Contraceptive potential of porcine and feline zona pellucida A, B and C subunits in domestic cats

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Abstract

Feral cat populations are a major problem in many urban regions throughout the world, threatening biodiversity. Immunocontraception is considered as an alternative and a more humane means to control overpopulation of pest animals than current methods including trapping, poisoning and shooting. In this study, we evaluate porcine zona pellucida (ZP) polypeptide (55 kDa) and feline ZP A, B and C subunits expressed by plasmid vectors as candidate vaccines against fertility in the female domestic cat. Cats were injected subcutaneously with three doses of the ZP vaccines. Vaccinated cats were compared with naïve cats for ZP-antibody response, ovarian histology and fertility after mating. Vaccination with native porcine ZP 55 kDa polypeptide induced anti-porcine ZP antibodies detected by ELISA. However, these antibodies did not cross-react with feline ZP as assessed by immunohistochemistry and no effect on fertility *in vivo* was observed after mating. However, vaccination of cats with feline ZPA or feline ZPB + C DNA vectors elicited circulating antibodies specific for feline ZP as assessed by ELISA, with reactivity to native feline ZP in ovarian follicles *in situ*. Vaccination with feline ZPA and ZPB + C DNA did not elicit changes in ovarian histology. Although sample sizes were small, conception rates in mated females were 25 and 20% in the ZPA and ZPB + C vaccinated groups respectively, compared with 83% in the control group. We conclude that feline ZPA and ZPB + C subunits are potential candidate antigens for immunocontraceptive vaccines in the domestic cat.

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Introduction

Despite many efforts, overpopulation of feral domestic cats (*Felis catus*) is a problem to control in many regions of the world. In Australia, feral cats have become well established, including remote arid areas. Feral cats have a wide range of prey and severely impact on native biodiversity (Dickman *et al.* 1993). For example, feral cats have been shown to be responsible for killing many re-introduced Rufous Hare wallabies (*Lagorchestes hirsutus*) released in the Northern Territory, and have significant impact on an isolated colony of Rock wallabies (genus *Petrogale*) in Queensland (Short *et al.* 1997). Present control measures include live trapping, shooting and poison baiting. However, these methods are not effective in the long-term and are labour intensive (Saunders *et al.* 1995, Short *et al.* 1997).

Immunocontraception is an alternative strategy, which offers a more humane approach with possible longer-term efficacy than present methods of control for animal populations. The objectives of immunocontraception are to elicit an effective autoimmune response against components of the reproductive system, which manifest in infertility. An attractive antigen target in females is the zona pellucida (ZP), a matrix composed of several

glycoprotein subunits that surrounds the oocyte. This ZP matrix serves several functions including sperm receptivity, prevention of polyspermy and protection of the fertilized oocyte in the initial stages of differentiation (Dean 1992).

ZP immunocontraception has been studied in many mammalian species including horse (Kirkpatrick *et al.* 1990), deer (Miller *et al.* 2000), rabbit (Sehgal *et al.* 1989) and mouse (Rhim *et al.* 1992, Sun *et al.* 1999). The immunization of females against ZP results in the formation of ZP-specific antibodies, which bind to native autologous ZP and offer contraception by blocking sperm binding to receptors. Studies have also shown ovarian destruction, either permanent or temporary, with possible correlation to ZP-specific antibody responses in the pig, rabbit, dog and squirrel monkey (Sacco *et al.* 1987, Jones *et al.* 1992, Kerr *et al.* 1999, Srivastava *et al.* 2002).

In the domestic cat, relatively few studies have been conducted on the suitability of a ZP vaccine strategy. The potential of heterologous ZP as an immunocontraceptive for cats is controversial (Jewgenow *et al.* 2000). Despite the wide application of porcine ZP in many species, porcine ZP proteins, although showing homology with feline ZP, have not been suitable for cat contraception

due to lack of immune cross-reactivity (Kaul *et al.* 1996, Ringleb *et al.* 2004). A study using porcine ZP (SpayVac) showed no effect on oestrus cycling or fecundity in cats despite production of high titre anti-porcine ZP antibodies in vaccinates (Gorman *et al.* 2002). The ZP of cats is composed of A, B and C protein subunits (Harris *et al.* 1994). Feline ZPB is the first zona glycoprotein to be expressed in maturing oocytes (Jewgenow & Fickel 1999) and antibodies directed against a cat ZPB peptide have been shown to inhibit sperm binding and fertilization *in vitro* (Ringleb *et al.* 2004).

In this study, porcine ZP was compared with feline ZP for suitability as an immunocontraceptive vaccine candidate antigen in domestic cats. DNA vaccines expressing autologous feline ZP A, B and C subunits were evaluated. We report on the immunogenicity of such ZP vaccines in eliciting feline ZP-specific antibodies, ovarian histology and infertility in cats after mating.

Results

Antibody responses of porcine ZP55-vaccinated cats

Serum antibody responses against soluble-isolated porcine ZP were assessed by ELISA in porcine ZP55 polypeptide (B+C) vaccinated cats (Fig. 1a). Sera from pre-bleeds of all cats were non-reactive against porcine ZP. No circulating antibody to porcine ZP was detected in the control group at any time point for the duration of the study. By contrast, within 2 weeks post-vaccination, a few of the porcine ZP55-vaccinated cats (2/5; Kte and Msh) had generated a detectable antibody response, which then declined at week 4. However, all porcine ZP55-vaccinated cats (5/5) produced an antibody response following the booster vaccination (week 4), which was observed out to week 10. One individual (Kte) displayed strong antibody reactivity, which was at a greater level than the endpoint titre for the rabbit anti-porcine ZP positive control serum (1:13 600). Furthermore, porcine ZP55-vaccinated cats developed circulating antibodies recognizing a 55 kDa component of soluble-isolated porcine ZP as assessed by western blot, indicating ZP polypeptide seroconversion (data not shown).

Next, the ability of porcine ZP55 vaccination to elicit antibodies cross-reactive to native feline ZP was investigated by immunohistochemical analysis using normal ovarian tissue from non-experimental cats. Rabbit anti-porcine ZP antibodies cross-reacted specifically against feline ZP of healthy late primary through to antral (Fig. 1b, top) and atretic follicles. Cross-reactivity with the ooplasm but not the primordial oocytes was observed. By contrast, immune sera (week 10 post-vaccination) from porcine ZP55-vaccinated cats showed no evidence of cross-reactivity to feline ZP in ovarian tissue by immunohistochemistry (Fig. 1b, bottom). Moreover, no endogenous IgG binding to feline ZP

Figure 1 Antibody responses of cats vaccinated with porcine ZP55 polypeptide. (a) IgG antibodies to solubilized porcine ZP in sera (diluted 1:100, $n=5$) from individual cats thrice vaccinated with porcine ZP55. Antibody levels determined by ELISA were expressed as a percentage of the antibody reactivity of rabbit anti-porcine ZP positive control (endpoint titre 1:13 600). Individual cats are designated with a three-letter code. No circulating antibody to porcine ZP was detected in the control group. (b) Immunohistochemistry for antibody cross-reactivity to native feline ZP in normal ovarian tissue. Top. Rabbit anti-porcine ZP serum (1:100) from rabbits immunized with solubilized whole porcine ZP. Arrows show antibody binding to ZP of oocytes in healthy follicles. 100 $\times$ magnification. Bottom. Immune serum (1:100) taken at week 10 from cats vaccinated with porcine ZP55 showing no reactivity. 400 $\times$ magnification. (c) Immunohistochemistry for antibody endogenously bound to ovarian tissue in a porcine ZP55-vaccinated cat. Top. Ovarian tissue from a porcine ZP-vaccinated cat at week 10 post-vaccination. Bottom. Ovarian tissue from a control cat at week 10 post-sham vaccination. 200 $\times$ magnification.

in the ovaries was observed *in situ* by immunohistochemistry, at post-mortem of the porcine ZP55 polypeptide-vaccinated cats (Fig. 1c, top). Sera from week 0 to 10 post-vaccination of cats in the control group did not display antibody reactivity to feline ZP (Fig. 1c, bottom).

Fertility of porcine ZP55-vaccinated cats

Cats were mated to assess effects of porcine ZP vaccination on fertility. Signs of oestrus were observed in varying degrees in all cats, including increased affection, calling and positioning. Only four out of the five control queens mated and three became pregnant (75% conception rate) with litters ranging from 2 to 4 live births (Table 1). One control cat was pseudopregnant, displaying initial characteristics of pregnancy but did not deliver a litter. All five porcine ZP55-vaccinated cats mated. Four of these vaccinated cats became pregnant (80% conception rate) with litter sizes ranging from 2 to 5 kittens, while the other vaccinated cat had histological evidence of ovulation but was pseudopregnant. There were no significant differences between the vaccinated and non-vaccinated mated cats ($P=1.00$). Also, antibody titres to porcine ZP, as assessed by ELISA, did not correlate with fertility outcomes of individual cats.

Ovarian histology of porcine ZP55-vaccinated cats

Ovaries from porcine ZP55-vaccinated and control cats were assessed post-mortem for structural changes (Fig. 2a). Queens were allowed to litter and ovaries were removed post-mortem after kittens were weaned. Both control and porcine ZP55-vaccinated cats ($n=5$ per group) revealed normal follicles ranging from early to antral stages, with evidence of ovulation in the form of corpora lutea. However, the porcine ZP55-vaccinated cats had a significantly higher proportion of tertiary follicles than the control group ($P<0.05$). Feral cats were also assessed for follicle distributions (Fig. 2b). Adult feral cats ($n=8$) showed a significantly higher proportion of primary follicles than juvenile feral cats ($n=6$, $P<0.05$). Overall, the distribution of follicle populations was found to be similar between experimental and adult feral cats except for a significantly smaller proportion of corpora lutea in the feral cats ($P<0.05$).

Table 1 Conception rate of porcine zona pellucida 55 (ZP55)-vaccinated cats.

Vaccine	Vaccinated ^a	Mated ^b	Conception rate (%) ^c	Litter size ^d
Control	5	4	75	3 ± 0.6
Porcine ZP55	5	5	80	3 ± 0.7

^aCats were either sham-vaccinated or vaccinated with porcine ZP55 polypeptide at week 0, 4 and 8 emulsified in adjuvant as described in Materials and Methods. The sample size is shown for the total number of cats introduced to a male. ^bCats were mated after 11th week post-vaccination. The number of cats that accepted a male and mated is shown. ^cConception rates defined as pregnancies with delivery of a litter from successfully mated queens. ^dAverage number of kittens per litter ± S.E.M. for mated females that delivered.

Figure 2 Distribution of follicle populations in cat ovary. (a) Ovarian tissue from control cats ($n=5$) and porcine ZP55-vaccinated cats ($n=5$). (b) Ovarian tissue from adult feral cats ($n=8$) and juvenile feral cats ($n=6$). The proportion of each follicle population is expressed as a percentage distribution per section (mean ± S.E.M.). * $P<0.05$.

Antibody responses of feline ZP DNA-vaccinated cats

Next, cats were vaccinated with either feline ZPA or a combination of feline ZPB and ZPC, delivered as DNA vaccines. Antibody responses against solubilized feline ZP were assessed by ELISA using pooled sera. Cats vaccinated with feline ZPA showed low titres of antibodies to feline ZP, peaking at week 4 ($1.5 \log_2$, Fig. 3a). Whereas, antibodies against feline ZP were not apparent in the feline ZPB+C DNA-vaccinated group until week 11, being 3 weeks after the second boost ($1.5 \log_2$). No circulating antibodies to feline ZP were detected in the control group at any time point.

There was no evidence of serum antibody binding to feline ZP in normal ovarian follicles from control (Fig. 3b, top, Fig. 4a, top), feline ZPA (Fig. 3b, bottom) and feline ZPB+C DNA-vaccinated groups (Fig. 4a, bottom). Examination of antibody binding *in situ* in feline ZP DNA-vaccinated cats was carried out post-mortem using immunohistochemistry. IgG antibody was bound to the ZP matrices of oocytes from all healthy follicles from the late primary/early secondary stage of folliculogenesis in feline ZPA and ZPB+C DNA-vaccinated cats (Fig. 3c, bottom, weeks 19–25

Figure 3 Antibody responses of cats vaccinated with feline ZPA DNA construct. (a) IgG antibodies to solubilized feline ZP in feline ZPA DNA-vaccinated cats detected by ELISA. Pooled sera (1:10) from seven cats thrice vaccinated with feline ZPA DNA construct were titrated in duplicate. Antibodies are expressed as endpoint titres using the OD for pre-bled sera as a negative control baseline $+3$ s.d. and expressed as log₂ at various weeks post-vaccination. No circulating antibody to feline ZP was detected in the control group. (b) Immunohistochemistry for antibody reactivity to native feline ZP in normal ovarian tissue. Top. Control cat serum (1:100) from cats sham-vaccinated with empty plasmid vector. Bottom. Immune serum (1:100) from cats vaccinated with feline ZPA DNA construct. 200 $\times$ magnification, representative of all cats per group. (c) Immunohistochemistry for antibody endogenously bound to ovarian tissue in feline ZPA DNA-vaccinated cats. Top. Ovarian tissue from a control cat. Bottom. Ovarian tissue from a feline ZPA DNA-vaccinated cat. Arrow shows antibody binding to ZP of oocyte. 200 $\times$ magnification, representative of all cats per group.

post-vaccination; Fig. 4b, weeks 24–32 post-vaccination respectively). Relative to the level of antibody binding of rabbit anti-porcine ZP antiserum (1:4000 dilution) to cat ovarian tissue, three feline ZPA vaccinated cats showed strong antibody binding. In the feline ZPB+C vaccinated group, two cats displayed moderate, two cats displayed weak, while one cat showed very weak levels of *in situ* antibody binding to feline ZP in the ovaries.

Collapsed ZP matrices from atretic follicles were more intensely stained for antibody binding relative to ZP from healthy follicles (Fig. 4b). By contrast, healthy follicles of control cats ($n=8$) were clear of antibody binding (Fig. 3c, top).

Fertility of feline ZP DNA-vaccinated cats

Conception rates of feline ZP DNA-vaccinated cats were next examined (Table 2). In the control group comprised of eight cats, seven mated with evidence of corpora lutea in ovaries and became pregnant with litter sizes of two to five kittens born, representing a 71% conception rate. When considering the total number of cats in the control group ($n=8$), the conception rate was 62% with an average litter size of two kittens. The one control cat, that did not mate, actively rejected the male and did not show signs of corpora lutea in the ovaries.

Of the seven feline ZPA DNA-vaccinated cats, four mated with production of only one litter of two kittens, representing a conception rate of 25% in mated females. However, the reduction in conception rate compared with the control group did not achieve significance due to low sample sizes ($P=0.24$). Indeed, the conception rate was further reduced to 14% ($P=0.12$) with an average litter size of 0.3 kittens when adjusted for total

Figure 4 Antibody responses of cats vaccinated with feline ZPB+C DNA construct. (a) Immunohistochemistry for antibody reactivity to native feline ZP in normal ovarian tissue. Top. Control cat serum (1:100) from cats sham-vaccinated with empty plasmid vector. Bottom. Immune serum (1:100) from cats vaccinated with feline ZPB+C DNA construct. 200 $\times$ magnification, representative of all cats per group. (b) Immunohistochemistry for antibody endogenously bound to ovarian tissue in feline ZPB+C DNA-vaccinated cats. Top. Ovarian tissue from a feline ZPB+C DNA-vaccinated cat. Arrow shows antibody binding to ZP of oocyte in a healthy follicle. Bottom. Atretic ovarian follicle from a feline ZPB+C DNA-vaccinated cat. Arrow shows antibody binding to ZP of oocyte in an atretic follicle. 200 $\times$ magnification, representative of all cats per group.

Table 2 Conception rate of feline zona pellucida (ZP) DNA-vaccinated cats.

Vaccine ^a Group (n)	Mated ^b (n)	Conception rate (%) ^c		Litter size ^d	
		Mated	All	Births	All
Control (8)	7	71	62	3.4±0.7	2.1±0.7
Feline ZPA (7)	4	25	14	2±0.0	0.3±0.3
Feline ZPB+C (5)	5	20	20	2±0.0	0.4±0.4

^aCats were either sham-vaccinated with empty vector (controls for separate vaccine trials) or vaccinated with feline ZP DNA constructs at weeks 0, 4 and 8 as described in Materials and Methods. The sample size is shown for the total number of cats introduced to a male. ^bCats were mated after 11th week post-vaccination. The number of cats that accepted a male and mated is shown. ^cConception rates defined as pregnancies with delivery of a litter for successfully mated females (mated) and from all cats per group (all). ^dAverage number of live kittens per litter ± s.e.m. for mated females that delivered (births) and for all cats per group (all).

number of ZPA-vaccinates ($n=7$). One ZPA-vaccinated cat had evidence of five empty sacs in the uterine horns at post-mortem, with small nodules of tissue distinguishable from the surrounding uterine wall in the sacs, suggesting foetus resorption. The ovaries appeared normal, with prominent corpora lutea. Two of the other ZPA-vaccinated cats became pseudopregnant, displaying torsioned and enlarged uterine horns with corpora lutea in the ovaries. Three vaccinated cats actively rejected the male and refused to mate. One of these cats did not show evidence of corpora lutea. Interestingly, the other two cats revealed reproductive anomalies, including very small and underdeveloped ovaries with no discernable follicular structures. In one of these cats, the corpora lutea were not evident. However, the uterine horns did not appear grossly abnormal. In the remaining cat, the uterine horns were very thin and translucent. Ovarian structures were translucent and vestigial in appearance and size.

In the feline ZPB+C DNA-vaccinated cat group ($n=5$), all five cats successfully mated with only one cat becoming pregnant and delivering a litter of two kittens, representing a conception rate of 20%. However, due to the small sample sizes, the reduction in conception was not statistically significant ($P=0.24$). The difference between adjusted conception rates of 20% for the vaccinated group and 62% for the controls, when using the total number of cats in each group, was also not significant ($P=0.26$). There was one incident of pseudopregnancy in the feline ZPB+C DNA-vaccinated group.

Ovarian histology of feline ZP DNA-vaccinated cats

Examination of post-mortem ovarian tissue from experimental cats showed normal histology despite feline ZP DNA-vaccination. Numbers of primary, secondary and

tertiary follicles, corpora lutea and atretic follicles were recorded (Fig. 5). Follicle cell subpopulations were expressed as a proportion of the total follicle population to account for any differences in ovary size between individuals. There were no significant differences ($P>0.05$) in either the cell subpopulation distribution or total number of follicles between control and feline ZP DNA-vaccinated cats, regardless of ZPA or ZPB+C vaccination. The distribution of primary follicle cell subpopulations in these feline ZP DNA-vaccinated cats was found to be similar to that observed in juvenile feral cats and significantly less than that found in adult feral cats ($P<0.05$).

Note, that the ovaries of two feline ZPA DNA-vaccinated cats were vestigial and underdeveloped with no recognizable follicular structures or oocytes. However, no significant antibody binding to ovarian tissue in these cats was observed by *in situ* immuno-histochemical analysis and no inflammation was evident (data not shown).

Figure 5 Distribution of follicle populations in cat ovary. Ovarian tissue from control cats ($n=8$), and feline ZPA DNA (f-ZPA; $n=5$) and ZPB+C DNA (f-ZPB/C; $n=7$) vaccinated cats. The proportion of each follicle population is expressed as a percentage distribution per section (mean ± s.e.m.). * $P<0.05$.

Discussion

Control of feral cat populations is a pertinent issue in many parts of the world, including Australia, as feral cats are regarded as significant predators to wildlife. Immun contraception using ZP proteins offers a humane approach to population control of pest animals. Few studies on the potential of immunological contraceptives in the domestic cat have been performed, despite large research efforts in other species, including wild deer and horse in North America (Willis *et al.* 1994, Miller *et al.* 2000), seals (Brown *et al.* 1997) and African elephants (Fayrer-Hosken *et al.* 2000). Here, in this study, we have examined both heterologous porcine ZP and autologous feline ZP antigens as candidate vaccines for immun contraception in the domestic cat.

In cats that were mated, vaccination of female domestic cats with three doses of porcine ZP55 polypeptide in Freund's adjuvant failed to elicit detectable antibody responses to feline ZP as assessed by immunohistochemistry, despite the production of antibodies to porcine ZP55. These results concur with the absence of antibody cross-reactivity between porcine ZP and cat ZP (Gorman *et al.* 2002). Furthermore, there was no evidence of ovarian pathology and no significant reduction in fecundity following mating of the porcine ZP-vaccinates ($P=1.00$). By contrast, vaccination of cats with plasmid vectors expressing autologous feline ZPA and ZPB+C subunits induced low levels of circulating antibodies to feline ZP. Ovarian tissue from feline ZP DNA-vaccinated cats displayed *in situ* IgG antibody bound specifically to ZP of oocytes in follicles as early as primary through to antral stages. However, this endogenously bound antibody did not interfere with folliculogenesis. In addition, *in situ* antibody binding to ZP of atretic follicles was observed in experimental cats and in some controls. This may suggest a role of autoantibodies in atresia (Kamo *et al.* 2004) or simply non-specific deposits of IgG to degenerated ZP giving rise to false-positives (Y Lou, personal communication). Ovarian histology was normal in most of the ZP DNA-vaccinated cats, despite the presence of feline ZP-specific antibodies. In two ZPA DNA-vaccinated cats, follicular structures and inflammation were absent in the ovaries, however, the cause of the pathology remains inconclusive.

A trend towards increased incidence of unsuccessful pregnancies was observed, although statistical significance was not achieved in this experimental study. Vaccination with feline ZPA DNA resulted in a 25% conception rate and vaccination with feline ZPB+C DNA resulted in a 20% conception rate compared with controls with fertility rates of 83%. We acknowledge that the sample sizes for the fertility outcomes are small as not all females (sham or ZP-vaccinated) accepted the male and mated during the experimental study. Also, an out bred population of animals may give variable

responses to ZP DNA vaccination and further vaccine trials are warranted. Nonetheless, our studies demonstrate the immun contraceptive potential for feline ZP subunits as candidate vaccine antigens for domestic cats.

There is increasing evidence that soluble-isolated whole porcine ZP, despite being efficacious for immun contraception in various species, is not suitable for either domestic or exotic felid species, possibly due to lack of feline antibody cross-reactivity (Harrenstein *et al.* 2004). Functional studies comparing feline ZPB with that of other species have shown that regions unique to feline ZPB appear to play a significant role in fertilization and sperm binding. Thus, the failure of porcine ZP as an immun contraceptive antigen appears to be due to lack of cross-reactivity of feline anti-porcine ZP antibodies with native feline ZPB epitopes. While ZP with sperm receptor function have been the major focus of immun contraceptive research, studies using porcine ZP in various species have shown that all ZP subunit proteins have contraceptive potential (Aitken *et al.* 1982, East *et al.* 1985, Henderson *et al.* 1988, Jewgenow *et al.* 1994). Cynomolgus monkeys vaccinated with recombinant human ZPA or C proteins were fertile, whereas vaccination with human ZPB resulted in infertility that was irreversible and associated with cycle disruptions (Martinez & Harris 2000).

Furthermore, heterologous ZP DNA constructs (recombinant dog ZP3 and bonnet monkey ZPB) have been used to immunize mice (Rath *et al.* 2002, 2003). Antibodies were produced that cross-reacted with recombinant ZP protein and native ZP in ovarian tissue of DNA-vaccinated mice, which inhibited sperm binding *in vitro*. Another study examined the ability of a truncated rabbit ZPC DNA construct to generate an immune response in female rabbits (Xiang *et al.* 2003). Female rabbits developed an antibody response capable of recognizing native ZP in ovarian tissues, with no associated changes to ovarian structure. However, no effects of such ZP DNA vaccination of rabbits on fertility *in vivo* were reported. However, studies on immun contraception in model species, for example mice, rabbits and monkeys, are not readily transferable to felid species.

ZP derived from several other species has been investigated in a cat study (Levy *et al.* 2005). Cats vaccinated with soluble ZP encapsulated in liposomes and emulsified in Freund's or alum adjuvant, showed that mink ZP was better than ferret, dog and feline ZP at inducing cross-reactive anti-feline ZP antibodies. However, mating trials at 20 weeks post-single vaccination showed that all cats ($n=3$ /group) became pregnant with normal litter sizes (four kitten/litter). Therefore, fertility was not impeded by ZP vaccination using this protocol. Several ZP immunization regimes have been shown to affect ovary structure and function, which may or may not manifest as infertility (Mahi-Brown *et al.* 1988, Bagavant *et al.* 1999). It has

been reported that ovarian damage is associated with CD4⁺T-cell epitopes on the immunizing antigen (Lou *et al.* 1995). However, oophoritis does not necessarily lead to infertility (Bagavant *et al.* 1999). Vaccination with a T-cell epitope that drives a Th1 response has been shown to induce autoantibodies against native ZP (Lou & Tung 1993, Lou *et al.* 1996). T- to B-cell epitope spreading is important in the generation of destructive autoimmune responses against healthy follicles (Lou & Borillo 2003). According to this relay model, ZP-based immunocontraception requires pro-inflammatory cell-mediated responses. Immunomodulation towards a Th1 bias, induced by feline ZP DNA vaccination, may fulfil both these requirements.

The ultimate goal for feline immunocontraceptive research in Australia would be in its application for control of feral cat populations. Delivery of vaccine to widespread and remote areas of Australia is a challenge. Live viral vectors have been experimentally investigated for delivery of ZP in mouse models, including recombinant ectromelia virus (Jackson *et al.* 1998) and recombinant mouse cytomegalovirus (Lloyd *et al.* 2003). The use of a disseminating viral vector has the advantage of transmission between individuals but disadvantages include environmental factors and public acceptability (Kerr *et al.* 1999, Barlow 2000). Ecological studies of feral mouse populations have helped define necessary parameters for successful disseminating vectored immunocontraception for that species (Singleton *et al.* 2002). However, the level of sterility required to reduce the impact of feral cats remains largely unknown.

Materials and Methods

Animals

Domestic female and male cats (*F. catus*), supplied by Culas, Sydney, New South Wales and Monash University, Victoria, Australia were used as breeding stock. All animals were maintained in the Animal House facility at Murdoch University. Progeny (~2–3 years of age) were used in vaccination studies. Females (5–8 cats) were housed next to a male, separated by wire mesh partitions. The rooms were maintained at 23 °C with artificial 12 h light:12 h darkness cycles to ensure continuous reproductive cycling. Commercial dry cat food and water was provided *ad libitum*, and tinned meat was given once a day. All cats were routinely vaccinated against feline calicivirus, rhinotracheitis and panleukopaemia virus (Protech F31 Vaccine, Fort Dodge, Southampton, UK). Experimental procedures were approved by the Murdoch University Animal Experimentation Ethics Committee in accordance with the Australian National Health and Medical Research Council guidelines. Cats were anaesthetized with Alfaxan (10 mg/kg, Jurox Pty Ltd, Rutherford, NSW, Australia) prior to procedures and euthanased at the end of the study with sodium-pentobarbitol (Lethobarb, Jurox Pty Ltd). Ovaries were obtained from feral cats caught in Western Australia by the government authority, Conservation

and Land Management. Feral cats were designated as either adult or juvenile based on body weight and size.

Porcine ZP vaccine

ZP protein was prepared as described previously (Jones *et al.* 1992) using pig ovaries from a local abattoir (Perth, WA, Australia). Briefly, ovaries were passaged through a descending series of molecular sieves and zonae solubilized and concentrated (×100) using an ultrafiltration cell. The major polypeptide band (55 kDa) after gel electrophoresis (Protoprep, National Diagnostics Inc., Atlanta, GA, USA) corresponded to the reported *M_r* for native porcine ZPB + C (Yurewicz *et al.* 1987). This band was dissolved and dialysed for the polypeptide (porcine ZP55) vaccine.

Preparation of native feline ZP

Ovaries from domestic cats (6 months to 2 years) were obtained from a local cat shelter (Shenton Park, Perth, WA, Australia), collected in PBS and stored frozen at –20 °C until processed. The extraction procedure was modified from Jones *et al.* (1992). Briefly, ovaries were homogenized and passed through a series of molecular sieves, centrifuged at 1800 *g* for 12 min at 4 °C and subjected to three rounds of freezing and thawing. Zonae were centrifuged at 2500 *g* for 15 min, washed and resuspended in water (pH 9), heat-solubilized and stored at –20 °C.

Feline ZP A, B and C DNA vaccines

Plasmids containing the full-length sequences for feline ZP A, B and C were obtained courtesy of Dr Jeff Harris (Zonagen, USA). The feline ZP gene inserts were subcloned into the pKCMVint mammalian expression vector (Vical Inc., San Diego, CA, USA). This vector contains the human cytomegalovirus immediate-early 1 gene enhancer/promoter and intron A for transcription initiation with the SV40 polyadenylation signal. Sequences of insert genes were verified with GenBank sequences for feline ZP A, B and C (Harris *et al.* 1994). Large-scale plasmid preparations were obtained from terrific broth cultures of transformed *Escherichia coli* (DH-5α) using standard DNA preparation procedures with LiCl precipitation. Plasmid integrity was verified by electrophoresis and DNA concentrations determined by spectrophotometric analysis. Transcription was confirmed by ZP-specific hybridization with ZP plasmid transfected COS-7 cell supernatant. Expression of ZP proteins was confirmed by immunohistochemical analysis of transfected COS-7 cells with rabbit anti-porcine ZP antibodies, which cross-react with feline ZP (Barber *et al.* 2001).

Vaccination protocols

Porcine ZP polypeptide (B + C) purified by electrophoresis in Protoprep synthetic melting gel containing the porcine ZP polypeptide band at 55 kDa was emulsified with Freund's complete (primary vaccine) or incomplete (booster vaccines) adjuvant (Sigma Chemical Co). The control group (*n* = 5)

received blank Protoprep gel (without porcine ZP), whereas the vaccine group ($n=5$) received the porcine ZP55 preparation (75 µg polypeptide) at weeks 0, 4 and 8. Cats were immunized subcutaneously, in the dorsum of the neck, over four sites (100 µl/site) to minimize scratching of the injection sites.

Feline ZP DNA vaccines were injected into the gastrocnemius and semitendinosus muscles of the hind leg of cats, using an insulin syringe. Cats in the control group ($n=8$) were injected with the blank pkCMVint vector without ZP gene inserts dissolved in saline (400 µg DNA/cat over four injection sites). The ZPA vaccine group ($n=7$) was immunized with 400 µg of the plasmid containing the full-length feline ZPA gene dissolved in saline. The ZPB+C vaccine group ($n=5$) was immunized with separate plasmids (400 µg of each construct) containing the full-length feline ZPB and ZPC genes dissolved in saline (total 800 µg DNA/cat). Two booster vaccinations were similarly given at 1-month interval.

Blood collection

Blood samples were obtained by jugular or cephalic venipuncture at fortnight intervals post-primary immunization, and prior to euthanasia. Plasma was collected from EDTA vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA).

ELISA

Soluble-isolated porcine and feline ZP (1 µg/µl) were used as coating antigens diluted in carbonate buffer and incubated overnight at 4 °C. Non-specific antibody binding sites were blocked with 5% skim milk in PBS for 1 h at 37 °C. Primary antibody was diluted (1:10 or 1:100 in duplicate) and incubated for 1 h at 37 °C. Wells were washed three times using PBS with 0.1% skim milk/0.05% Tween-20 after all antibody reactions. Rabbit anti-cat IgG (Nordic Immunology, Tilburg, The Netherlands, 1:2000) and goat anti-rabbit IgG (H+L) conjugated with HRP (Sigma Chemical Co., 1:3500) were incubated for 1 h at 37 °C. TMB One substrate solution (Promega Corp.) was developed for up to 10 min at room temperature and stopped with 1 M HCl (50 µl/well). Absorbancies were read at 450 nm using a spectrophotometer (Bio-Rad Model 680). Absorbancies from vaccinated cat sera that were greater than the mean OD + 3 s.d. of control cat sera were considered positive. Rabbit anti-porcine ZP serum was used as a positive control (endpoint titre of 1:13 600).

Mating trials

Female cats were mated at 11 weeks post-vaccination. The cat reproductive cycle was monitored through observation of behavioural changes indicative of impending oestrus, including changes in the call sounds, rolling motions, increased scent-marking, positioning of the body and head when grasped in a mating hold and increased interest of the male to the female. Mating was arranged by introducing a single female to a male for one to three mates per day for over three consecutive days. Mating was

confirmed by visual observation and post-coital behaviour in the female. Initial determination of pregnancy was made by abdominal palpation at 2–3 weeks post-coitus with confirmation by ultrasound examination at 4–6 weeks. If the queen and litter were to be euthanased before parturition, an overdose of sodium-pentobarbitone was given no later than 6 weeks. If the queen was to carry the litter to term, she was segregated from non-pregnant females. A littering box was provided for birthing. Litter details were recorded as number of live births (average $\pm$ s.e.m.). Queens were killed once the kittens had weaned.

Immunohistochemistry

Ovaries were kept in cold PBS until placed in 10% buffered formalin and embedded in paraffin prior to sectioning (4 µm) for immunohistochemical analysis. Antigen retrieval was achieved by heating (15–30 min) in 50 mM citric acid (pH 6) for the porcine vaccine trial or Tris/EDTA/sucrose buffer (10 mM Tris; 1 mM EDTA; 1% sucrose, pH 9) for the feline ZP DNA vaccine trials. Briefly, endogenous peroxidase activity was quenched with H₂O₂. Tissue sections were incubated for 1 h with 1% BSA/5% normal goat or horse serum. Primary cat antibody (1:100) or rabbit anti-porcine ZP antiserum (1:150) was incubated for 1.5 h at room temperature. Rabbit anti-cat antibody and then goat-anti-rabbit IgG conjugated to HRP were applied for 1.5 h at room temperature and developed with 3,3'-diaminobenzidine substrate (Sigma Chemical Co.) for 10 min. Slides were counterstained with haematoxylin (Dako, Botany, NSW, Australia) and viewed using light microscopy.

Histological analysis of ovarian tissue

Follicle populations at different stages of development including primary, secondary, tertiary follicles and atretic follicles were counted in cat ovarian sections that had been fixed in 10% buffered formalin, embedded in paraffin and stained with haematoxylin and eosin. An oocyte with a single layer of granulosa cells was termed as a primary follicle. Growth of surrounding granulosa cells was used to characterize secondary follicles. Progression of the secondary follicle with an antrum was used to characterize tertiary follicles. In addition, evidence of corpora lutea was noted. Primordial follicles are not presented.

Statistical analysis

Statistical differences for the ovarian histology were assessed by Students *t*-test assuming unequal variances with *P* values <0.05 considered significant. Significance of the conception rates from the vaccine groups of the mating trials was assessed by the two-tailed Fisher's exact test with *P* values <0.05.

Declaration of interest

We declare that there is no conflict of interest with this research.

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