Evaluation of the immunocontraceptive potential of *Escherichia coli*-expressed recombinant dog ZP2 and ZP3 in a homologous animal model

N. Srivastava¹, R. Santhanam¹, P. Sheela¹, S. Mukund¹,
S. S. Thakral², B. S. Malik² and S. K. Gupta^{1*}

¹Gamete Antigen Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi-110067, India; and ²Central Military Veterinary Laboratory, Merrut Cantt, UP, India

Dog zona pellucida glycoprotein 2 (dZP2), excluding the N-terminal signal sequence and the C-terminal transmembrane-like domain, was cloned and expressed as a polyhistidine fusion protein in *Escherichia coli* to evaluate the immunocontraceptive efficacy of ZP glycoproteins. The recombinant dZP2 (rec-dZP2) revealed a 70 kDa band corresponding to the full length transcript, as well as several low molecular mass fragments in western blot analysis. In addition to rec-dZP2, *E. coli* expressed recombinant dog ZP glycoprotein 3 (rec-dZP3), which has also been evaluated for its efficacy to block fertility in a homologous system. Three groups of female dogs ($n = 4$ per group) were immunized with rec-dZP2 conjugated to diphtheria toxoid (rec-dZP2–DT), rec-dZP3 conjugated to DT (rec-dZP3–DT) and DT alone. Immunization of female dogs with rec-dZP2–DT and rec-dZP3–DT led to

generation of antibodies against the respective ZP proteins as well as to DT. Subsequent to mating, the four female dogs immunized with rec-dZP2–DT all conceived, which is indicative of failure of the anti-rec-dZP2 antibodies to block fertility. In the group of dogs immunized with rec-dZP3–DT, three of four animals did not conceive when mated with males of proven fertility. The block in fertility was associated with anti-dZP3 antibody titres. Ovarian histopathology revealed that the block in fertility in the group immunized with rec-dZP3–DT is probably manifested by inhibition in the development of follicles and is due to atretic changes in the zona pellucida. These results, although preliminary, indicate that immunization with dZP3 may be a feasible proposition to control dog populations provided that adequate antibody titres are achieved.

Introduction

The zona pellucida (ZP) glycoproteins play a vital role in mammalian fertilization. In most species, the zona pellucida is composed of at least three biochemically distinct glycoproteins: ZP1, ZP2 and ZP3. In the mouse model, spermatozoa initially bind to ZP3, which triggers the acrosome reaction (Bleil and Wassarman, 1983). Subsequent to the acrosome reaction, ZP2 acts as the secondary receptor and helps in the maintenance of binding of acrosome-reacted spermatozoa to the egg (Bleil *et al.*, 1988). ZP1 has been postulated to cross-link the ZP2–ZP3 heterodimer. ZP2 is proteolytically cleaved after fusion of the sperm membrane with the oolemma and this modification, together with presumed changes in ZP3, is postulated to play an important role in the post-fertilization block to polyspermy (Moller and Wassarman, 1989). This critical role during fertilization has made the ZP glycoproteins potential candidate antigens for immunocontraception.

Antibodies generated against the ZP glycoproteins

purified from a given species show variable degrees of immunological crossreactivity with the ZP glycoproteins from other species (Sacco *et al.*, 1981). The immunological crossreactivity among ZP glycoproteins from different species is a result of a variable degree of amino acid sequence homology and this property has allowed the possibility of heterologous immunization. Initially, pig ZP glycoproteins were used because of the availability of a large number of pig ovaries; the immunological cross-reactivity of antibodies generated in this way with zonae pellucidae from various species was demonstrated. Studies in several animal models have demonstrated that the active immunization of female subjects with ZP glycoproteins leads to a block of fertility (Skinner *et al.*, 1984; Dunbar *et al.*, 1989; Sacco *et al.*, 1989; Jones *et al.*, 1992; Paterson *et al.*, 1992; Bagavant *et al.*, 1994).

In several countries, including India, the increasing population of street dogs is a significant problem. It is also associated with a high incidence of rabies. It may be worth exploring the potential of ZP glycoproteins as immunocontraceptive vaccines to control the canine population. Instead of pig ZP glycoproteins, immunization with dog zona proteins *per se* may generate optimum immune responses with better recognition of native dog zona pellicu-

*Correspondence
Email: skgupta@nii.res.in

cida. This effort will demand the availability of large amounts of purified dog zona proteins without contaminants of any other ovarian-associated proteins.

In the present study, the construction of a vector for high expression and single-step purification of recombinant dog ZP2 (rec-dZP2) in *Escherichia coli* is described. The efficacy of rec-dZP2 conjugated to diphtheria toxoid (DT) as a carrier for regulation of fertility was evaluated in female dogs. In addition, recombinant dog ZP3 (rec-dZP3) expressed in *E. coli* as described by Santhanam *et al.* (1998) and conjugated to DT was evaluated for its immunocontraceptive potential. Animals were assessed for antibody response, progesterone concentration, vaginal cytology and conception.

Materials and Methods

Cloning of dZP2 cDNA in a prokaryotic expression vector

A female dog (aged 1 year) reared at the Animal Facility, National Institute of Immunology, New Delhi, was ovariectomized as per the guidelines and approval of the Institutional Animals Ethics Committee, and her ovaries were snap-frozen in liquid nitrogen. Total RNA was isolated from the frozen ovaries and the poly(A)⁺ fraction was purified using the poly A tract mRNA isolation system (Promega, Madison, WI). cDNA was synthesized from the purified poly(A)⁺ RNA using a Riboclone cDNA synthesis system (Promega). Attempts to directly amplify the full length dZP2 cDNA by PCR failed. Hence, dZP2 was amplified as two fragments by PCR and subsequently assembled as shown (Fig. 1a). The dF1ZP2 fragment (305–1204 bp, excluding the signal sequence) was amplified by PCR using a forward primer with a *SacI* restriction site and a reverse primer without a restriction site. The dF2ZP2 fragment (1181–2110 bp, excluding the transmembrane-like domain following the putative furin cleavage site) was amplified by PCR using a forward primer without a restriction site and a reverse primer with a *KpnI* restriction site. Primers were designed based on the published sequence of dZP2 (Harris *et al.*, 1994). The dZP2 cDNA was assembled from the purified fragments by a second PCR using *Vent* DNA polymerase in a 50 µl reaction. The cycling conditions for PCR were: (i) one cycle of 94°C for 2 min, 50°C for 2 min and 72°C for 2 min; (ii) addition of 1 µmol l⁻¹ of each of the forward (5'-CCGAGCTCACTTCAGTGAATC-AGTA-3' incorporating a *SacI* restriction site) and the reverse (5'-CGGGGTACCGGATGATACAGGGCAAGT-3' incorporating a *KpnI* restriction site) primers; (iii) 30 cycles of 94°C for 2 min, 54°C for 2 min and 72°C for 2 min; and (iv) a final extension at 72°C for 15 min. The amplified and purified fusion product was initially cloned into the pCR Script SK(+) cloning vector. The positive pCR Script clone was selected by restriction digestion analysis with *SacI* and *KpnI*, and the purified dZP2 fragment was further cloned in-frame downstream of the (His)₆ tag under T5 promoter–*lac* operator control in the pQE-30 expression vector (QIA

Fig. 1. (a) Schematic representation of the construction of recombinant dog zona pellucida glycoprotein 2 (rec-dZP2) cDNA in pQE-30 expression vector and immunoblot analysis of transformed bacterial cell lysates. The cDNA corresponding to dZP2 (nucleotides 305–2110), excluding the signal sequence and transmembrane-like domain, was assembled by PCR using previous PCR-amplified fragments corresponding to F1 (nucleotides 305–1204) and F2 (nucleotides 1181–2110) as templates. The *SacI*–*KpnI*-digested 1806 bp dZP2 fragment from the pCR Script SK (+) vector was cloned in-frame downstream of His₆ under the phage T5 (pT5) promoter in the pQE-30 vector. *Escherichia coli* SG13009 [pREP4] transformed with the pQE-dZP2 plasmid was induced with 1 mmol isopropyl-β-D-thiogalactopyranoside (IPTG) l⁻¹ and processed for immunoblotting. (b) M: molecular mass marker (kDa); lanes 1 and 2: uninduced and induced wild-type cells; lanes 3 and 4: uninduced and induced SG13009 [pREP4] cells harbouring pQE-dZP2 plasmid.

Express; Qiagen GmbH, Hilden). The nucleotide sequence of pQE-dZP2 was confirmed using a forward sequencing primer (5'-GGCGTATCACGAGGCCCTTTCG-3') and a reverse sequencing primer (5'-CATTACTGGATCTATCA-ACAGG-3') corresponding to pQE-30 vector and the internal primers.

Expression of rec-dZP2 in *E. coli*

The SG13009[pREP4] strain of *E. coli* was transformed with pQE-dZP2 plasmid. A positive clone was isolated and

checked for expression of recombinant protein by western blot analysis. In brief, a single transformed colony was inoculated into 1 ml Luria broth (LB; Difco Laboratories, Detroit, MI) containing 100 µg ampicillin ml⁻¹ and 25 µg kanamycin ml⁻¹, and was grown overnight at 37°C. The next day, the cells were subcultured (1:100 dilution) into fresh LB (1 ml) and grown at 37°C until the A₆₀₀ reached a value of approximately 0.5–0.6. The culture was induced with an optimized concentration of 1.0 mmol isopropyl-β-D-thiogalactopyranoside l⁻¹ (IPTG; Sigma Chemical Co., St Louis, MO) for 2 h. The cells were collected by centrifugation at 1000 g for 2 min and the pellet was stored at –70°C until used.

SDS-PAGE and immunoblotting

The cell pellet obtained from 1 ml culture was solubilized by boiling for 5 min in 100 µl double-strength sample buffer (0.0625 mol Tris l⁻¹, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.001% (w/v) bromophenol blue). The proteins were resolved by 0.1% (w/v) SDS–10% (w/v) polyacrylamide gel electrophoresis (Laemmli, 1970). For immunoblotting, the proteins were transferred by electrophoresis to 0.45 µm nitrocellulose membrane (BioRad, Hercules, CA) overnight at a constant voltage of 15V in Tris glycine buffer with 20% (v/v) methanol (Towbin *et al.*, 1979). Blots were developed with 1:1000 dilution of polyclonal antibodies generated in rabbits against the pig solubilized zona pellucida (pSIZP) as described by Kaul *et al.* (1997).

Purification of rec-dZP2 and rec-dZP3

For purification of rec-dZP2 fusion protein from the inclusion bodies, SG13009[pREP4] cells transformed with the pQE-dZP2 plasmid were grown (A₆₀₀ = 0.7) at shake flask level (250 ml culture flask, total volume 2 l) and induced with 1 mmol IPTG l⁻¹ for 2 h. The cells were harvested by centrifugation at 1500 g for 30 min at 4°C and the pellet was stored at –70°C until required. The cell pellet was sonicated in sonication buffer (50 mmol phosphate buffer l⁻¹, 300 mmol NaCl l⁻¹, pH 7.8) and inclusion bodies were solubilized in buffer A (6 mol guanidine hydrochloride l⁻¹, 100 mmol NaH₂PO₄ l⁻¹, 10 mmol Tris l⁻¹, pH 8.0; 5 ml buffer A/g inclusion bodies). The total cell lysate was centrifuged at 10 000 g for 5 min at 4°C and the supernatant containing the recombinant fusion protein was purified using nickel–nitrilotriacetic acid (Ni–NTA) resin (Qiagen GmbH) as described by Kaul *et al.* (1997). The eluted fractions, showing the presence of rec-dZP2 in western blot analysis, were concentrated in an Amicon concentrator using a YM30 membrane (Amicon, Lexington, MA) and dialysed against 100 mmol phosphate buffer l⁻¹, pH 7.4, containing 4 mol urea l⁻¹. The purified recombinant protein was quantified by bicinchoninic acid (Sigma Chemical Co.).

The cDNA corresponding to dZP3, excluding the N-terminal signal sequence and the C-terminus transmembrane-like domain, was cloned in pQE-30 vector and the

recombinant protein was expressed as His₆-fusion protein in *E. coli* as described by Santhanam *et al.* (1998). The rec-dZP3 was purified essentially as described for rec-dZP2 and analysed by western blot analysis using murine monoclonal antibody (MA-451) generated against pig ZP3β (a homologue of dZP3) and immunologically crossreactive with dog zona pellucida as described by Santhanam *et al.* (1998).

Conjugation of rec-dZP2 and rec-dZP3 to DT

Rec-dZP2 (10 mg) was conjugated to 8.0 mg DT using the 'one-step' glutaraldehyde coupling procedure at a molar ratio of 1:1. In brief, conjugation was performed in 100 mmol phosphate buffer l⁻¹, pH 7.4 with 4 mol urea l⁻¹ using 0.1% (v/v) glutaraldehyde, overnight at 4°C with gentle end-to-end mixing. Unreacted sites were blocked by incubation with 100 mmol lysine l⁻¹ for 3 h at room temperature. The conjugate was dialysed against PBS (10 mmol phosphate buffer l⁻¹, 150 mmol NaCl l⁻¹, pH 7.4) containing 0.3 mol urea l⁻¹. The same coupling procedure was used to conjugate 10.0 mg rec-dZP3 to 6.0 mg DT (molar ratio 2:1).

Immunization of rabbits with rec-dZP2–DT and reactivity of the immune sera with dog zona pellucida

Two female New Zealand white rabbits (Small Experimental Animal Facility, National Institute of Immunology, New Delhi) were immunized intradermally at multiple sites with 250 µg rec-dZP2–DT emulsified in complete Freund's adjuvant (CFA). The animals were given two i.m. booster injections at 4 week intervals with an equivalent amount of rec-dZP2–DT in incomplete Freund's adjuvant (IFA). Blood was collected from the immunized animals through the ear vein before initiation of immunization and at 2 weeks after the second booster. The reactivity of the pre-immune and immune serum samples with dog zona pellucida was assessed by an indirect immunofluorescence assay on dog ovarian cryo-sections essentially as described by Santhanam *et al.* (1998).

Immunization of female dogs

The female dogs (*n* = 12; aged 3–5 years) used in the present study were reared at the Central Military Veterinary Laboratory (Meerut Cantt, Meerut, India). Active immunization studies were initiated according to the guidelines and with the approval of the Institutional Animals Ethics Committee. The animals were divided randomly into three groups (*n* = 4 dogs per group). The animals were immunized i.m. at two sites with rec-dZP2–DT (dogs 6, 7, 11 and 12; equivalent to 250 µg rec-dZP2 per animal), rec-dZP3–DT (dogs 1, 3, 4 and 5; equivalent to 250 µg rec-dZP3 per animal) or DT (dogs 2, 8, 9 and 10; equivalent to 250 µg DT per animal), emulsified in Squalene (Sigma Chemical Co.) and Arlacel-A (Sigma Chemical Co.) in a 4:1 ratio. Sodium phthalyl derivative of lipopolysaccharide (1 mg; SPLPS), prepared as described by Elin *et al.* (1981), was also included in the first injection as an additional

adjuvant. Primary immunization of dogs comprised three injections of the respective proteins at monthly intervals. Blood samples (2–3 ml) were collected before and at day 15 after each injection from the ante-cubital vein for determination of progesterone concentrations and antibody titres. The dogs were subsequently given booster injections with the respective immunogens (equivalent amount to first injection) to maintain the desired antibody titre until the dogs entered oestrus. The immunized female dogs were kept for 3 days with males of proven fertility for mating at day 5 after pro-oestrous bleeding. Bleeding at the time of pro-oestrus was associated with vaginal swelling and the ability of female dogs to attract the male dogs. In addition, vaginal smears were taken and examined for the presence of cornified epithelial cells. Rates of conception, serum progesterone concentrations and antibody titres were recorded for the female dogs.

ELISA

The microtitre plates were coated with optimized concentrations of rec-dZP2 (250 ng per well), rec-dZP3 (250 ng per well) and DT (500 ng per well) in 50 mmol PBS I⁻, pH 7.4 at 37°C for 1 h and incubated overnight at 4°C. The plates were blocked with 1% (w/v) BSA in PBS and processed for determination of antibody titres as described by Santhanam *et al.* (1998). For each serum sample tested, a reciprocal of the serum dilution giving an absorbance of 1.0 was calculated by regression analysis and is represented as antibody units (AU). One serum sample from each assay was used in the next assay as an internal control. The inter-assay coefficient of variation was < 10%.

Radioimmunoassay for progesterone

Serum progesterone concentration was determined by radioimmunoassay using the WHO matched assay reagent programme as described by Sufi *et al.* (1999).

Ovarian histology

Animals immunized with rec-dZP2-DT (dogs 7 and 12), rec-dZP3-DT (dogs 1, 3 and 5) or DT (dogs 2, 8 and 9) were administered with xylazine and ketamine hydrochloride (2 mg kg⁻¹ body weight) i.v. on day 380 after the initiation of immunization, as general anaesthesia. The ovaries were surgically removed, fixed in buffered 10% (v/v) formalin for 48 h and processed for paraffin wax sections. The sections were stained with haematoxylin and eosin using standard protocols. For each ovary, six to eight non-adjacent sections were chosen randomly and examined under a light microscope for ovarian pathology with respect to follicular development, cystic changes or infiltration by lymphocytes.

Results

Expression and purification of rec-dZP2 and rec-dZP3

The dZP2 cDNA (305–2110 bp) encoding a polypeptide of 602 amino acids, excluding the signal sequence and

Fig. 2. Electrophoretic analysis (SDS-PAGE) and western blot analysis of purified recombinant dog zona pellucida glycoprotein 2 (rec-dZP2) and its conjugate with diphtheria toxoid (rec-dZP2-DT). The *Escherichia coli*-expressed rec-dZP2 was purified on a nickel-nitrilotriacetic acid agarose affinity column. The purified recombinant protein was conjugated to DT using 0.1% (v/v) glutaraldehyde as coupling agent. Left panel: M: molecular mass markers (kDa); lane 1: purified rec-dZP2. Right panel: M: molecular mass markers (kDa); lane 1: purified rec-dZP2; lane 2: rec-dZP2-DT.

transmembrane-like domain, was assembled by PCR and cloned in-frame under the control of T5 promoter and *lac* operator control in the pQE-30 expression vector. The nucleotide sequence of dZP2 revealed three changes (C to G, G to C and C to T at positions 1079, 1080 and 1087, respectively) compared with the previously published dZP2 sequence (Harris *et al.*, 1994). These changes in the sequence led to one change in the amino acid sequence (arginine to alanine at position 292). The numbering of the nucleotides and amino acids is as for the dZP2 precursor protein.

SG13009[pREP4] cells were transformed with the recombinant plasmid. The typical immunoblot result of one of the transformed clones is shown (Fig. 1b). The rec-dZP2 protein had an apparent molecular mass of 70 kDa. In addition, several low molecular mass fragments were also observed. Optimum expression of rec-dZP2 was observed when the cells were grown for 2 h with 1 mmol IPTG I⁻ (data not shown). The expression of rec-dZP2 was tightly regulated, as expression was not observed in the non-induced transformed cells. Cellular localization studies revealed that the rec-dZP2 was present in the inclusion bodies and was not secreted in the periplasm (data not shown). The SDS-PAGE profile of Ni-NTA-purified rec-dZP2 revealed that, in addition to the 70 kDa band corresponding to the full length transcript, three major bands of 60, 35 and 25 kDa and two minor bands of 65 and 31 kDa were also observed (Fig. 2, left panel, lane 1). The 25 kDa band, as observed in SDS-PAGE, did not react in western blot analysis (Fig. 2, right panel, lane 1). With the shake-flask method, approximately 7.0 mg rec-dZP2 was obtained from 1 l of culture.

Fig. 3. Electrophoretic analysis (SDS-PAGE) and western blot analysis of purified recombinant dog zona pellucida glycoprotein 3 (rec-dZP3) and its conjugate with diphtheria toxoid (rec-dZP3-DT). The rec-dZP3 was purified on a nickel–nitrilotriacetic acid agarose affinity column. Left panel: M: molecular mass markers (kDa); lane 1: purified rec-dZP3. Right panel: M: molecular mass markers; lane 1: purified rec-dZP3; lane 2: rec-dZP3-DT.

Expression of rec-dZP3 in *E. coli* strain SG13009[pREP4] was reported by Santhanam *et al.* (1998). The rec-dZP3 His₆ fusion protein was also purified by Ni-NTA resin. The purified rec-dZP3 in SDS-PAGE (Fig. 3, left panel, lane 1), as well as western blot analysis (Fig. 3, right panel, lane 1), revealed a 42 kDa band corresponding to the full length transcript. In addition, a 32 kDa band was observed. Conjugation of purified rec-dZP2 and rec-dZP3 with DT was confirmed by a shift in the mobility of the respective bands in western blot analysis (Fig. 2, right panel, lane 2; Fig. 3, right panel, lane 2).

Recognition of native dog zona pellucida by polyclonal rabbit antibodies against rec-dZP2-DT or rec-dZP3-DT

Rabbits immunized with rec-dZP2-DT had high antibody titres against rec-dZP2 (R-190, 26 300 AU; and R-191, 32 400 AU) as observed in ELISA. Before assessing the immunocontraceptive potential of the rec-dZP2 in dogs, the ability of the rabbit polyclonal antibodies against rec-dZP2-DT to recognize dog zona pellucida was evaluated by an indirect immunofluorescence assay (Fig. 4). Anti-rec-dZP2 antibodies showed positive fluorescence with dog zona pellucida and did not recognize other ovarian cells. No fluorescence was observed when pre-immune serum from the same immunized animal was used. Rabbit polyclonal anti-rec-dZP3 antibodies also recognized the native dog zona pellucida as described by Santhanam *et al.* (1998).

Immunization of female dogs with rec-dZP2-DT, rec-dZP3-DT or DT, and their effect on fertility

Immunization with rec-dZP2-DT. Immunization of four female dogs (dogs 6, 7, 11 and 12) with rec-dZP2-DT

Fig. 4. Reactivity of rabbit anti-rec-dZP2 (anti-recombinant dog zona pellucida glycoprotein 2) serum with native dog zona pellucida by indirect immunofluorescence. Dog ovarian cryosections (5 μ m thickness) were incubated with 1:200 dilution of rabbit anti-rec-dZP2-DT (anti-rec-dZP2 conjugated with diphtheria toxoid) (a) immune or (b) preimmune serum followed by incubation with 1:2000 dilution of fluorescein isothiocyanate (FITC)-labelled anti-rabbit immunoglobulins. Representative immunofluorescence patterns are shown. Scale bar represents 120 μ m.

conjugate induced an antibody response against rec-dZP2 in all the immunized animals, range $0.4\text{--}99.2 \times 10^3$ AU (Fig. 5). Dog 6 had higher anti-rec-dZP2 antibody titres than dogs 7, 11 and 12. All the immunized animals also showed high antibody titres against DT. The antibody titres against DT were at least 10–40-fold higher than titres against rec-dZP2 (Fig. 5, Table 1). Animals were given boosters to maintain the antibody titres until the dogs entered oestrus. An increase in the antibody titre was observed in all the animals after the booster injections. Immunization of female dogs with rec-dZP2-DT did not lead to a block of oestrus. During oestrus, the immunized females attracted the males for mating. Oestrus was confirmed by an increase in serum progesterone concentrations as well as by vaginal cytology (for example, presence of cornified epithelial cells). All four of the dogs immunized with rec-dZP2-DT became pregnant, thereby indicating that the antibodies against rec-dZP2 failed to block fertility (Fig. 5, Table 1).

Fig. 5. Antibody titres, progesterone profile and status of fertility in four female dogs immunized with recombinant dog zona pellucida glycoprotein 2 conjugated to diphtheria toxoid (rec-dZP2-DT). The immunization schedule is indicated by arrows along the x axis. The anti-rec-dZP2 (∇) and anti-DT ($\circ$) antibody (Ab) titres are expressed as antibody units (AU) $\times 10^{-3}$. $\square$: Progesterone; M: time of mating; P: pregnancy.

Table 1. Effect of immunization with rec-dZP2-DT, rec-dZP3-DT and DT on fertility in female dogs

Experimental group	Antibody titre range (AU $\times 10^3$) against		Number of matings	Anti-dZP2 or anti-dZP3 titre at time of mating (AU $\times 10^3$)	Pregnancy (number of pups)
	dZP2 or dZP3	DT			
Immunization with rec-dZP2–DT					
Dog 6	1.1–99.2	13.7–595.0	2	~99.0	Pregnant (4)
Dog 7	0.6–26.1	20.0–461.0	1	~5.0	Pregnant (6)
Dog 11	0.7–12.2	20.0–474.0	1	~2.0	Pregnant (4)
Dog 12	0.4–13.4	12.0–180.0	1	~2.1	Pregnant (3)
Immunization with rec-dZP3–DT					
Dog 1	0.7–26.0	10.0–461.0	1	~6.0	No pregnancy
Dog 3	0.7–18.8	10.0–1760.0	1	~0.7	Pregnant (5)
Dog 4	0.8–12.8	20.4–608.0	3	~2.0	No pregnancy
Dog 5	0.7–93.1	11.4–1083.0	1	~8.8	No pregnancy
Immunization with DT					
Dog 2	nd	4.8–267.0	2	nd	Pregnant (4)
Dog 8	nd	4.6–153.0	1	nd	No pregnancy
Dog 9	nd	10.9–291.0	1	nd	Pregnant (5)
Dog 10	nd	12.8–365.0	1	nd	Pregnant (3)

rec-dZP2-DT: recombinant dog zona pellucida glycoprotein 2 conjugated to diphtheria toxoid; rec-dZP3-DT: recombinant dog zona pellucida glycoprotein 3 conjugated to diphtheria toxoid; AU: antibody units; nd: not detected.

Immunization with rec-dZP3-DT. Immunization of four female dogs (dogs 1, 3, 4 and 5) with rec-dZP3-DT conjugate induced an antibody response against rec-dZP3 in the immunized animals, range $0.7\text{--}93.1 \times 10^3$ AU (Fig. 6,

Table 1). Dogs 1 and 5 had a higher anti-rec-dZP3 antibody response than dogs 3 and 4. The anti-DT antibody response ranged from 10 to 1760×10^3 AU. All the dogs in the rec-dZP3-DT immunized group showed normal oestrus. Three

Fig. 6. Antibody titres, progesterone profile and status of fertility in four female dogs immunized with recombinant dog zona pellucida glycoprotein 3 conjugated to diphtheria toxoid (rec-dZP3-DT). The immunization schedule is indicated by arrows along the x axis. The anti-rec-dZP3 (Δ) and anti-DT ($\circ$) antibody (Ab) titres are expressed as antibody (Ab) units (AU) $\times 10^{-3}$. $\square$: Progesterone; M: time of mating; P: pregnancy; NP: not pregnant.

of four immunized females (dogs 1, 4 and 5) did not conceive when mated during oestrus. Dog 3 became pregnant subsequent to mating. The anti-dZP3 antibody titre in dog 3 was lower than that of the immunized animals that failed to conceive (Fig. 6). At the time of mating, dog 3 had an anti-rec-dZP3 antibody titre of 0.7×10^3 AU.

Immunization with DT. The four animals (dogs 2, 8, 9 and 10) immunized with DT showed anti-DT antibody titres ranging from 4.6 to 365.0×10^3 AU (Fig. 7, Table 1). In the DT-immunized group, three of four animals became pregnant subsequent to mating (Fig. 7, Table 1). The fourth animal (dog 8) did not conceive, probably due to the low concentration of serum progesterone at the time of oestrus.

Ovarian morphology of the female dogs immunized with DT, rec-dZP2-DT and rec-dZP3-DT

Histopathology of the ovaries from the DT-immunized group revealed follicles at the various stages of development without any degenerated follicles (Fig. 8a,b). The zona pellucida appeared normal. The ovarian histology of the dogs immunized with rec-dZP2-DT also showed normal patterns of follicle development (Fig. 8c,d). However, in the dogs immunized with rec-dZP3-DT, inhibition of follicular development and degenerative changes in the zona pellu-

cida were observed (Fig. 8e,f). No infiltration by lymphocytes was observed in atretic follicles. These changes were associated with the antibody titres, as dogs 1 and 5 showed a more pronounced increase in the number of atretic follicles compared with dog 3 (data not shown).

Discussion

ZP glycoproteins will be required in large quantities, preferably free from other ovarian-associated proteins, for evaluation of their immunocontraceptive potential. Moreover, large-scale production of the antigen has to be economically viable to produce a vaccine for practical use. Attempts have been made by several groups to express ZP2, the secondary sperm receptor, from various species such as rabbits (Lee *et al.*, 1993; Vandevoort *et al.*, 1995), pigs (Tsubamoto *et al.*, 1999) and bonnet monkeys (Jethanandani *et al.*, 1998) using various types of expression systems to overcome these problems. It is thought that the processing of ZP glycoproteins within maturing oocytes for incorporation within the zona matrix involves the removal of the signal peptide by signal peptidase and the transmembrane-like domain at the upstream furin cleavage site by furin proteases (von Heijne, 1986; Hosaka *et al.*, 1991). Hence, rec-dZP2, excluding the N-terminal signal sequence and C-terminal transmembrane-like domain, was

Fig. 7. Antibody titres, progesterone profile and status of fertility in female dogs immunized with diphtheria toxoid (DT). The immunization schedule is indicated by arrows along the x axis. The anti-DT antibody (Ab) titres (○) are expressed as antibody units (AU) $\times 10^{-3}$. □: Progesterone; M: time of mating; P: pregnancy; NP: not pregnant.

expressed in *E. coli* as His₆ fusion protein, which allowed it to be purified easily. The presence of lower molecular mass fragments in addition to the protein (70 kDa) corresponding to the full length transcript may be due to either degradation of the expressed protein or premature termination during the translation of the dZP2 mRNA.

The availability of rec-dZP2 expressed in *E. coli*, which is devoid of other ovarian proteins as contaminants, prompted us to evaluate its efficacy to block fertility using a homologous animal model. In addition, rec-dZP3 expressed in *E. coli*, as reported by Santhanam *et al.* (1998), was included for evaluation of its efficacy to block fertility. The rec-dZP2 and rec-dZP3 will be recognized essentially as self-antigens in female dogs. Rec-dZP2 and rec-dZP3 were conjugated to DT as a carrier protein to make them immunogenic. Previous clinical trials of the contraceptive vaccine in women, consisting of a heterospecies dimer of the β -subunit of hCG associated non-covalently with the α -subunit of ovine LH, have demonstrated that tetanus toxoid (TT) and DT are excellent carrier proteins (Talwar *et al.*, 1994). Rec-dZP2 and rec-dZP3 were conjugated with DT using glutaraldehyde, a commonly used coupling agent. Several heterobifunctional reagents such as 1-ethyl-3-(3-diethylaminopropyl)-carbodiimide (EDCI) and M-maleimidobenzoyl-*N*-hydroxysuccinamide ester (MBS) have also been used to prepare conjugates with defined stoichiometry. Previous studies from our laboratory have

demonstrated that conjugation of either recombinant zona proteins or their corresponding synthetic peptides with DT, using glutaraldehyde as a coupling agent, generates effective antibody responses leading to a block in fertility (Govind and Gupta, 2000; Kaul *et al.*, 2001). Hence, glutaraldehyde was preferred as a coupling agent. It is a simple and effective method of coupling, although has a risk of formation of homopolymers and heterogeneous populations of the resulting conjugate. The conjugation of rec-dZP2 and rec-dZP3 with DT was confirmed by western blot analysis.

Antisera raised in rabbits against rec-dZP2-DT recognized the dog native ZP and did not react with any other type of cell in the ovary. Evaluation of the immunoreactivity of the antibodies generated in female dogs against rec-dZP2-DT and rec-dZP3-DT with dog zona pellucida could not be performed with certainty because of the high immunofluorescence background on sections of dog ovary with dog pre-immune serum samples.

Immunization of the female dogs with the rec-dZP2-DT emulsified with Squalene and Arlacel-A (SPLPS as an additional adjuvant only in the first injection) generated an adequate antibody response against rec-dZP2 as well as DT in all the animals immunized. However, 10- to 40-fold higher anti-DT antibody titres compared with antibody titres against rec-dZP2 could be a result of the fact that dZP2 might have been recognized essentially as a self-antigen.

Fig. 8. Photomicrographs of ovarian sections of female dogs immunized with diphtheria toxoid (DT), recombinant dog zona pellucida glycoprotein 2 conjugated to DT (rec-dZP2-DT) and rec-dZP3-DT. The sections were stained with haematoxylin and eosin. (a,b) Ovarian morphology from dogs immunized with DT (a: dog 2; b: dog 8) showing normal folliculogenesis with primordial (p) and developing (d) follicles. (c,d) Ovarian sections from dogs immunized with rec-dZP2-DT (c: dog 7; d: dog 12) showing follicles in different stages of development. (e,f) Ovarian sections from dogs immunized with rec-dZP3-DT (e: dog 1; f: dog 5) showing atretic follicles. Scale bar represents 100 μm .

Despite the presence of anti-rec-dZP2 antibodies, all the immunized females conceived when mated with a male during oestrus. The mean number of pups born to dogs immunized with rec-dZP2-DT was not significantly different (Student's *t* test) from the number born to the dogs immunized with DT. The ovarian histology of the dogs immunized with rec-dZP2-DT revealed normal follicular development. Immunization of cynomolgus monkeys with bacterially expressed 75 kDa rabbit ZP protein (homologue of dZP2) conjugated to protein A generated antibodies that interfered with follicular development and ovarian cyclicity

(Vandevoort *et al.*, 1995); however, the status of fertility was not evaluated by these workers. Failure to block fertility and apparent normal ovarian morphology in female dogs immunized with rec-dZP2-DT may be a result of either low antibody titres or qualitative differences in the antibodies generated.

Active immunization of bitches with crude pig zona pellucida together with CFA induced infertility (Mahi-Brown *et al.*, 1982). Immunization resulted in abnormal oestrous cycles characterized by prolonged pro-oestrus and oestrus. Oocytes recovered from the ovaries of the

immunized animals failed to bind spermatozoa. Histological examination of the ovaries revealed that the animals with the highest antibody titres showed depletion of oocytes (Mahi-Brown *et al.*, 1988). Subsequently, these investigators immunized bitches with pig zona pellucida adsorbed on alum together with CP20, 961, a synthetic lipid amine, as an emulsifier, resulting in a variable antibody response (Mahi-Brown *et al.*, 1985). One of the animals developed a temporary inflammatory response to the adjuvant at the site of injection, had the lowest antibody response and conceived after mating, whereas the other animals had higher antibody titres, remained infertile and did not show any inflammatory response. However, these studies used solubilized zona pellucida and the contraceptive efficacy could not be related to a particular zona glycoprotein. The potential of purified pig ZP glycoproteins to control canine populations was further demonstrated by Fayrer-Hosken *et al.* (2000).

In the present study, three of four female dogs immunized with rec-dZP3-DT did not conceive after mating during oestrus. Low anti-rec-dZP3 antibody titres (approximately 0.7×10^3 AU) in the fourth dog at the time of mating may not have been adequate to prevent conception. It is difficult to assess the protective threshold level of anti-rec-dZP3 antibody titres from this study, but failure to conceive, when the antibody titres were approximately 2.0×10^3 AU indicates that antibody titres $\geq 2.0 \times 10^3$ AU will be required to achieve the contraceptive effect. Between six and seven injections of rec-dZP3-DT, over a period of > 350 days, were administered to study its contraceptive efficacy. It is desirable that the number of injections required to achieve contraception should be reduced to make it a practical proposition. Further optimization to enhance the antibody response against rec-dZP3 could be achieved by incorporating more potent adjuvants. In addition to conventional immunization, various other strategies such as viral vector or liposomes could also be used. Immunization of mice with recombinant ectomelia virus (a natural pathogen for mice) expressing mouse ZP3 led to a decrease in fertility as well as litter size (Jackson *et al.*, 1998). A single injection of pig solubilized zona pellucida using a liposome delivery system decreased pup production by grey seals (*Halichoerus grypus*) by 90% over a period of 5 years (Brown *et al.*, 1997). Single injection of TT entrapped in polylactide-co-glycolide and polylactide polymer particles generated an antibody response for > 5 months in rats (Raghuvansi *et al.*, 2001).

The failure of the dogs to conceive was not due to failure of mating, as all the four female dogs immunized with rec-dZP2-DT and three of four animals immunized with DT alone became pregnant subsequent to mating. The ovarian histology of the dogs immunized with rec-dZP3-DT revealed a decrease in the number of primordial follicles, suppression in follicular development and atretic changes in the zona pellucida. In another study, active immunization of marmoset monkeys with purified recombinant human ZP3 also resulted in long-term infertility associated

with ovarian dysfunction, characterized by suppression of folliculogenesis and depletion of the pool of primordial follicles (Paterson *et al.*, 1998). These studies indicate that the main mechanism by which anti-rec-dZP3 antibodies cause infertility is by follicular atresia and degenerative changes in the zona pellucida. It is not clear whether these changes are solely a result of anti-rec-dZP3 antibodies or whether there is an involvement of oophoritogenic T cells.

To our knowledge, these studies have evaluated, for the first time, the efficacy of active immunization with rec-dZP2 and rec-dZP3 expressed in *E. coli* and coupled to DT as a carrier protein to block fertility in female dogs. The observed block in fertility, subsequent to immunization with rec-dZP3-DT, although preliminary, is potentially useful. Attempts are being made in our laboratory to express rec-dZP3 in a glycosylated form using a baculovirus expression system to enhance the immunogenicity of rec-dZP3.

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