Immunogenicity and Contraceptive Efficacy of *Escherichia coli*-Expressed Recombinant Porcine Zona Pellucida Proteins

Neha Gupta[1], Kausiki Chakrabarti[1], Krishna Prakash[1], Neerja Wadhwa[2], Tripti Gupta[1], Satish K. Gupta[1]

[1]Reproductive Cell Biology Laboratory, National Institute of Immunology, New Delhi, India;
[2]Embryo Biotechnology Laboratory, National Institute of Immunology, New Delhi, India

Keywords
Contraceptive vaccine, immunogenicity, *in vitro* fertilization, promiscuous T-cell epitope, recombinant porcine ZP3/ZP4, zona pellucida proteins

Correspondence
Satish Kumar Gupta PhD, Deputy Director, Chief, Reproductive Cell Biology Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi-110 067, India.
E-mail: skgupta@nii.ac.in

Submission November 19, 2012;
accepted January 19, 2013.

Citation
Gupta N, Chakrabarti K, Prakash K, Wadhwa N, Gupta T, Gupta SK. Immunogenicity and contraceptive efficacy of *Escherichia coli*-expressed recombinant porcine zona pellucida proteins. Am J Reprod Immunol 2013; 70: 139–152

doi:10.1111/aji.12095

Problem
To overcome availability of the purified native zona pellucida (ZP) glycoproteins for immunocontraception, porcine ZP3, and ZP4 were expressed in *E. coli*.

Method of study
Purified recombinant proteins were characterized by SDS-PAGE and Western blot, and immunogenicity and contraceptive efficacy determined in FvB/J female mice.

Results
Purified ZP3, ZP3 with promiscuous T-cell epitope of tetanus toxoid, ZP4 and ZP4 incorporating promiscuous T-cell epitope of bovine RNase revealed ~44-, ~49-, ~53-, and ~55-kDa bands by SDS-PAGE and Western blot, respectively. Immunization of female mice with recombinant proteins elicited high antibody titers as well as T-cell responses. Immune sera recognized mouse oocyte ZP and also inhibited *in vitro* fertilization. Immunized mice showed significant decrease in fertility. Recombinant proteins were able to recall memory antibody response in female mice primed with porcine native ZP.

Conclusion
Availability of recombinant porcine proteins will be useful in the development of contraceptive vaccine.

Introduction

Zona pellucida (ZP) glycoproteins by virtue of their critical role during fertilization have been proposed as candidates for the development of contraceptive vaccine.[1,2] In mammals, ZP matrix is comprised of either 3 or 4 glycoproteins. For example, in mice, it is composed of ZP glycoprotein-1 (ZP1), -2 (ZP2), and -3 (ZP3)[3]; whereas pig ZP matrix is also composed of 3 glycoproteins, but instead of ZP1, ZP glycoprotein-4 (ZP4) is present.[4] *Zp4* is a pseudogene in mice.[5] Human ZP matrix has complement of all the four ZP glycoproteins.[6] These proteins are evolutionary conserved and share variable degree of amino acid (aa) sequence identity among various species; for example, at aa level, mouse ZP3 has 68, 66, and 69 percent identity with porcine, canine, and human ZP3. This property of ZP glycoproteins has made heterologous immunization as feasible proposition to develop contraceptive vaccine. Studies from various species suggest that ZP3 is the putative primary sperm receptor and is also responsible for induction of acrosome reaction.[7–10] In addition to ZP3, in humans and porcine model, ZP4 also binds to spermatozoa[9–11] and in humans it has been shown to induce acrosome reaction.[9,10] However, subsequent studies showed that in porcine model, ZP3 (previously designated as ZP3β/ZPC)-ZP4 (previously

designated as ZP3α/ZPB) heterocomplexes bind to boar sperm membrane vesicles but not to free glycoprotein subunits.[12] In mouse model, ZP1 has been postulated to cross-link the filaments formed by ZP2–ZP3 heterodimers and may provide structural stability to ZP matrix.[13] However, recent studies suggest that in humans, ZP1 also binds to spermatozoa and induces acrosome reaction.[14] ZP2 during fertilization acts as a secondary sperm receptor and maintains the binding of the acrosome-reacted spermatozoa to ZP[15]; however, recent studies using transgenic mice suggest its direct involvement in sperm–egg binding.[16]

Contraceptive vaccines based on ZP glycoproteins have been used successfully to control population of feral horses (*Equus caballus*)[17], white-tailed deer (*Odocoileus virginianus*)[18], African elephant (*Loxodonta africana*)[19], koalas (*Phascolarctos cinereus*),[20] and grey seal (*Halichoerus grypus*).[21] Long-term follow-up of the immunized horses and white-tailed deer did not reveal any significant debilitating effects on their health.[22–24] Oophoritis often associated with ZP-based contraceptive vaccine may not be a major concern in the management of wildlife population as either long-term infertility or permanent sterility is desirable. In the present manuscript, attempts have been made to clone and express porcine ZP3 and ZP4 in *E. coli*. Recombinant *E. coli*-expressed porcine ZP3 and ZP4 have been evaluated for their immunogenicity and contraceptive efficacy in female mice.

Materials and methods

Expression of Recombinant Porcine ZP3 and ZP4 in *E. coli*

Porcine ZP3 and ZP4 were expressed as His_6-tag fusion proteins without or with promiscuous T-cell epitope. The commercially available porcine ovarian total RNA (Zyagen, San Diego, CA, USA) was employed as starting material for cDNA synthesis using Superscript-3 cDNA synthesis kit (Invitrogen Corp., Carlsbad, CA, USA) following manufacturer's instructions. Primers were designed for porcine ZP3 and ZP4 on the basis of the sequences published in the GenBank having the accession numbers NM_213893 and NM_214045, respectively, to amplify the cDNAs encoding ZP3 [amino acid (aa) residues 20–344] and ZP4 (aa residues 22–462) without the signal peptide and transmembrane-like domain following consensus furin cleavage site. In addition, ZP3 was also expressed as chimeric fusion protein encompassing promiscuous T-cell epitope of tetanus toxoid (TT; aa residues 830–844) at its N-terminus and separated from ZP3 by dilysine linker (TT-KK-ZP3). Similarly, ZP4 was also expressed as chimeric fusion protein incorporating promiscuous T-cell epitope of bovine RNase (bRNase; aa residues 94–104; bRNase-KK-ZP4). The constructs' designs are shown in Fig. 1.

The PCR amplification was performed in 50-μL final reaction volume using 10 ng of template cDNA, 0.2 mM deoxyribonucleotide triphosphates (dNTPs), 50 pmol of primers, 5 μL of 10X Taq buffer B ($MgCl_2$ free), 3 μL of 25 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Invitrogen Corp.). Table I lists the sequence of primers used to amplify cDNA encoding various recombinant proteins. PCR amplification involved initial denaturation at 94°C for 10 min and 30 cycles of 94°C for 1 min, appropriate annealing temperature as shown in Table I for 1 min and 72°C for 1 min followed by a final extension at 72°C for 10 min. The PCR amplified fragments were initially cloned in pGEMT-Easy (Promega Corporation, Madison, WI, USA) sequencing vector followed by pRSET-A expression vector (Invitrogen Corp.) downstream of His_6-tag under T7 promoter, and proteins were expressed in BL21(DE3)pLysS bacterial cells deficient in *lon* and *ompT* proteases (Stratagene, La Jolla, CA, USA) following standard protocols.

To check the expression of recombinant proteins, the transformants with respect to the four constructs

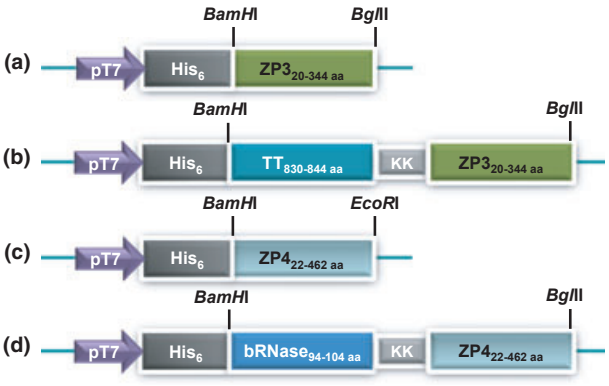

Fig. 1 Porcine ZP constructs cloned in pRSET-A expression vector. (a and b) Porcine ZP3 (aa residues 20–344) and ZP3 linked to T-cell epitope of tetanus toxoid (TT) by dilysine linker at N-terminus, respectively. (c and d) ZP4 (aa residues 22–462) and ZP4 linked to T-cell epitope of bovine RNase by dilysine linker at N-terminus, respectively. The respective restriction sites used for cloning various inserts are shown. pT7, T7 promoter; His_6, histidine tag.

Table I Sequence of the primers used for the amplification of cDNA-encoding recombinant porcine ZP3 and ZP4

Construct	Primers	Annealing temperature (in °C)	Sequence of the primers[a]
ZP3	Forward	60	5′-CG*GGATCC*TGCAGCCCGCAGCCC-3′
	Reverse		5′-GA*AGATCT*TTCAGGGAGCAGACTGTCTCTT-3′
TT-KK-ZP3	Forward	62	5′-CG*GGATCC*CAGTATATAAAAGCAAATTCTAAATTTATA··· GGTATAACTGAACTAAAGAAGTGCAGCCCGCAGCCC-3′
	Reverse		5′-GA*AGATCT*TTCAGGGAGCAGACTGTCTCTT-3′
ZP4	Forward	56	5′-CG*GGATCC*AGCCCAAAGCAGCAGAT-3′
	Reverse		5′-GA*GAATTC*TCATCTGGCAGCAGGACAGGTTGT-3′
bRNase-KK-ZP4	Forward	58	5′-CG*GGATCC*AACTGTGCATACAAGACAACACAGGCTAA··· CAAGCAAGAAGAGCCCAAAGCAGCAGAT-3′
	Reverse		5′-GA*AGATCT*TCATCTGGCAGCAGGACAGGTTGT-3′

[a]Restriction sites are represented by italic and underlined nucleotides.

were inoculated into 3 mL of Luria broth (LB) medium (Difco Laboratories, Detroit, MI, USA) containing 100 μg/mL of ampicillin and 37 μg/mL of chloramphenicol and grown overnight at 37°C in an orbital shaker (New Brunswick Scientific, Edison, NJ, USA) set at 200 rpm. The cultures were diluted 1:100 into fresh medium and grown until the cell density reached an absorbance of approximately 0.5–0.6 (mid-log phase) at 600 nm (A_{600}). Expression of the respective proteins was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Biosynth International, Inc., Itasca, IL, USA) at 37°C for 3 hr. Simultaneously, BL21[DE3]pLysS host cells induced with same concentration of IPTG were used as negative control. The cells were centrifuged at 6500 × *g* in a microfuge for 5 min, and the resulting pellets were stored at −20°C until used.

SDS-PAGE and Immunoblotting

The cell pellet obtained from 1-ml culture was boiled for 10 min in 5X sample buffer (0.15 M Tris-HCl, pH-6.8, 5% sodium dodecyl sulfate (SDS), 25% glycerol, 12.5% β-mercaptoethanol, 0.025% bromophenol blue), and proteins were resolved by 0.1% SDS-10% PAGE and stained with 0.2% Coomassie blue. Alternatively, SDS-PAGE resolved proteins were processed for Western blot analysis essentially as described previously.[25] In brief, after transfer of resolved proteins on nitrocellulose membrane, the membrane was washed once with phosphate-buffered saline (PBS; 50 mM phosphate and 150 mM NaCl, pH 7.4), and non-specific sites were blocked with 3% BSA in PBS for 1 hr at room temperature (RT). All the subsequent incubations were carried out for 1 hr at RT followed by three washings with PBS containing 0.1% Tween-20. Expression of the recombinant protein was detected by mouse monoclonal antibodies (MAbs): MA-451 for ZP3 and MA-410 for ZP4.[26–28] Murine MAb, MA-451 (IgG_1), was generated against native porcine ZP3, which reacted specifically with glycosylated, deglycosylated and reduced and carboxyamidomethylated forms of ZP3 both in ELISA and Western blot. [26] It recognized an epitope corresponding to aa residues 166–171 of ZP3 (EEKLVF).[27] MA-410 (IgG_1) was developed against native porcine ZP4, which reacted specifically with glycosylated as well as deglycosylated ZP4 and showed no reactivity with ZP3 in ELISA and Western blot.[28] The bound antibody was revealed using goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (HRPO; Pierce, Rockford, IL, USA) at a predetermined dilution of 1:5000. The blot was developed with 0.1% (w/v) 3,3′-diaminobenzidine (Sigma-Aldrich Inc., St. Louis, MO, USA) in 50 mM PBS and 0.06% H_2O_2. The reaction was stopped by washing the membrane extensively with milliQ water.

Purification of the Recombinant Porcine ZP3 and ZP4 Proteins

To purify recombinant ZP3, TT-KK-ZP3, ZP4, and bRNase-KK-ZP4, the respective clones were grown at a shake-flask level (250 mL culture/flask; total volume 1 L) as described above. The cells were induced with 1 mM IPTG for 3 hr at 37°C and centrifuged at 6500 × *g* for 15 min at 4°C, and cell pellet

stored at −20°C until used. The bacterial cell pellet (1 gm) was suspended in 5 mL of solubilization buffer (6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0) and lysed by sonication using a Branson Sonifier-450 (Branson Ultrasonic Corp., Danbury, CT, USA) for 8 to 10 cycles of 30 s each at a 40-watt output, with 2-min pause intervals on ice and centrifuged at 10,000 ***g*** for 20 min at 4°C. The recombinant proteins from the respective supernatants were purified by nickel nitrilotriacetic acid (Ni-NTA; Qiagen, GmbH, Hilden, Germany) affinity chromatography under reducing conditions followed by renaturation essentially as described previously.[25] The refolded proteins were finally dialyzed against 20 mM PBS, pH 8.0. The purified proteins were characterized by SDS-PAGE and Western blotting as described above. The concentration of purified proteins was estimated by bicinchoninic acid assay (Pierce) using BSA as the standard.

Active Immunization of Mice With the Recombinant Porcine Zona Proteins

Inbred FvB/J female mice were used for active immunization studies with various recombinant porcine zona proteins as they have higher litter size as compared to BALB/cJ. Mice, 8–10 weeks of age, kept under the conventional containment levels at the Small Experimental Animal Facility, National Institute of Immunology, New Delhi, India, were used. These studies were conducted as per the guidelines and approval of the Institutional Animals Ethics Committee. Female inbred FvB/J mice were immunized subcutaneously with 25 μg of recombinant protein in 100 μL of 20 mM PBS (pH 8.0)/injection/mouse using synthetic polymer Montanide™ PetGel A (5%; Seppic, Paris, France) as an adjuvant.[29,30] Immunization groups comprised of the following:

Experiment I

(i) PetGel A only (n = 9)
(ii) ZP3 (n = 8)
(iii) TT-KK-ZP3 (n = 9)
(iv) ZP4 (n = 9)
(v) bRNase-KK-ZP4 (n = 10)

Experiment II

(i) PetGel A only (n = 14)
(ii) TT-KK-ZP3 (n = 14)
(iii) bRNase-KK-ZP4 (n = 14)

Primary immunization was followed by two booster doses administered intraperitoneally on days 21 and 49 of the same amount of the recombinant protein as used in the first injection. Immunized mice (Experiment II) were also followed up for estrus cycles by daily examination of vaginal cytology. Animals were bled retro-orbitally on days 0, 35, and 63, and serum samples were processed for the determination of antibody titer by ELISA as described below.

In addition, FvB/J female inbred mice (n = 7) were also immunized subcutaneously with 50 μg of porcine heat-solubilized isolated zona pellucida (SIZP) in 100 μL of saline/injection/mouse along with 5% PetGel A as an adjuvant. Another group of mice (n = 7) was immunized with 5% PetGel alone. Subsequently, both groups of mice were injected intraperitoneally on day 21, with the physical mixture (1:1) of TT-KK-ZP3 and bRNase-KK-ZP4. Each animal received 25 μg of TT-KK-ZP3 and 25 μg of bRNase-KK-ZP4, respectively. Animals were bled retro-orbitally on days 0, 20, and 35, and serum samples were processed for quantitation of antibody titers against SIZP, ZP3, and ZP4 by ELISA.

Determination of the Antibody Titers by Enzyme-Linked Immunosorbent Assay (ELISA)

Microtitration plates (Nunc a/s, Roskilde, Denmark) were coated with the optimized concentration of SIZP, recombinant ZP3, and ZP4 (200 ng/well) in 0.2 M carbonate buffer, pH 9.5 at 37°C for 1 hr and then at 4°C overnight. The plates were washed once with PBS and incubated with 1% BSA in PBS (200 μL/well) for 2 hr at 37°C for blocking the non-specific sites. Each incubation was followed by washings done with PBS containing 0.05% Tween-20 (PBST). Post-blocking, the respective coated plates were incubated with doubling dilutions of serum samples (100 μL/well) obtained from various groups of immunized mice for 1 hr at 37°C. Bound antibodies were revealed with goat anti-mouse immunoglobulins conjugated to horseradish peroxidase at an optimized dilution of 1:10,000 in PBST. Estimation of the enzyme activity was carried out with 0.05% orthophenylenediamine in 50 mM citrate phosphate buffer, pH 5.2, with 0.06% hydrogen peroxide as the substrate. The reaction was stopped with 50 μL of 5 N H_2SO_4, and absorbance read at 490 nm with 630 nm as reference filter; values are represented as antibody titers. Antibody titers were calculated by

regression analysis and are expressed as antibody units (AU), that is, reciprocal of the dilution of the serum giving an absorbance of 1.0. The antibody response generated in a group of mice was represented as arithmetic mean ± standard error (S.E.) of the antibody titers of the individual animals. Immune sera were also checked for their reactivity with the respective recombinant proteins by Western blot.

T-Cell Proliferation Assay

To analyze T-cell proliferation, on day 64, mice immunized with TT-KK-ZP3 and bRNase-KK-ZP4 were euthanized and their spleen removed aseptically. Splenocytes (pooled from two immunized mice) were used to perform T-cell proliferation with recombinant porcine ZP3, TT-KK-ZP3, ZP4, and bRNase-KK-ZP4, respectively. Splenocytes were diluted to a final concentration of 5×10^6 cells/mL in RPMI-1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS; Gibco, Grand Island, NY, USA). The cells (0.5×10^6/well) were dispensed into a 96-well plate containing serial dilutions of the recall antigens. Three days after addition of cells, cultures were pulsed with 1 μCi/well of [^{3}H]-thymidine (NEN, Life Science Products, Boston, USA) for 16 hr. Cells were lysed and harvested onto glass fiber filaments for liquid scintillation counting (Betaplate, Wllac, Turku, Finland). Phytohaemagglutinin (2.5 μg/well; Sigma) was used as positive control. All assays were carried out in triplicates. Data were represented as Stimulation Index (SI), which is calculated by dividing the mean counts per minute (cpm) observed in the presence of stimulating antigen by cpm observed in unstimulated cells.

Reactivity of Antibodies with Mouse Oocytes by Indirect Immunofluorescence

FvB/J female mice (4–5 weeks old) were superovulated by injecting intraperitoneally pregnant mare serum gonadotropin (PMSG; 5 IU/mice; Intervet, Boxmeer, Netherlands) followed by human chorionic gonadotropin (hCG; 5 IU/mice; Intervet India Pvt. Ltd.) after 48 hr. The next day, animals were euthanized by cervical dislocation, and ovaries removed surgically. Oocytes retrieved from ampulla region were washed once with Brister modified oocyte culture (BMOC) medium (Gibco) followed by treatment for 5 min with hyaluronidase (0.1 mg/mL; Sigma) at RT to obtain denuded oocytes. The oocytes were subsequently incubated with 50 mM PBS, pH 7.4, supplemented with 1% BSA for 45 min at 37°C. The oocytes ($n = 5$) were washed twice with PBS followed by incubation with 1:20 dilution of serum samples from mice immunized with adjuvant only, TT-KK-ZP3, and bRNase-KK-ZP4 for 45 min at 37°C. After washing 4 times with PBS, oocytes were further incubated with 1:400 dilution of goat anti-mouse IgG conjugated to fluorescein isothiocyanate (Alexa Fluor® 488; Invitrogen Corp.) for 45 min at 37°C. After several washes in PBS, the oocytes were observed under fluorescence microscope (Nikon Optiphot; Nikon Corp., Tokyo, Japan). To determine specificity of reactivity of the immune serum samples with mouse ZP, the mouse oocytes ($n = 4$) were incubated as described above with the serum samples (1:20 dilution) pre-incubated for 30 min at RT with 5 μg/mL of the recombinant ZP3/ZP4.

In Vitro Fertilization Assay

Mouse sperm were collected by flushing caudae epididymis from male FvB/J mice in BMOC medium and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 10 min to allow the sperm to swim-up. Sperm were further incubated for 1 hr to allow capacitation, before use. Cumulus-free unfertilized oocytes (18–30 oocytes/treatment group) obtained as described above were transferred to 100 μL BMOC medium droplet under mineral oil (Sigma) and incubated for 1 hr with 1:20 dilution of decomplemented serum samples from mice immunized with either recombinant TT-KK-ZP3/bRNase-KK-ZP4 or adjuvant alone. After incubation, sperm ($0.5–1.0 \times 10^6$/droplet) were transferred to the medium droplet containing oocytes and further incubated at 37°C in a humidified atmosphere of 5% CO_2 for 4–6 hr. After incubation, the oocytes were washed 2–3 times with BMOC medium, transferred to fresh medium and incubated overnight. Next day, numbers of 2-cell embryos formed were counted. The experiment was repeated 3 times.

Mating Studies

Immunized female mice comprising of the above groups were mated with the healthy males of proven fertility next day after obtaining the last bleed as described above. Mating was performed by housing

two immunized female mice along with a male mouse per cage. Male mice were rotated every third day. Mating was confirmed by the presence of vaginal plug. The male and female mice were co-habitated for 15 consecutive days to assess the contraceptive efficacy.

Ovarian Histology

Animals immunized with *E. coli*-expressed recombinant ZP3, TT-KK-ZP3, ZP4, and bRNase-KK-ZP4 were euthanized by cervical dislocation on day 120 after the initiation of immunization. In addition, mice immunized with recombinant TT-KK-ZP3 and bRNase-KK-ZP4 (Experiment II) were also euthanized on day 64 after the initiation of immunization. The ovaries were surgically removed, fixed in buffered 10% (v/v) formalin for 24 hr, and processed for paraffin wax sections (5 μm). The sections were stained with hematoxylin and eosin using standard protocols. For each ovary, four to six sections were chosen randomly and examined under a light microscope for ovarian pathology with respect to follicular development, cystic changes, or infiltration by lymphocytes.

Statistical Analysis

The statistical significance for the contraceptive efficacy of the animals immunized with various recombinant proteins and the immune serum samples to inhibit *in vitro* fertilization was performed by comparing the results obtained in experimental groups with the respective controls by using one-way analysis of variance. A P-value of <0.05 was considered to be statistically significant.

Results

Characteristics of the Recombinant Porcine ZP3 and ZP4

PCR amplified cDNAs encoding ZP3, TT-KK-ZP3, ZP4, and bRNase-KK-ZP4 were cloned in pGEMT-Easy cloning vector (Promega Corporation) and were used to determine the nucleotide sequence. The deduced amino acid sequence of the cloned constructs did not reveal any changes as compared to the GenBank sequences (ZP3, NM_213893; ZP4, NM_214045). These constructs were then further cloned into the expression vector pRSET-A, and respective proteins expressed as His_6-tag fusion protein in *E. coli* as described in *Materials and Methods*. The BL21[DE3]pLysS *E. coli* cells harboring the plasmid encoding ZP3, TT-KK-ZP3, ZP4, and bRNase-KK-ZP4 when induced with 1 mM IPTG revealed by SDS-PAGE, major band corresponding to ~44, ~49, ~53, and ~55 kDa, respectively (Figs 2a, lane 2; 2b, lane 4; 3a, lane 2 and 3b, lane 4).

The expressed proteins were purified using Ni-NTA affinity chromatography. SDS-PAGE analysis of the purified recombinant ZP3 revealed a dominant band of ~44 kDa (Fig. 2c, lane 5) which reacted with MA-451 generated against porcine ZP3β (now designated as ZP3) in Western blot (Fig. 2d, lane 5). Recombinant TT-KK-ZP3 revealed a major band of

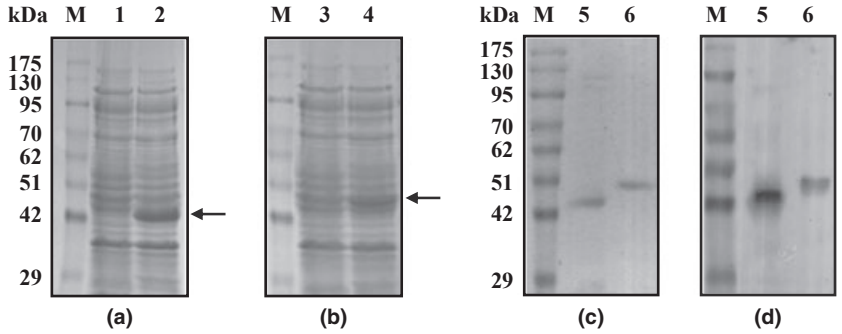

Fig. 2 SDS-PAGE and immunoblot analysis of the *E. coli*-expressed porcine ZP3 and TT-KK-ZP3. Recombinant porcine ZP3 and TT-KK-ZP3 were expressed in *E. coli*, purified and renatured, as described in *Materials and Methods*. Total cell lysate of *E. coli*-expressed recombinant protein and wild-type host cells induced with 1 mm IPTG were analyzed by SDS-PAGE, and the purified proteins were analyzed by SDS-PAGE and Western blot. (a and b) Coomassie-stained SDS-PAGE profile of total cell lysate of recombinant ZP3 and TT-KK-ZP3, respectively. (c) Represents Coomassie-stained SDS-PAGE profile of purified recombinant ZP3 (lane 5) and TT-KK-ZP3 (5 μg/lane; lane 6), and (d) Western blot of the same (3 μg/lane). Various lanes represent the following: M, Molecular weight markers; lanes 1 and 3, wild-type BL21[DE3]pLysS cell lysate; lane 2, recombinant ZP3 cell lysate; lane 4, TT-KK-ZP3 cell lysate; lane 5, purified recombinant ZP3; and lane 6, purified TT-KK-ZP3.

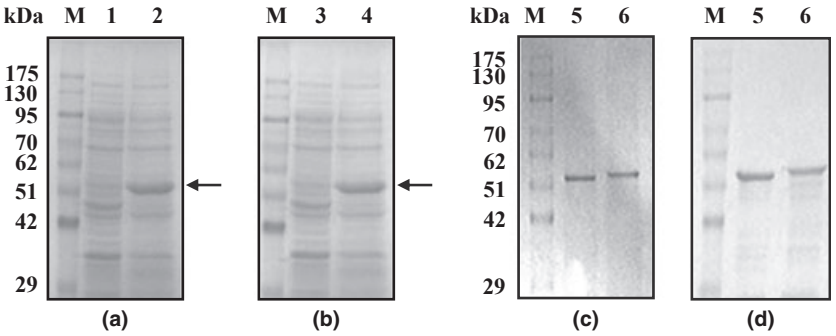

Fig. 3 SDS-PAGE and immunoblot analysis of the *E. coli*-expressed porcine ZP4 and bRNase-KK-ZP4. Recombinant porcine ZP4 and bRNase-KK-ZP4 were expressed in *E. coli*, purified and renatured, as described in *Materials and Methods*. Total cell lysate of *E. coli*-expressed recombinant protein and wild-type host cells induced with 1-mM IPTG were analyzed by SDS-PAGE, and the purified proteins were analyzed by SDS-PAGE and Western blot. (a and b) Coomassie-stained SDS-PAGE profile of total cell lysate of recombinant ZP4 and bRNase-KK-ZP4, respectively. (c) Coomassie-stained SDS-PAGE profile of purified recombinant ZP4 (lane 5) and bRNase-KK-ZP4 (5 μg/lane; lane 6), and (d) Western blot of the same (3 μg/lane). Various lanes represent; M, Molecular weight markers; lanes 1 and 3, wild-type BL21[DE3]pLysS cell lysate; lane 2, recombinant ZP4 cell lysate; lane 4, bRNase-KK-ZP4 cell lysate; lane 5, purified recombinant ZP4; and lane 6, purified bRNNase-KK-ZP4.

~49 kDa in both SDS-PAGE and Western blot (Fig. 2c,d, lane 6). Ni-NTA purified recombinant ZP4 resolved in SDS-PAGE as ~53-kDa band (Fig. 3c, lane 5) which reacted with MA-410 generated against porcine ZP3α (now designated as ZP4; Fig. 3d, lane 5). Purified recombinant bRNase-KK-ZP4 revealed a dominant band of ~55 kDa in SDS-PAGE as well as Western blot (Fig. 3c,d, lane 6). The yield of the above recombinant proteins was in the range of 2 to 5 mg per liter of the culture when induced with 1 mM IPTG for 3 hr.

Immunogenicity of the Recombinant Porcine ZP Proteins

Female FvB/J mice were immunized with the various recombinant porcine ZP proteins following an immunization schedule as described in *Materials and Methods*. Serum samples were analyzed for antibody titers against respective protein in an ELISA. The results are summarized in Table II. Immunization of mice with ZP3 led to generation of antibodies against ZP3 which showed mean antibody titers of $278.0 \pm 25.4 \times 10^3$ AU on day 35 and $383.4 \pm 65.5 \times 10^3$ AU on day 63. Immunization with recombinant TT-KK-ZP3 led to generation of higher antibody titers as compared to animals immunized with recombinant ZP3 at both 35 and 63 days of immunization (Table II). The mean antibody titers on day 63 in the group of animals immunized with TT-KK-ZP3 were $508.5 \pm 62.9 \times 10^3$ AU in Experiment I and $625.0 \pm 46.0 \times 10^3$ AU in Experiment II. Similarly, animals immunized with recombinant bRNase-KK-ZP4 also generated higher antibody titers as compared to those immunized with recombinant ZP4 (Table II). The immune serum samples from the mice immunized with recombinant ZP3 reacted with recombinant ZP3 and TT-KK-ZP3 in Western blot (Fig. 4, lanes 1, 2). Similarly, the immune serum samples from mice immunized with ZP4 reacted in immunoblot with recombinant ZP4 and bRNase-KK-ZP4 (Fig. 4, lanes 3, 4).

Recombinant TT-KK-ZP3 and bRNase-KK-ZP4 Boost Antibody Production in Female Mice Primed With the Porcine SIZP

Group of female FvB/J mice immunized with porcine SIZP when boosted with physical mixture (1:1) of recombinant TT-KK-ZP3 and bRNase-KK-ZP4 elicited significantly higher antibody titers against SIZP, recombinant ZP3 and ZP4 as compared to adjuvant control group (Fig. 5), suggesting thereby that recombinant proteins are competent to act as booster in SIZP-primed mice.

Immunization with Recombinant Porcine Zona Proteins also Elicit T-Cell Response

In vitro stimulation of splenocytes obtained from mice immunized with TT-KK-ZP3 with either recombinant ZP3 or TT-KK-ZP3 showed generation of T-cell responses (Fig. 6). The SI observed with recombinant TT-KK-ZP3 (SI = 16.5) was higher as

Table II Immunogenicity and contraceptive efficacy of the recombinant porcine ZP proteins in female FvB/J mice

Immunogen	Antibody titers (AU[a] × 10^3)			Percentage of animals failed to conceive	Pups born/ mated mouse (mean ± S.E.M.)
	Day 0	Day 35	Day 63		
Experiment I					
Nil (n = 9)	<0.05[b]	<0.05[b]	<0.05[b]	0.0	5.60 ± 0.17
ZP3 (n = 8)	<0.05[b]	278.0 ± 25.4	383.4 ± 65.5	87.5	0.63 ± 0.62 ($P = 1.5 \times 10^{-6}$)[c]
TT-KK-ZP3 (n = 9)	<0.05[b]	542.5 ± 55.2	508.5 ± 62.9	88.8	0.55 ± 0.55 ($P = 4.1 \times 10^{-6}$)[c]
ZP4 (n = 9)	<0.05[b]	227.7 ± 22.8	257.8 ± 18.1	55.5	1.89 ± 0.75 ($P = 2.2 \times 10^{-4}$)[c]
bRNase-KK-ZP4 (n = 10)	<0.05[b]	477.5 ± 37.0	406.0 ± 32.8	50.0	1.73 ± 0.58 ($P = 2.2 \times 10^{-5}$)[c]
Experiment II					
Nil (n = 10)	<0.05[b]	<0.05[b]	<0.05[b]	0.0	6.90 ± 0.31
TT-KK-ZP3 (n = 10)	<0.05[b]	500.5 ± 52.1	625.0 ± 46.0	80.0	0.70 ± 0.47 ($P = 2.25 \times 10^{-9}$)[c]
bRNase-KK-ZP4 (n = 10)	<0.05[b]	403.2 ± 68.7	564.2 ± 41.7	60.0	1.93 ± 0.43 ($P = 1.54 \times 10^{-5}$)[c]

[a]AU represents antibody titers as reciprocal of the serum dilution giving an absorbance of 1.0 in ELISA.
[b]serum samples when tested at 1:50 dilution showed an absorbance of <1.0.
[c]*P* values calculated with respective control group.

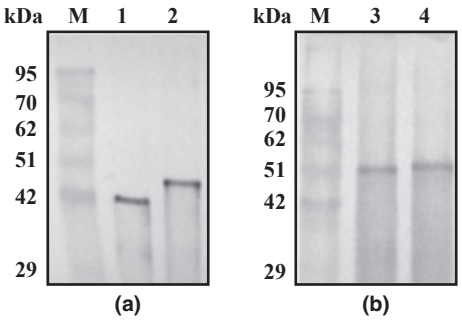

Fig. 4 Reactivity of immune serum samples from ZP3/ZP4-immunized mice with recombinant porcine zona proteins in Western blot. Immune serum samples (day 63 bleed) collected from mice immunized with either recombinant porcine ZP3 or ZP4 were assessed for their reactivity with recombinant porcine zona proteins (2 μg/lane) in Western blot. (a) Western blot profile of recombinant ZP3 (lane 1) and TT-KK-ZP3 (lane 2) probed with sera (1:5000 dilution) from the mice immunized with recombinant ZP3 protein. (b) The reactivity profile of the immune serum (1:5000 dilution) collected from ZP4-immunized mice with recombinant ZP4 (lane 3) and bRNase-KK-ZP4 (lane 4). Lane M represents molecular weight markers.

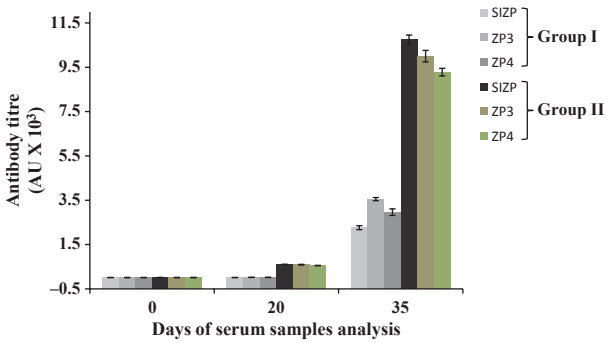

Fig. 5 Antibody titers in mice primed with porcine SIZP and boosted with combination of recombinant ZP3 and ZP4. Female FvB/J mice were primed with porcine SIZP (50 μg/animal) followed by booster on day 21 with physical mixture of recombinant TT-KK-ZP3 (25 μg/animal) and bRNase-KK-ZP4 (25 μg/animal). The sera were analyzed for antibody titers against SIZP, ZP3, and ZP4 by ELISA as described in *Materials and Methods*. Group I comprised of animals that did not receive SIZP, whereas Group II comprised of animals that were primed with SIZP followed by booster with recombinant proteins.

compared with recombinant ZP3 (SI = 10.1). Similarly, splenocytes obtained from mice immunized with bRNase-KK-ZP4 also elicited T-cell response against recombinant bRNase-KK-ZP4 (SI = 16.1) which was higher as compared to recombinant ZP4 (SI = 6.9). The dose–response studies revealed that lower concentrations of TT-KK-ZP3 (50 μg/mL)/ bRNase-KK-ZP4 (25 μg/mL) were required to obtain optimum T-cell responses as compared to ZP3 (100 μg/mL)/ZP4 (50 μg/mL).

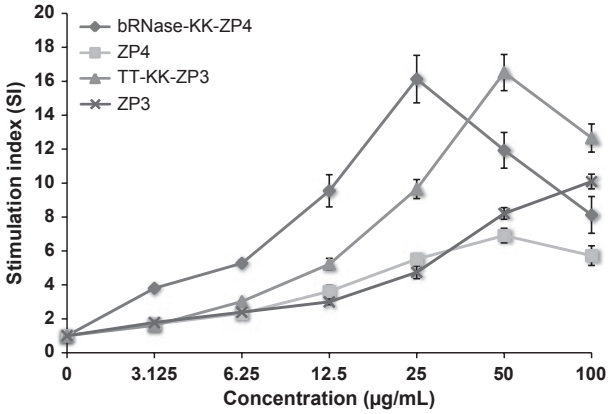

Fig. 6 *In vitro* proliferation of T cells isolated from mice immunized with recombinant porcine zona proteins. Single-cell suspension of splenocytes was prepared from mice immunized with TT-KK-ZP3 and *in vitro* stimulated with recombinant ZP3/TT-KK-ZP3 and processed for T-cell proliferation. Similarly, splenocytes isolated from mice immunized with bRNase-KK-ZP4 were stimulated with ZP4 and bRNase-KK-ZP4. The graph represents stimulation index (SI) versus concentration of recombinant protein used for *in vitro* stimulation. Data are expressed as mean ± S.E. of triplicate cultures.

Antibodies Against Recombinant Porcine Zona Proteins React with ZP of Mouse Oocytes

The immune serum samples (day 63 bleed) from mice immunized with recombinant TT-KK-ZP3 (Fig. 7b) as well as recombinant bRNase-KK-ZP4 (Fig. 7c) reacted with ZP matrix surrounding the mouse oocytes in an indirect immunofluorescence assay, whereas the serum sample from the animal immunized with adjuvant alone failed to show any reactivity (Fig. 7a). Prior incubation of the respective immune serum samples with either recombinant ZP3 (Fig. 7b′) or ZP4 (Fig. 7c′) abrogated their reactivity with the ZP of mouse oocytes.

Table III Inhibition of *in vitro* fertilization by antibodies against recombinant porcine zona proteins

Treatment groups	Number of mouse oocytes	Number of 2 cell embryos	Percent fertilization
Control	76	46	60.53
TT-KK-ZP3	60	5	8.33
bRNase-KK-ZP4	76	9	11.84

The denuded mouse oocytes were treated with serum samples (1:20 dilution) obtained from mice immunized with adjuvant only (control group), TT-KK-ZP3, and bRNase-KK-ZP4.

Antibodies Against Recombinant Porcine Zona Proteins Inhibit *In Vitro* Fertilization (IVF)

To investigate the *in vitro* contraceptive efficacy of antibodies generated against porcine recombinant zona proteins, mouse IVF assay was performed. The serum samples had no effect on either the viability or the motility of the sperm. Serum sample from control mice (immunized with adjuvant alone) showed 60.53% fertilized oocytes, while serum samples from mice immunized with TT-KK-ZP3 and bRNase-KK-ZP4 resulted in a significant reduction in fertilization of oocytes, that is, 8.33% ($P = 0.00009$) and 11.84% $P = 0.00005$), respectively (Table III).

Effect of Immunization of Female Mice with the Recombinant Porcine ZP Proteins on Fertility

Immunized mice corresponding to Experiment I/II were mated with males of proven fertility as described in *Materials and Methods* to assess *in vivo* contraceptive efficacy of the recombinant zona proteins. All animals immunized with adjuvant alone conceived with an average litter size of 5.60 ± 0.17

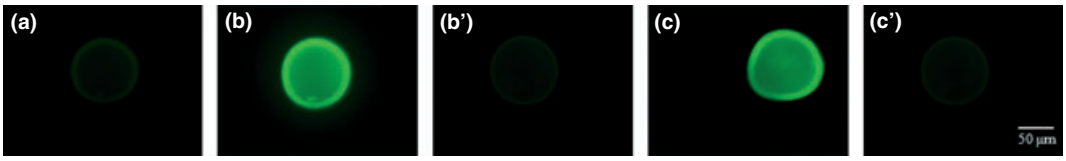

Fig. 7 Reactivity with mouse oocytes of the immune sera from mice immunized with recombinant porcine zona proteins. Mouse oocytes were incubated with 1:20 dilution of the immune serum samples (day 63 bleed) from mice immunized with either recombinant TT-KK-ZP3 or bRNase-KK-ZP4 and processed for their reactivity by an indirect immunofluorescence assay as described in *Materials and Methods*. Serum sample obtained from mouse immunized with adjuvant alone was used as negative control. Representative immunofluorescence profiles are shown. (a) Shows reactivity profile with serum sample from mouse immunized with adjuvant alone. (b and c) Reactivity profile of serum samples from TT-KK-ZP3 and bRNase-KK-ZP4-immunized mice and (b′ and c′) the same when the respective serum samples (1:20 dilution) were pre-incubated with either recombinant ZP3 or ZP4 (5 μg/mL). The scale bar represents 50 μm.

pups/animal (Experiment I) and 6.90 ± 0.31 pups/animal (Experiment II). In contrast, only one of 8 female mice immunized with recombinant ZP3 conceived (Table II). Recombinant TT-KK-ZP3 was also efficient in curtailing the fertility in the immunized animals. The average litter size ranged from 0.55 ± 0.55 (Experiment I) to 0.70 ± 0.47 (Experiment II), which was significantly lower ($P < 0.001$) than the respective control groups (Table II). Group of animals immunized with recombinant porcine ZP4 also resulted in significant sterility as compared to the control group (55.5% versus 0.0%). The litter size was also significantly reduced as compared to adjuvant control group. Further, immunization with bRNase-KK-ZP4 also resulted in significant curtailment of fertility as well as litter size as evident by two independent series of active immunization studies (Table II). Moreover, immunized animals (Experiment II) when assessed for estrus cycle did not reveal any significant effect of immunization with recombinant TT-KK-ZP3/bRNase-KK-ZP4 on cyclicity as compared to adjuvant control group (data not shown).

Mating studies with group of animals primed with porcine SIZP followed by booster with mixture of recombinant ZP3 and ZP4 proteins revealed that of 7 immunized animals, only one became pregnant (data not shown). In comparison, all the 7 female mice that were not primed with SIZP conceived (data not shown).

Immunization with Recombinant Porcine ZP Proteins Does not Lead to Ovarian Pathology

Histology of the ovaries from the PetGel A–immunized group revealed follicles at the various stages of development without any degenerative changes in the zona pellucida matrix (Fig. 8). The ovarian histology of the mice immunized with ZP3, TT-KK-ZP3, ZP4, and bRNase-KK-ZP4 assessed on day 120

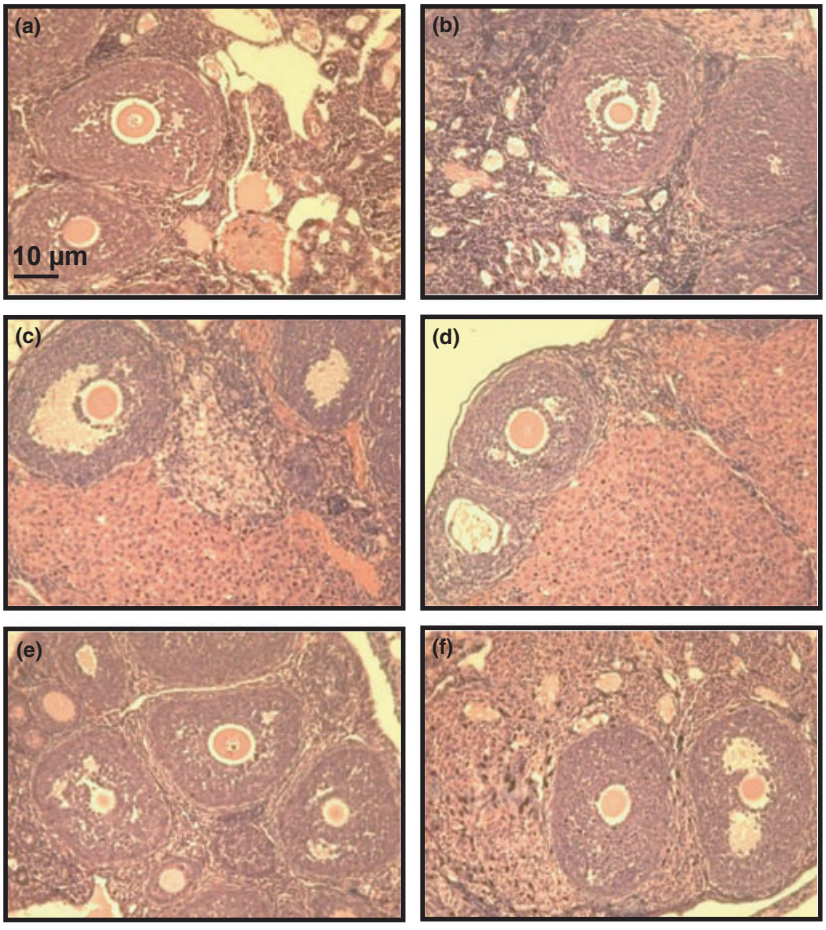

Fig. 8 Photomicrographs of ovarian sections of mice immunized with porcine ZP proteins. Ovaries were surgically removed, and 5-µm sections were cut and stained as described in *Materials and Methods*. (a and b) Ovarian section of mice from control group. (c, d, e, and f) Show representative ovarian sections of ZP3, TT-KK-ZP3, ZP4, and bRNase-KK-ZP4-immunized mice, respectively. The scale bar in (a) 10 µm, which is same for rest of the panels.

after initiation of immunization (Fig. 8) and of mice immunized with TT-KK-ZP3 and bRNase-KK-ZP4 on day 64 after immunization (data not shown) also showed normal patterns of follicle development along with the presence of corpora lutea without infiltration of lymphocytes in the ovarian follicles.

Discussion

Immunization studies with porcine zonae pellucidae, SIZP as well as purified native proteins in various animal models established the efficacy of this procedure to curtail fertility in the immunized animals.[17–24] By and large, active immunization studies employing either zonae pellucidae or SIZP revealed that infertility is invariably associated with concomitant ovarian pathology, suggesting that immunological intervention is not only at the level of sperm–egg interaction but may also involve inhibition of follicular development.[31,32] Ovarian pathology was considerably reduced when purified porcine zona proteins were used supplemented with adjuvants other than complete Freund's adjuvant (CFA)/incomplete Freund's adjuvant (IFA).[33,34] Use of synthetic peptides, devoid of oophoritogenic T-cell epitopes, corresponding to zona proteins and DNA vaccine based on mouse ZP3 has revealed that it is feasible to inhibit fertility without any ovarian pathology.[35–37]

To overcome the limitation of availability of purified native porcine zona proteins from pig oocytes and possibility of contamination by other ovarian-associated proteins, porcine ZP3 and ZP4 were cloned and expressed in *E. coli*. A His-tag was added at the N-terminus of the porcine ZP3 and ZP4 to aid in their convenient purification. Purified recombinant ZP3 revealed an apparent molecular weight of 44 kDa, which is higher than the calculated molecular weight of 36 kDa. Expression of porcine ZP3 excludes N-terminus signal peptide and C-terminus transmembrane-like domain in *E. coli*, which has also been reported previously.[38] However, the molecular weight reported was 38 kDa in SDS-PAGE. The reasons of apparently high molecular weight obtained by us are not clear at this stage. Expression of porcine ZP4 has been reported earlier using baculovirus expression system and *Pichia pastoris*.[39,40] To the best of our knowledge, expression of porcine ZP4 in *E. coli* is being reported for the first time. Surprisingly, baculovirus-expressed ZP3 and ZP4 did not bind to porcine sperm but bound to bovine sperm.[39] It is likely due to the amendment of glycosylation by insect cells (Sf9), which are similar to bovine rather than porcine zona proteins. Similarly, recombinant ZP4 has an apparent molecular weight of 54 kDa, which is also higher than the calculated molecular weight of 48 kDa. His_6-tag proteins often show a slightly retarded mobility on SDS-PAGE, and this discrepancy between the apparent and theoretical value could be explained by the same phenomenon.[41]

In addition, recombinant porcine ZP3 and ZP4 have also been expressed by incorporating promiscuous T-cell epitopes of TT and bRNase. Promiscuous T-cell epitope binds to various major histocompatibility complex (MHC) molecules and thereby likely to elicit immune response in larger proportion of the immunized outbred population. Promiscuous T-cell epitopes from a variety of antigens have a potential to provide T-cell help that can be employed to generate humoral immune response against self proteins.[42–44] This will also avoid chemical conjugation of the recombinant porcine ZP3 and ZP4 with 'carrier' protein. Dilysine linker used in the expressed recombinant proteins is the target site for lysosomal protease cathepsin B, which is an important protease in the context of MHC II antigen presentation.[45] The successful cleavage at dilysine linker site by cathepsin B ensures generation of antibodies against the fusion partners separately, concurrently avoiding the generation of antibodies against the new epitope that may result due to the fusion of two fragments via linker.[46] The proteins expressed in a *lon*- and *ompT*-protease-deficient *E. coli* strain, BL21[DE3] pLysS, have permitted the reduction/elimination of proteolytic degradation of the expressed proteins.

Immunization studies in female mice with the recombinant porcine ZP3 and ZP4 along with PetGel A revealed these to be immunogenic as evident by generation of both humoral- and cell-mediated immune responses. The polyclonal antibodies thus generated reacted with the respective recombinant proteins both in ELISA and in Western blot. Studies with PetGel A have shown that it is a safe adjuvant and can be used for vaccines meant for animal application.[29] Incorporation of T-cell epitope of TT in recombinant ZP3 (TT-KK-ZP3) as well as bRNase in recombinant ZP4 (bRNase-KK-ZP4) led to generation of higher antibody titers as compared to the respective protein devoid of T-cell epitope (Table II). Recombinant TT-KK-ZP3 and bRNase-KK-ZP4 also elicited higher T-cell proliferation as compared to

recombinant ZP3/ZP4 thereby confirming that the promiscuous T-cell epitope of TT, and bRNase are also contributing to T-cell proliferation. The polyclonal antibodies generated in mice against porcine ZP3 also reacted with mouse oocyte ZP, which may be due to 68% sequence identity of the mouse ZP3 with the porcine ZP3 at the amino acid level. Although *Zp4* is a pseudogene in mice[5], the reactivity of mouse polyclonal antibodies against recombinant porcine ZP4 may be due to its cross-reactivity with the mouse ZP1 that has a sequence identity of 50% with porcine ZP4 at amino acid level. The reactivity of the antibodies against recombinant porcine ZP3/ZP4 to the mouse oocyte ZP is specific as it can be inhibited by prior incubation of the immune serum samples with the respective recombinant proteins. Interestingly, antibodies generated against porcine SIZP showed reactivity with recombinant porcine ZP3 and ZP4 (Fig. 5). Further, recombinant proteins can act as booster in animals primed with porcine SIZP and increased the antibody titers against SIZP (Fig. 5). These observations suggest that the recombinant porcine ZP3 and ZP4 described herein may be useful in the efforts for the population management of wild horses and white-tailed deers by currently used contraceptive vaccine based on native porcine ZP.[17,18] These may act as good candidates to boost the immune responses generated by native proteins.

Antibodies against the native porcine zona proteins have been shown to inhibit *in vitro* sperm–egg binding in various animal models.[47–49] The antibodies elicited in female mice by immunization with recombinant porcine TT-KK-ZP3 as well as bRNase-KK-ZP4 also inhibited *in vitro* fertilization using mouse oocytes and sperm, suggesting their contraceptive potential. Further, immunization with recombinant porcine ZP3 and ZP4 also led to curtailment of fertility in the immunized mice. ZP3 has been proposed as primary sperm receptor in mice.[7] The cross-reactivity of antibodies generated against recombinant porcine ZP3 with mouse ZP3 is likely to be responsible for the curtailment of fertility. The probable cross-reactivity of antibodies generated against recombinant porcine ZP4 with mouse ZP1 may explain the curtailment of fertility in the recombinant ZP4–immunized groups. In mouse model, ZP1 has no direct functional role during fertilization and has been proposed to primarily act as cross-linker of filaments formed by ZP2-ZP3 heterodimers.[13] At this stage, it is a conjecture that antibodies against recombinant porcine ZP4 cross-react with mouse ZP1 and bring out the contraceptive effect by steric hindrance. This may explain lower contraceptive efficacy of recombinant porcine ZP4 as compared to ZP3 (Table II). Recombinant porcine ZP4 may have better contraceptive efficacy in mammalian species that have ZP4 instead of ZP1 such as dogs, pigs as compared to mice, which remains to be investigated.

These preliminary active immunization studies with the recombinant porcine zona proteins did not reveal any apparent ovarian pathology. However, further long-term active immunization studies should be undertaken that not only include ovarian histopathology but also various endocrine parameters to determine the safety of this procedure. Normal ovarian histology observed in the present study may be due to the following reasons: (i) recombinant proteins are devoid of other ovarian-associated proteins, (ii) the use of PetGel A as adjuvant instead of CFA/IFA: it has been shown that animals immunized with the zona proteins along with CFA showed ovarian pathology as compared to animals immunized with same preparation of zona protein in alum[50], and it has been demonstrated that Montanide™gel can be used safely with various antigens and in several animal species[29], and (iii) timing of observations: 64 or 120 days after initiating immunization may be a short period to bring about adverse effects on ovaries by antibodies generated against recombinant porcine zona proteins.

To conclude, we report expression of recombinant porcine ZP3 and ZP4 in *E. coli*, which are immunogenic and competent to curtail fertility in murine model. The availability of these recombinant proteins will facilitate our efforts to develop porcine ZP–based contraceptive vaccine for wildlife population management.

Acknowledgments

We would like to sincerely acknowledge and appreciate the encouragement and guidance from Dr. Andrew N Rowan, Executive Vice President-Operation and Chief Scientific Officer, The Humane Society of the United States, Washington, DC. The financial support from Humane Society of United States, Washington, DC, and Tata Innovation Fellowship awarded to SKG by Department of Biotechnology, Government of India, is gratefully acknowledged. PetGel A was kindly provided by Dr. V. A. Srinivasan, Indian Immunological Limited, Hyderabad, India.

References

1 Gupta SK, Bansal P: Vaccines for immunological control of fertility. *Reprod Med Biol* 2010; 9:61–71.

2 Gupta SK, Srinivasan VA, Suman P, Rajan S, Nagendrakumar SB, Gupta N, Shrestha A, Joshi P, Panda AK: Contraceptive vaccines based on the zona pellucida glycoproteins for dogs and other wildlife population management. *Am J Reprod Immunol* 2011; 66:51–62.

3 Bleil JD, Wassarman PM: Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Dev Biol* 1980; 76:185–202.

4 Hedrick JL, Wardrip NJ: On the macromolecular composition of the zona pellucida from porcine oocytes. *Dev Biol* 1987; 121:478–488.

5 Goudet G, Mugnier S, Callebaut I, Monget P: Phylogenetic analysis and identification of pseudogenes reveal a progressive loss of zona pellucida genes during evolution of vertebrates. *Biol Reprod* 2008; 78:796–806.

6 Lefievre L, Conner SJ, Salpekar A, Olufowobi O, Ashton P, Pavlovic B, Lenton W, Afnan M, Brewis IA, Monk M, Hughes DC, Barratt CL: Four zona pellucida glycoproteins are expressed in the human. *Hum Reprod* 2004; 19:1580–1586.

7 Bleil JD, Wassarman PM: Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell* 1980; 20:873–882.

8 Beebe SJ, Leyton L, Burks D, Ishikawa M, Fuerst T, Dean J, Saling P: Recombinant mouse ZP3 inhibits sperm binding and induces the acrosome reaction. *Dev Biol* 1992; 151:48–54.

9 Chakravarty S, Kadunganattil S, Gupta SK: Baculovirus expressed recombinant human zona pellucida glycoprotein-B induces acrosomal exocytosis in capacitated spermatozoa in addition to zona pellucida glycoprotein-C. *Mol Hum Reprod* 2005; 11:365–372.

10 Chiu PC, Wong BS, Chung MK, Lam KKW, Pang RT, Lee KF, Sumitro SB, Gupta SK, Yeung WS: Effects of native human zona pellucida glycoprotein-3 and -4 on acrosome reaction and zona pellucida binding of human spermatozoa. *Biol Reprod* 2008; 79:869–877.

11 Yurewicz EC, Pack BA, Armant DR, Sacco AG: Porcine zona pellucida ZP3 alpha glycoprotein mediates binding of the biotin-labeled M(r) 55,000 family (ZP3) to boar sperm membrane vesicles. *Mol Reprod Dev* 1993; 36:382–389.

12 Yurewicz EC, Sacco AG, Gupta SK, Xu N, Gage DA: Hetero-oligomerization-dependent binding of pig oocyte zona pellucida glycoproteins ZPB and ZPC to boar sperm membrane vesicles. *J Biol Chem* 1998; 273:7488–7494.

13 Greeve JM, Wassarman PM: Mouse egg extracellular coat is a matrix of interconnected filaments possessing a structural repeat. *J Mol Biol* 1985; 181:253–264.

14 Ganguly A, Bukovsky A, Sharma RK, Bansal P, Bhandari B, Gupta SK: In humans, zona pellucida glycoprotein-1 binds to spermatozoa and induces acrosomal exocytosis. *Hum Reprod* 2010; 25:1643–1656.

15 Bleil JD, Greve JM, Wassarman PM: Identification of a secondary sperm receptor in the mouse egg zona pellucida: role in maintenance of binding of acrosome-reacted sperm to eggs. *Dev Biol* 1988; 128:376–385.

16 Baibakov B, Boggs NA, Yauger B, Baibakov G, Dean J: Human sperm bind to the N-terminal domain of ZP2 in humanized zonae pellucidae in transgenic mice. *J Cell Biol* 2012; 197:897–905.

17 Kirkpatrick JF, Liu IKM, Turner JW Jr: Remotely-delivered immunocontraception in feral horses. *Wildl Soc Bull* 1990; 18:326–330.

18 McShea WJ, Monfort SL, Hakim S, Kirkpatrick JF, Liu IKM, Turner JW, Chassy L, Munson L: Immunocontraceptive efficacy and the impact of contraception on the reproductive behaviors of white-tailed deer. *J Wildl Manage* 1997; 61:560–569.

19 Fayrer-Hosken RA, Grobler D, Van Altena JJ, Bertschinger HJ, Kirkpatrick JF: Immunocontraception of African elephants. *Nature* 2000; 407:149.

20 Kitchener AL, Kay DJ, Walters B, Menkhorst P, McCartney CA, Buist JA, Mate KE, Rodger JC: The immune response and fertility of koalas (*Phascolarctos cinereus*) immunized with porcine zonae pellucidae or recombinant brushtail possum ZP3 protein. *J Reprod Immunol* 2009; 82:40–47.

21 Brown RG, Bowen WD, Eddington JD, Kimmins WC, Mezei M, Parsons JL, Pohajdak B: Evidence for a long-lasting single administration contraceptive vaccine in wild grey seals. *J Reprod Immunol* 1997; 35:43–51.

22 Kirkpatrick JF, Turner A: Reversibility of action and safety during pregnancy of immunization against porcine zona pellucida in wild mares (*Equus caballus*). *Reprod Suppl* 2002; 60:197–202.

23 Kirkpatrick JF, Turner A: Absence of effects from immunocontraception on seasonal birth patterns and foal survival among barrier island wild horses. *J Appl Anim Welf Sci* 2003; 6:301–308.

24 Curtis PD, Richmond ME, Miller LA, Quimby FW: Pathophysiology of white-tailed deer vaccinated with porcine zona pellucida immunocontraceptive. *Vaccine* 2007; 25:4623–4630.

25 Kaul P, Afzalpurkar A, Gupta SK: Expression of bonnet monkey (*Macaca radiata*) zona pellucida glycoprotein-3 (ZP3) in a prokaryotic system and its immunogenicity. *Mol Reprod Dev* 1997; 47:140–147.

26 Gupta M, Gupta SK: Characterization of antigenic determinants on porcine zona pellucida-3β (ZP3β) glycoprotein by monoclonal antibodies. *Indian J Exp Biol* 1994; 32:376–381.

27 Afzalpurkar A, Gupta SK: Identification of epitopes of monoclonal antibodies to porcine zona pellucida 3 beta glycoprotein, a homologue of the mouse/human sperm receptor. *Am J Reprod Immunol* 1997; 38:26–32.

28 Chadha K, Gupta M, Gupta SK: Generation and characterization of monoclonal antibodies to porcine zona pellucida -3 alpha glycoprotein. *Indian J Exp Biol* 1993; 31:583–586.

29 Parker R, Deville S, Dupuis L, Bertrand F, Aucouturier J: Adjuvant formulation for veterinary vaccines: Montanide™Gel safety profile. *Procedia Vaccinol* 2009; 1:140–147.

30 Vialle R, Dupuis L, Deville S, Bertrand F, Gaucheron J, Aucouturier J: Microgel particulate adjuvant: characterisation and mechanisms of action. *Procedia Vaccinol* 2010; 2:12–16.

31 Skinner SM, Mills T, Kirchik HJ, Dunbar BS: Immunization with zona pellucida proteins results in abnormal ovarian follicular differentiation and inhibition of gonadotropin-induced steroid secretion. *Endocrinology* 1984; 115:2418–2432.

32 Mahi-Brown CA, Huang TT Jr: Yanagimachi R: infertility in bitches induced by active immunization with porcine zonae pellucidae. *J Exp Zool* 1982; 222:89–95.

33 Sacco AG, Pierce DL, Subramanian MG, Yurewicz EC, Dukelow WR: Ovaries remain functional in squirrel monkeys (*Saimiri sciureaus*) immunized with porcine zona pellucida 55,000 macromolecule. *Biol Reprod* 1987; 36:481–490.

34 Bagavant H, Thillai-Koothan P, Sharma MG, Talwar GP, Gupta SK: Antifertility effects of porcine zona pellucida-3 immunization using permissible adjuvants in female bonnet monkeys (*Macaca radiata*): reversibility, effect on follicular development and hormonal profiles. *J Reprod Fertil* 1994; 102:17–25.

35 Lou Y, Ang J, Thai H, McElveen F, Tung KS: A zona pellucida 3 peptide vaccine induces antibodies and reversible infertility without ovarian pathology. *J Immunol* 1995; 155:2715–2420.

36 Kaul R, Sivapurapu N, Afzalpurkar A, Srikanth V, Govind CK, Gupta SK: Immunocontraceptive potential of recombinant bonnet monkey (*Macaca radiata*) zona pellucida glycoprotein-C expressed in *Escherichia coli* and its corresponding synthetic peptide. *Reprod Biomed Online* 2001; 2:33–39.

37 Li J, Jin H, Zhang A, Li Y, Wang B, Zhang F: Enhanced contraceptive response by co-immunization of DNA and protein vaccines encoding the mouse zona pellucida 3 with minimal oophoritis in mouse ovary. *J Gene Med* 2007; 9:1095–1103.

38 Xu WX, Qiu DY, Xiong Y, Sheng QX, Gu SH, Cao GT, Ying K, Xie Y: Study on the expression of pig zona pellucida-3 beta excluding N-terminus signal peptide and C-terminus transmembrance-like domain in *Escherichia coli*. *Sheng Wu Gong Cheng Xue Bao* 2001; 17: 16–19.

39 Yonezawa N, Kudo K, Terauchi H, Kanai S, Yoda N, Tanokura M, Ito K, Miura K, Katsumata T, Nakano M: Recombinant porcine zona pellucida glycoproteins expressed in Sf9 cells bind to bovine sperm but not to porcine sperm. *J Biol Chem* 2005; 280:20189–20196.

40 Gao YR, Pan SP, Xie QX, Xiao LJ, Ke Q, Li WX, Shi WW: Secretory expression of recombinant porcine zona pellucida glycoprotein-3 alpha (rpZP3alpha) in *Pichia pastoris*. *Sheng Wu Gong Cheng Xue Bao* 2006; 22:499–503.

41 The QIAexpressionist: The High Level Expression and Protein Purification System. QIAGEN Gmbh, Hilden, Germany, 1992, pp 1–70.

42 Ho PC, Mutch DA, Winkel KD, Saul AJ, Jones GL, Doran TJ, Rzepczyk CM: Identification of two 'promiscuous' T cell epitopes from tetanus toxin. *Eur J Immunol* 1990; 20:477–483.

43 Lairmore MD, DiGeorge AM, Conrad SF, Trevino AV, Lal RB, Kaumaya PT: Human T-lymphotropic virus type 1 peptides in chimeric and multivalent constructs with promiscuous T cell epitopes enhance immunogenicity and overcome genetic restriction. *J Virol* 1995; 69:6077–6089.

44 O'Hern PA, Liang ZG, Bambra CS, Goldberg E: Colinear synthesis of an antigen-specific B-cell epitope with a 'promiscuous' tetanus toxin T-cell epitope: a synthetic peptide immunocontraceptive. *Vaccine* 1997; 16:1761–1766.

45 Lennon-Dum'enil AM, Bakker AH, Wolf-Bryant P, Ploegh HL, Lagaudri'ere-Gesbert C: A closer look at proteolysis and MHC class-II restricted antigen presentation. *Curr Opin Immunol* 2002; 14:15–21.

46 Oishi Y, Onozuka A, Kato H, Shimura N, Imai S, Nisizawa T: The effect of amino acid spacers on the antigenicity of dimeric peptide inducing cross-reacting antibodies to a cell surface protein antigen of *Streptococcus mutans*. *Oral Microbiol Immunol* 2001; 16:40–44.

47 Sacco AG, Yurewicz EC, Subramanian MG, DeMayo FJ: Zona pellucida composition: species cross reactivity and contraceptive potential of the antiserum to a purified pig zona antigen (PPZA). *Biol Reprod* 1981; 25:997–1008.

48 Koyoma K, Hasegawa A, Inoue M, Isojima S: Blocking of sperm zona interaction by monoclonal antibodies to a glycoprotein family (ZP4) of porcine zona pellucida. *Biol Reprod* 1991; 45:727–735.

49 Bagavant H, Yurewicz EC, Sacco AG, Talwar GP, Gupta SK: Block in porcine gamete interaction by polyclonal antibodies to a pig ZP3β fragment having partial sequence homology to human ZP3. *J Reprod Immunol* 1993; 25:277–283.

50 Upadhyay SN, Thillai-Koothan P, Bamezai A, Jayaraman S, Talwar GP: Role of adjuvants in inhibitory influence of immunization with porcine zona pellucida antigen (ZP3) on ovarian folliculogenesis in bonnet monkeys: a morphological study. *Biol Reprod* 1989; 41: 665–673.