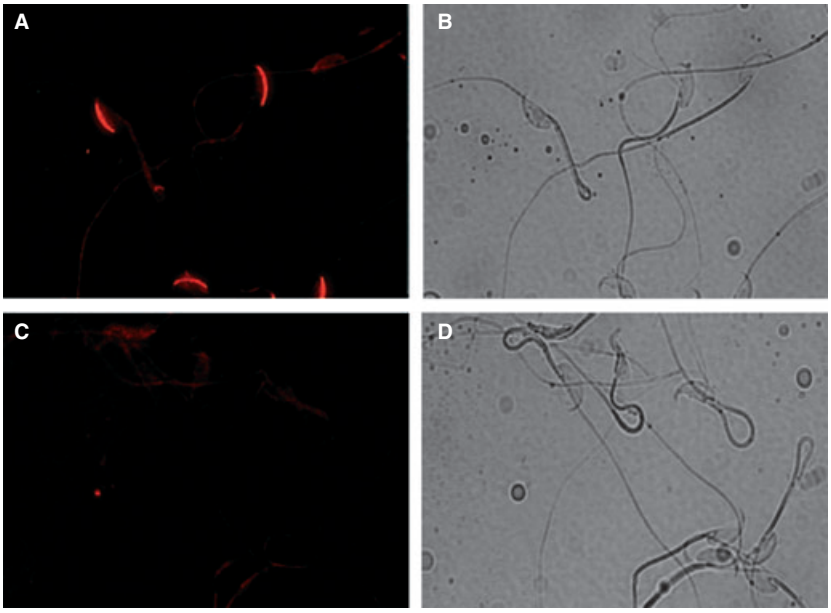

Fig. 3 Binding of antibodies from pSF2-Acro-immunized mice to mouse spermatozoa. Mouse spermatozoa were incubated with sera from immunized (pSF2-Acro) (panel a) or control (pSF2) (panel c) mice followed by cell incubation with Cy3-labeled anti-mouse IgG. Corresponding brightfield images: panels b and d, respectively.

of mating. Litter size and fertility rates were compared between pSF2-Acro-immunized and pSF2 control males in two independent mating experiments. A mouse was considered fertile if it had at least 1 pup.

The number of fertile mice was lower among pro-acrosin immunized animals than in the control group (2/18 versus 9/18, respectively; $P = 0.027$). Mating behavior seemed not to be affected by the immunization treatment, because all mice showed evidence of mating in at least 8 days. Litter size was significantly lower in the treated group than in control group, in both experiments (6 versus 35 and 0 versus 22, $P < 0.05$). Moreover, antibody levels in the pSF2-Acro group showed a significant negative correlation with the number of pups ($r = -0.696$, $P < 0.05$, Spearman's correlation test) and animals exhibiting an OD/ODpre-immune ratio over 18 were all infertile (Fig. 4).

Five mice, three injected with pSF2-Acro plasmid (mouse 1, 2 an 3) and two with pSF2 control plasmid (mouse 4 and 5), were mated again at week 44. Mouse 1 (see Table I) was infertile at week 15 but regained its fertility at week 44, coincident with a decrease in anti-acrosin antibody levels. Mouse 2, which maintained high antibody levels at week 44, was infertile in both mating assays. Mouse 3, which

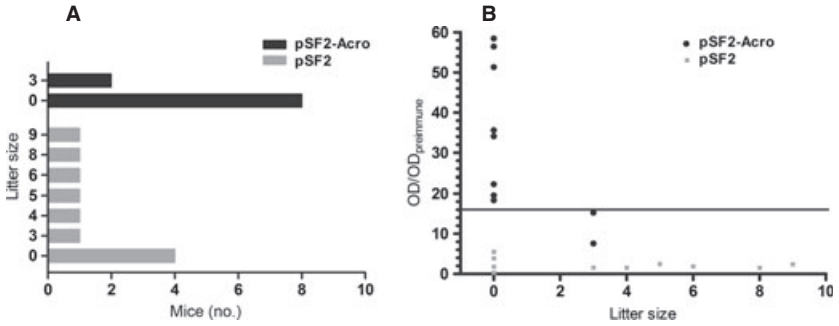

Fig. 4 Fertility of mice immunized with pSF2-Acro. Immunized male mice were mated with female mice of proven fertility until evidence of mating. Number of pups/animal was registered in two independent mating experiments. Results are shown for one of them. (A) Litter size from female mice mated to pSF2- and pSF2-Acro-immunized males. (B) Relationship between antibody levels and litter size for pSF2-Acro- and pSF2-injected male mice.

showed OD/ODpre-immune below 18 throughout the period studied, was fertile in both experiments, as did control mice.

Proacrosin Immunization does not Affect Testis Histopathology and Routine Sperm Parameters

After mating, male mice were killed and the testes, as well as spermatozoa from the *cauda* epididymis, were obtained. Testis histopathology and spermatogenesis were not affected in either treated or control group (pSF2-Acro- and pSF2-injected mice). In particular, no lymphomononuclear cell infiltrates were observed in the testicular interstitium of any of the animals studied (Fig. 5A). Moreover, spermatozoa from *cauda* epididymis showed cell number, motility, and ability to undergo Ca^{+2}-ionophore-induced AE similar to controls (Fig. 5B–E).

Anti-proacrosin Antibodies Inhibit Sperm Binding to ZP

The effects of anti-acrosin antibodies on early events of fertilization were also assessed by evaluating sperm–ZP binding in the presence of immune and control sera in three independent experiments. When positive sera were added at a 1:1000 dilution in culture medium, binding was significantly impaired compared with control (pSF-Acro: 9.4 ± 0.6; pSF2: 15.6 ± 0.7; medium: 17.5 ± 1.2 sperm/ZP; mean±S.E.M; $P < 0.0001$, medium versus pSF2-Acro and pSF2 versus pSF2Acro; no. of oocytes: pSF2-Acro: 44; pSF2: 34; medium: 24) (42% decrease relative to control pSF2). Sera added at a 1:5000 dilution did not affect sperm–ZP binding (pSF2-Acro: 12.3 ± 0.7 versus pSF2: 13.8 ± 0.7 sperm/ZP; mean ± S.E.M; no. of oocytes: 46 and 36, respectively) (11% decrease relative to control pSF2) (Fig. 6).

Anti-proacrosin Antibodies Impair Ca^{+2}-ionophore-induced Acrosomal Exocytosis

In a previous study from our group, a monoclonal antibody toward h-proacrosin was found to impair ZP-induced AE.[35] The ability of sera obtained after DNA immunization against h-proacrosin to inhibit mouse sperm Ca^{+2}-ionophore-induced AE was assessed. Spermatozoa from untreated mice were incubated with sera from pre-immune or immune mice before adding the AE inducer; two different pools of sera were tested. As shown in Fig. 7, the number of AR spermatozoa was significantly lower in sperm suspensions incubated with a 1:500 dilution of the immune sera when compared with those exposed to the same dilution of pre-immune sera, at zero, 30, 60, and 90 min ($P < 0.05$). This effect was not observed when sera were assayed at a 1:5000 dilution.

Discussion

Several sperm proteins have been used as immunogens in the development of animal models to modulate their fertility and to study the molecular basis of fertilization. Experimental designs with selected antigens have been applied to both male and female animals and, in a large proportion of the cases, target antigens have been mainly surface sperm proteins synthesized during spermatogenesis or acquired during maturation in the epididymis (reviewed in 7 and

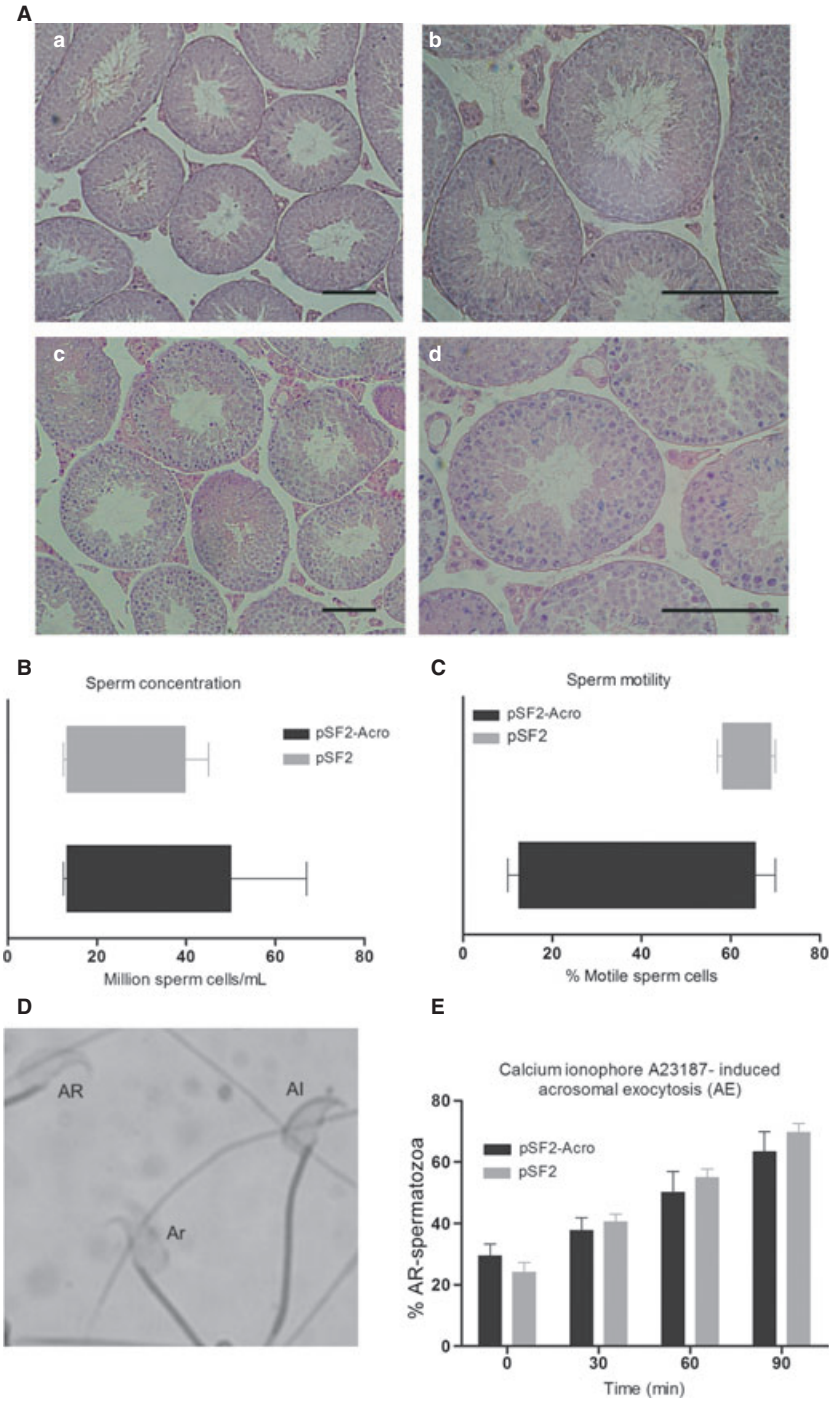

Fig. 5 Effects of immunization on testis histology and epididymal sperm parameters. Testicular tissue and *cauda* epididymal spermatozoa were obtained from immunized (pSF2-Acro) or control (pSF2) mice. (A) Representative images of haematoxylin and eosin-stained tissue sections from testis of a mouse injected with pSF2-Acro (a, b) or pSF2 (c, d) (control) plasmids (a, c: 100× magnification, bar: 100 μm; b, d: 400× magnification, bar: 100 μm). (B) Sperm count in cell suspensions recovered from the *cauda* epididymis. (C) Progressive motility of *cauda* epididymal spermatozoa. (D) Sperm acrosomal status evaluated after cell staining with Coomassie Brilliant Blue G-250; representative microscopic field with an acrosome-reacted, acrosome-intact (AI), and acrosome-reacting (Ar) spermatozoa. (E) Quantitative analysis of Ca^{2+}-ionophore-induced acrosomal exocytosis (AE) on *cauda* epididymal spermatozoa. AE was assessed by Coomassie Brilliant Blue staining (panel d). Results are expressed as mean ± S.E.M.

8). Acrosomal proteins have been used in a lesser extent to that purpose, although there are some examples, such as SP-10 protein.[36–38] With regard to the sperm acrosomal protease proacrosin/acrosin system, a previous study from our group has demonstrated that antibodies to h-proacrosin were able to inhibit protein functions as well as animal fertility in a female mouse DNA immunization model.[29] The present study aimed at evaluating the ability of DNA immunization to induce an immune response against proacrosin/acrosin in male mice and the effect of the immune response upon animal fertility.

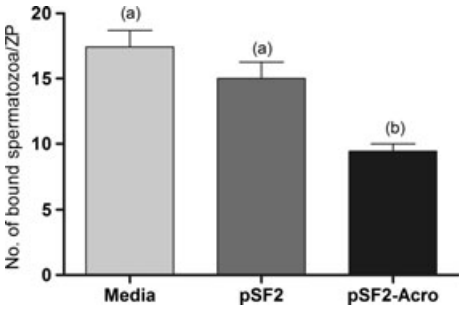

Fig. 6 Effects of antibodies upon sperm–*zona pellucida* (ZP) binding. Spermatozoa from untreated mice were incubated under conditions to promote capacitation, placed with sera from immunized (pSF2-Acro) or control (pSF2) animals, and co-incubated with matured oocytes; at the end of the gamete co-incubation assay, the number of spermatozoa bound to the ZP was scored. Results are expressed as mean ± S.E.M. Different superscripts denote significant differences.

DNA immunization is a simple and effective method to induce strong and long-lasting immune responses and has been found advantageous over protein immunization. In particular, antigen purification and care to avoid its degradation are not required; moreover, protocol standardization and scaling-up is easily achieved, and induction of both humoral and cellular immunity is attained (reviewed in 31). Its effectiveness in eliciting an immune response to sperm antigens was previously reported.[39,40] In the present study, an experimental model of male mouse DNA immunization with the coding sequence for h-proacrosin was developed. Despite using the human sequence, it was expected that antibodies raised in male mice could recognize the murine protease, based on the high similarity between the mouse and human proacrosin sequences. Similarities of 91.3, 82.5, and 51.2% were previously reported for domains I (residues 1–23), II (24–322), and III (333–402), respectively.[41] In agreement with the *in silico* data, antibodies from sera of immunized animals were able to bind to the acrosomal region of permeabilized mouse spermatozoa, as observed by the immunocytochemistry results. The highest response found for the recombinant proenzyme (Rec-40) would indicate expression of the full-length protein in the immunized animals. Moreover, reactivity toward proacrosin fragments suggests the presence of epitopes along the whole sequence that would be capable of triggering an immune response.

Serum antibodies against proacrosin/acrosin were detected in all animals inoculated with the plasmid containing the coding sequence of h-proacrosin but not in those injected with the empty plasmid; these findings indicate that the protein was efficiently expressed, processed, and presented to the mouse immune system. DNA injection led to a sustained immune response up to 5 months after the last immunization dose. In this regard, long-lasting responses after DNA inoculation have been well documented for other antigens.[42–44] The DNA immunization protocol induced a response that lasted longer than that obtained when male mice were inoculated with recombinant acrosin (Rec-30), in which OD/ODpre-immune values started to drop markedly since week 24 (data not shown).

Gene immunization against proacrosin resulted in lower fertility rates in immunized male mice compared with controls; these findings agree with previous studies reported by us in a model of DNA immunization against proacrosin in female mice.[29] Infertility was associated with antibody levels, as previously described for other protein targets.[45,46] In

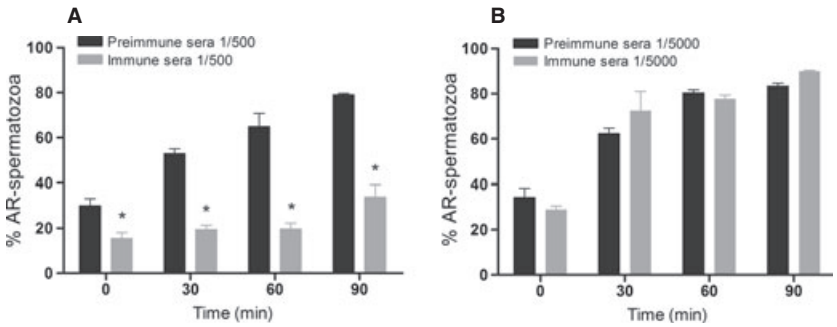

Fig. 7 Acrosomal exocytosis (AE) in the presence of sera from immunized mice. Spermatozoa from *caudae* epididymidis of non-treated mice were incubated under conditions to promote capacitation and placed in culture medium containing sera of immunized (pSF2-Acro) mice added at a 1:500 (A) and 1:5000 (B) dilution. AE was scored after sperm staining with with Coomassie Brilliant Blue G-250. Results are expressed as mean ± S.E.M. (*) Indicates statistical significance.

this work, a decreased fertility was evidenced by the significantly lower number of born pups in pSF2-Acro-immunized mice. In this regard, other studies have demonstrated that immunization with Izumo,[45] a polyepitope vaccine containing peptides from proliferin, SP56, ZP1 and ZP3,[47] and tsNHE,[48] among others, led to similar outcomes. Indeed, total suppression of fertility was hardly ever reported, a phenomenom that is not surprising because fertilization is a complex process, in which several proteins appear to be involved to assure success. Moreover, evidence supporting a role of the oviduct microenvironment in rescuing a deficient sperm function has been presented.[49]

Immunity to sperm antigens has been found to lead to a variety of deleterious effects on reproductive organs, in addition to sperm agglutination and impairment of sperm motility, sperm–mucus and sperm–oocyte interaction.[50–52] Antibodies developed against proacrosin/acrosin by gene immunization did not affect animal general health or sexual behavior, as well as they did not induce testis damage or local inflammation; moreover they seemed not to affect *cauda* epididymal sperm motility or morphology. However, anti-acrosin antibodies were found to impair fertilization-related events, as evidenced by their inhibitory effect upon sperm–ZP binding and Ca^{+2}-ionophore-induced sperm AE. Regarding ZP-binding blockage, it could be speculated that antibodies produced in this study recognized epitopes located in the mouse proacrosin catalytic domain II, a protein region described to account for part of the mouse acrosin–ZP binding activity[21,22,53] and inhibited gamete interaction. With regard to their effect upon AE, findings from this study are in line with the role attributed to proacrosin during the AE[20] and specifically agree with a recent report from our group showing the inhibitory effect of the anti-proacrosin monoclonal antibody AcrC5F10 upon ZP-induced AE in human spermatozoa.[35]

Classical studies have indicated that only acrosome-intact spermatozoa are able to bind to ZP and undergo AE after this binding; however, the site of initiation of AE is currently under revision and evidence has been presented, indicating that acrosomal components are exposed early before full exocytosis occurs.[54–56] In the present study, antibodies against proacrosin blocked sperm–oocyte binding. Altogether, recent findings and our results support the hypothesis that antibodies may interact with acrosomal components during early steps of gamete interaction and negatively modulate their function, by inhibiting fertilization-related events.

In summary, the results presented in this report have shown that DNA immunization against proacrosin elicits high antibody levels in male mice, without inducing testis inflammation. The immune response was significantly correlated with infertility and antibodies inhibited sperm–ZP binding and AE.

Financial support

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT2004 5-26110) and CONICET (PIP2120) to MHVL and from the Universidad Nacional del Litoral (CAI+D 2009, PI 15-91) to CV.

References

1 Choudhury SR, Knapp LA: Human reproductive failure I: immunological factors. *Hum Reprod Update* 2001; 7:113–134.
2 Menge AC, Medley NE, Mangione CM, Dietrich JW: The incidence and influence of antisperm antibodies in infertile human couples on sperm-cervical mucus interactions and subsequent fertility. *Fertil Steril* 1982; 38:439–446.
3 Bronson RA: Antisperm antibodies: a critical evaluation and clinical guidelines. *J Reprod Immunol* 1999; 45:159–183.
4 Fijak M, Meinhardt A: The testis in immune privilege. *Immunol Rev* 2006; 213:66–81.
5 Mital P, Hinton BT, Dufour JM: The blood-testis and blood-epididymis barriers are more than just their tight junctions. *Biol Reprod* 2011; 84:851–858.
6 Lu JC, Huang YF, Lu NQ: Antisperm immunity and infertility. *Expert Rev Clin Immunol* 2008; 4:113–126.
7 Naz RK: Status of contraceptive vaccines. *Am J Reprod Immunol* 2009; 61:11–18.
8 McLaughlin EA, Aitken RJ: Is there a role for immunocontraception? *Mol Cell Endocrinol* 2011; 335:78–88.
9 Fijak M, Iosub R, Schneider E, Linder M, Respondek K, Klug J, Meinhardt A: Identification of immunodominant autoantigens in rat autoimmune orchitis. *J Pathol* 2005; 207:127–138.
10 Naz RK: Antisperm contraceptive vaccines: where we are and where we are going? *Am J Reprod Immunol* 2011; 66:5–12.
11 Talwar GP, Vyas HK, Purswani S, Gupta JC: Gonadotropin-releasing hormone/human chorionic gonadotropin beta based recombinant antibodies and vaccines. *J Reprod Immunol* 2009; 83:158–163.
12 Killian G, Kreeger TJ, Rhyan J, Fagerstone K, Miller L: Observations on the use of GonaCon in captive female elk (*Cervus elaphus*). *J Wildl Dis* 2009; 45:184–188.
13 Klemm U, Muller-Esterl W, Engel W: Acrosin, the peculiar sperm-specific serine protease. *Hum Genet* 1991; 87:635–641.
14 Tesarik J, Drahorad J, Testart J, Mendoza C: Acrosin activation follows its surface exposure and precedes membrane fusion in human sperm acrosome reaction. *Development* 1990; 110:391–400.
15 Kashiwabara S, Arai Y, Kodaira K, Baba T: Acrosin biosynthesis in meiotic and postmeiotic spermatogenic cells. *Biochem Biophys Res Commun* 1990; 173:240–245.

16 Escalier D, Gallo JM, Albert M, Meduri G, Bermudez D, David G, Schrevel J: Human acrosome biogenesis: immunodetection of proacrosin in primary spermatocytes and of its partitioning pattern during meiosis. *Development* 1991; 113:779–788.
17 Baba T, Kashiwabara S, Watanabe K, Itoh H, Michikawa Y, Kimura K, Takada M, Fukamizu A, Arai Y: Activation and maturation mechanisms of boar acrosin zymogen based on the deduced primary structure. *J Biol Chem* 1989; 264:11920–11927.
18 Zahn A, Furlong LI, Biancotti JC, Ghiringhelli PD, Marin-Briggiler CI, Vazquez-Levin MH: Evaluation of the proacrosin/acrosin system and its mechanism of activation in human sperm extracts. *J Reprod Immunol* 2002; 54:43–63.
19 Urch UA, Wardrip NJ, Hedrick JL: Limited and specific proteolysis of the zona pellucida by acrosin. *J Exp Zool* 1985; 233:479–483.
20 Yamagata K, Murayama K, Okabe M, Toshimori K, Nakanishi T, Kashiwabara S, Baba T: Acrosin accelerates the dispersal of sperm acrosomal proteins during acrosome reaction. *J Biol Chem* 1998; 273:10470–10474.
21 Furlong LI, Harris JD, Vazquez-Levin MH: Binding of recombinant human proacrosin/acrosin to zona pellucida (ZP) glycoproteins. I. Studies with recombinant human ZPA, ZPB, and ZPC. *Fertil Steril* 2005; 83:1780–1790.
22 Richardson RT, O'Rand MG: Site-directed mutagenesis of rabbit proacrosin. Identification of residues involved in zona pellucida binding. *J Biol Chem* 1996; 271:24069–24074.
23 Sasanami T, Yoshizaki N, Dohra H, Kubo H: Sperm acrosin is responsible for the sperm binding to the egg envelope during fertilization in Japanese quail (*Coturnix japonica*). *Reproduction* 2011; 142:267–276.
24 Peknicova J, Capkova J, Geussova G, Ivanova M, Mollova M: Monoclonal antibodies to intra-acrosomal proteins inhibit gamete binding in vitro. *Theriogenology* 2001; 56:211–223.
25 Syner FN, Kuras R, Moghissi KS: Active immunization of female rabbits with purified rabbit acrosin and effect on fertility. *Fertil Steril* 1979; 32:468–473.
26 Morton DB, McAnulty PA: The effect on fertility of immunizing female sheep with ram sperm acrosin and hyaluronidase. *J Reprod Immunol* 1979; 1:61–73.
27 Moreno RD, Bustamante E, Schatten G, Barros C: Inhibition of mouse in vitro fertilization by an antibody against a unique 18-amino acid domain in the polysulfate-binding domain of proacrosin/acrosin. *Fertil Steril* 2002; 77:812–817.
28 Howe SE, Grider SL, Lynch DM, Fink LM: Antisperm antibody binding to human acrosin: a study of patients with unexplained infertility. *Fertil Steril* 1991; 55:1176–1182.
29 Veaute C, Furlong LI, Cameo M, Harris JD, Vazquez-Levin MH: Antiacrosin antibodies and infertility. II. Gene immunization with human proacrosin to assess the effect of immunity toward proacrosin/acrosin upon protein activities and animal fertility. *Fertil Steril* 2009; 91:1256–1268.
30 Veaute C, Furlong LI, Bronson R, Harris JD, Vazquez-Levin MH: Acrosin antibodies and infertility. I. Detection of antibodies towards proacrosin/acrosin in women consulting for infertility and evaluation of their effects upon the sperm protease activities. *Fertil Steril* 2009; 91:1245–1255.
31 Liu MA: DNA vaccines: an historical perspective and view to the future. *Immunol Rev* 2011; 239:62–84.
32 Furlong LI, Hellman U, Krimer A, Tezon JG, Charreau EH, Vazquez-Levin MH: Expression of human proacrosin in *Escherichia coli* and binding to zona pellucida. *Biol Reprod* 2000; 62:606–615.
33 ILAR: Guide for the Care and Use of Laboratory Animals, 7th edn. Washington, DC, National Academy Press, 1996.
34 Larson JL, Miller DJ: Simple histochemical stain for acrosomes on sperm from several species. *Mol Reprod Dev* 1999; 52:445–449.
35 Veaute C, Liu de Y, Furlong LI, Biancotti JC, Baker HW, Vazquez-Levin MH: Anti-human proacrosin antibody inhibits the zona pellucida (ZP)-induced acrosome reaction of ZP-bound spermatozoa. *Fertil Steril* 2010; 93:2456–2459.
36 Kurth BE, Digilio L, Snow P, Bush LA, Wolkowicz M, Shetty J, Mandal A, Hao Z, Reddi PP, Flickinger CJ, Herr JC: Immunogenicity of a multi-component recombinant human acrosomal protein vaccine in female *Macaca fascicularis*. *J Reprod Immunol* 2008; 77:126–141.
37 Hamatani T, Tanabe K, Kamei K, Sakai N, Yamamoto Y, Yoshimura Y: A monoclonal antibody to human SP-10 inhibits in vitro the binding of human sperm to hamster oolemma but not to human zona pellucida. *Biol Reprod* 2000; 62:1201–1208.
38 Coonrod SA, Herr JC, Westhusin ME: Inhibition of bovine fertilization in vitro by antibodies to SP-10. *J Reprod Fertil* 1996; 107:287–297.
39 Naz RK: Effect of sperm DNA vaccine on fertility of female mice. *Mol Reprod Dev* 2006; 73:918–928.
40 Naz RK: Effect of fertilization antigen (FA-1) DNA vaccine on fertility of female mice. *Mol Reprod Dev*, 2006; 73:1473–1479.
41 Vazquez-Levin MH, Furlong LI, Veaute C, Ghiringhelli PD: An overview of the proacrosin/acrosin system in human spermatozoa. In Endocrinología Molecular, J Reventós (ed.). Barcelona, Treballs de la SCB, 2005, pp 1–16.
42 Laylor R, Porakishvili N, De Souza JB, Playfair JH, Delves PJ, Lund T: DNA vaccination favours memory rather than effector B cell responses. *Clin Exp Immunol* 1999; 117:106–112.
43 Rigato PO, MacielMJr, Goldoni AL, Piubelli O, de Brito CA, Fusaro AE, Eurico deAlencar LX, August T, Azevedo MarquesETJr, da SilvaDuarte AJ, Sato MN: Immunization of neonatal mice with LAMP/p55 HIV gag DNA elicits robust immune responses that last to adulthood. *Virology* 2010; 406:37–47.
44 Patel V, Valentin A, Kulkarni V, Rosati M, Bergamaschi C, Jalah R, Alicea C, Minang JT, Trivett MT, Ohlen C, Zhao J, Robert-Guroff M, Khan AS, Draghia-Akli R, Felber BK, Pavlakis GN: Long-lasting humoral and cellular immune responses and mucosal dissemination after intramuscular DNA immunization. *Vaccine* 2010; 28:4827–4836.
45 An G, Huang TH, Wang DG, Xie QD, Ma L, Chen DY: In vitro and in vivo studies evaluating recombinant plasmid pCXN2-mIzumo as a potential immunocontraceptive antigen. *Am J Reprod Immunol* 2009; 61:227–235.
46 Wang M, Shi JL, Cheng GY, Hu YQ, Xu C: The antibody against a nuclear autoantigenic sperm protein can result in reproductive failure. *Asian J Androl* 2009; 11:183–192.
47 Hardy CM, Clydesdale G, Mobbs KJ: Development of mouse-specific contraceptive vaccines: infertility in mice immunized with peptide and polyepitope antigens. *Reproduction* 2004; 128:395–407.
48 Liu T, Huang JC, Lu CL, Yang JL, Hu ZY, Gao F, Liu YX: Immunization with a DNA vaccine of testis-specific sodium-hydrogen exchanger by oral feeding or nasal instillation reduces fertility in female mice. *Fertil Steril* 2010; 93:1556–1566.
49 Yamashita M, Honda A, Ogura A, Kashiwabara S, Fukami K, Baba T: Reduced fertility of mouse epididymal sperm lacking Prss21/Tesp5 is rescued by sperm exposure to uterine microenvironment. *Genes Cells* 2008; 13:1001–1013.
50 Bohring C, Krause W: Immune infertility: towards a better understanding of sperm (auto)-immunity. The value of proteomic analysis. *Hum Reprod* 2003; 18:915–924.

51 Suescun MO, Rival C, Theas MS, Calandra RS, Lustig L: Involvement of tumor necrosis factor-{alpha} in the pathogenesis of autoimmune orchitis in rats. *Biol Reprod* 2003; 68:2114–2121.
52 Qu N, Terayama H, Naito M, Ogawa Y, Hirai S, Kitaoka M, Yi SQ, Itoh M: Caput epididymitis but not orchitis was induced by vasectomy in a murine model of experimental autoimmune orchitis. *Reproduction* 2008; 135:859–866.
53 Howes E, Pascall JC, Engel W, Jones R: Interactions between mouse ZP2 glycoprotein and proacrosin; a mechanism for secondary binding of sperm to the zona pellucida during fertilization. *J Cell Sci* 2001; 114:4127–4136.
54 Kim KS, Gerton GL: Differential release of soluble and matrix components: evidence for intermediate states of secretion during spontaneous acrosomal exocytosis in mouse sperm. *Dev Biol* 2003; 264:141–152.
55 Liu DY, Martic M, Clarke GN, Grkovic I, Garrett C, Dunlop ME, Baker HW: An anti-actin monoclonal antibody inhibits the zona pellucida-induced acrosome reaction and hyperactivated motility of human sperm. *Mol Hum Reprod* 2002; 8:37–47.
56 Tardif S, Cormier N: Role of zonadhesin during sperm-egg interaction: a species-specific acrosomal molecule with multiple functions. *Mol Hum Reprod* 2011; 17:661–668.