Differences between antigenic determinants of pig and cat zona pellucida proteins

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Despite many efforts, the control of reproduction in feral cat populations is still a problem in urban regions around the world. Immunocontraception is a promising approach; thus the present study examined the suitability of the widely used pig zona pellucida proteins (pZP) for contraception in feral domestic cats. Purified zona pellucida proteins obtained from pig and cat ovaries were used to produce highly specific antisera in rabbits. Antibodies against pZP raised in rabbits or lions were not effective inhibitors of either in vitro sperm binding (cat spermatozoa to cat oocytes) or in vitro fertilization in cats, whereas antibodies against feline zona pellucida proteins (fZP) raised in rabbits showed a dose-dependent inhibition of in vitro fertilization. Immunoelectrophoresis, ELISA and immunohistology of ovaries confirmed these results, showing crossreactivity of anti-fZP sera to fZP and to a lesser extent to pZP, but no interaction of anti-pZP sera with fZP. It is concluded that cat and pig zonae pellucidae express a very small number of shared antigenic determinants, making the use of pZP vaccine in cats questionable. A contraceptive vaccine based on feline zona pellucida determinants will be a better choice for the control of reproduction in feral cats if immunogenity can be achieved.

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Introduction

Feral domestic cats are a problem in urban regions around the world. Their large populations not only threaten many populations of birds and small mammals (Churcher and Lawton, 1985; May, 1988), but stray cats can also act as vectors for diseases and thus compromise human or animal health (Patronek, 1998; Olsen, 1999). The reproductive capacity of feral cats is high. They become sexually mature within 10 months and can give birth to two to five kittens twice a year. Theoretically, two cats producing eight kittens per year could be the progenitors of 781 250 cats in 7 years (assuming a 50% sex ratio and no kitten mortality; Olson and Johnston, 1993).

Populations can be controlled either by increasing the mortality rate or by reducing the birth rate (Tuyltens and Macdonald, 1998). Fertility control, an environmentally benign and humane form of birth rate reduction, would also satisfy many of the ethical, environmental and ecological criteria required of an effective and acceptable control method in mammals (Bomford, 1990). Therefore, there has been a substantial increase in interest in the application of fertility control to the management of feral cat populations. Ovariohysterectomy in female animals is currently the most common method of fertility control. The advantage of surgery is that irreversible infertility is attained immediately.

However, disadvantages include cost, changes in behaviour and risk of infection and mortality. Surgery also requires the capture of animals, which is often expensive, time-consuming, difficult and stressful for the animal (Asa et al., 1996). Thus, surgical methods of fertility control are not useful for the control of large populations of wild or feral animals. Innovative methods are required to avoid the problems of ovariohysterectomy and to make feasible the effective control of fertility in large populations (Carter, 1990).

Alternative methods for fertility control based on the use of gamete antigens as immunogens have been reported (Bradley, 1994). The fertility-associated protein used to promote an immune response can come from the egg, the spermatozoon or other parts of the reproductive tract. At present, the best prospect appears to be a peptide from the zona pellucida gene, which causes long-lasting contraception in several feral and wildlife species (Kirkpatrick et al., 1996). Even in abundant species it is important to target the gametogenic function of the gonad without affecting its endocrine function to render the target animal infertile but not impotent (Tyndale-Biscoe, 1994).

The aim of the present study was to examine the suitability of the widely used pig zona pellucida proteins (pZP) for immunocontraception in feral cats. Antibodies against pZP and feline zona pellucida proteins (fZP) were tested for their crossreactivity to both pig and cat zonae pellucidae to determine whether the pig zona pellucida shares antigenic determinants with the cat zona pellucida. In
addition, the contraceptive efficiency of anti-pZP and anti-
fZP antibodies was tested in a feline in vitro fertilization system.

**Materials and Methods**

Pig ovaries were collected from a local abattoir, whereas cat ovaries and testes were obtained from domestic cats castrated at local veterinary clinics. After excision, ovaries were placed in Dulbecco’s PBS (DPBS) at 4°C, washed and stored at −70°C until use. All chemicals were obtained from Sigma (Sigma Chemie GmbH, Deisenhofen) unless stated otherwise and were of the highest purity available.

**Isolation of feline and pig zona pellucida proteins (fZP and pZP)**

Cat zonae pellucidae were isolated as described by Pukazhenti *et al.* (1996). Briefly, approximately 40 thawed cat ovaries were placed immediately into centrifuge tubes together with 10 ml homogenization buffer and were homogenized with an Ultra Turrax T 25 (IKA Labortechnik, Staufen i. Br.). After homogenization, 0.001% (w/v) trypsin inhibitor (type I-S from soybean), 100 μl DNase I and 100 μl hyaluronidase were added to the homogenate. After further homogenization, 0.2% (v/v) Triton-X100, 1 mmol phenylmethanesulfonyl fluoride l⁻¹ (PMSF, 100 mmol l⁻¹ stock solution in 95% ethanol) and 500 μl of 20% (v/v) deoxycholic acid were added. The homogenate (5 ml) was then overlaid with 72.5% (v/v) Percoll in homogenization buffer and ultracentrifuged at 30 000 g for 15 min at 4°C. The white layer of the Percoll gradient containing the zonae pellucidae was transferred into an equal volume of freeze–thaw buffer (19.37 mmol NaCl l⁻¹, 4.78 mmol KCl l⁻¹, 1.7 mmol CaCl₂, 2H₂O l⁻¹, 1.19 mmol KH₂PO₄ l⁻¹, 1.19 mmol MgSO₄·7H₂O l⁻¹, 1.2% (v/v) penicillin–streptomycin, 25 mmol NaHCO₃ l⁻¹, 10 mmol Hepes l⁻¹, 5.6 mmol glucose l⁻¹, 0.02% (w/v) BSA, 0.2% (v/v) Triton-X100, 10 mmol EDTA l⁻¹, 1 mmol PMSF l⁻¹, 0.001% (w/v) trypsin inhibitor, 1% (w/v) polysorbate 20 and 1% (w/v) polyvinylpyrrolidone K25 (PVP; Fluka AG, Buchs)). The zonae pellucidae were subjected to three cycles of freezing and thawing at −20°C and room temperature, respectively. After centrifugation (2600 g, 15 min) and washing with homogenization buffer, the pellet was resuspended in 0.5 ml PBS and stored at −70°C.

The zonae pellucidae (1 ml thawed preparation) were dissolved in 2 ml of 0.3 mol lithium-3,5-diiodosalicylate l⁻¹ in 50 mmol Tris–HCl l⁻¹ (pH 7.5) stirred magnetically for 15 min at room temperature as described by Dietl (1986). After addition of 3 ml distilled water and 10 min incubation at room temperature, the preparation was centrifuged (45 000 g, 90 min, 4°C). The supernatant was stirred with an equal volume of 60% (v/v) phenol–water solution (50 min, 4°C) and centrifuged (4000 g, 60 min). After 48 h dialysis against distilled water at 4°C and lyophilization, the zona pellucida protein was dissolved in 200 μl PBS.

Approximately 20 frozen pig ovaries were thawed slowly at 4°C and dissected in 10 ml homogenization buffer (150 mmol NaCl l⁻¹, 1 mmol MgCl₂ l⁻¹, 1 mmol CaCl₂ l⁻¹, 1% (v/v) PVP, 25 mmol triethanolamine hydrochloride l⁻¹ (Merck, Darmstadt); pH 8.8) using the ovary dissection device reported by Jewgenow and Pitra (1991). After removal of minced ovaries, the buffer containing pig oocytes, somatic cells, erythrocytes and tissue shreds was transferred into centrifuge tubes and treated as described for the cat ovaries.

For biotin labelling, an aliquot (5 μg) of both pZP and fZP was incubated with 2.4 ng biotinamidocaproate N-hydroxysuccinimide ester in 220 μl PBS for 4 h at room temperature followed by the addition of 20 μl glycine. After 30 min, the reaction was stopped with an equal volume of 1% (w/v) BSA in PBS. The biotin-labelled ZP was dialysed against PBS and the protein titre was determined in a microtitre well plate.

**Antisera against cat and pig zona pellucida proteins**

Purified pZP and fZP were used to obtain polyclonal antibodies in rabbits. For both zona proteins two rabbits were treated. Immunization (100 μg protein per injection) and boosting (100 μg protein per injection) were performed by BioGenes GmbH (Berlin). The anti-titre testing was performed using a modified ELISA technique. Briefly, anti-rabbit IgG-coated microtitre well plates (Nunc, Wiesbaden) were co-incubated with increasing dilutions of antisera (100 μl) and the corresponding biotinylated zona pellucida protein (40 μl). After washing, 150 μl streptavidin–horseradish peroxidase conjugate (100 μg assay buffer ml⁻¹; Boehringer Mannheim, Mannheim) was added to each well for 45 min at room temperature. The amount of peroxidase retained in the immunocomplex after washing was determined photometrically at 450 nm with enzyme–substrate solution containing tetramethylbenzidin (TMB, Mallinckrodt Baker, Griesheim).

Blood samples of two lions (*Panthera leo*) from Rotterdam Zoo treated with pZP vaccine in 1993 and 1994 were kindly provided by W. Schaftenaar. The antibody titres of the sera were determined by species-independent pZP antibody titre ELISA (Wegner *et al*., 1998). Both pre-immune samples expressed no titre, whereas after two booster injections the pZP titre was 1:875 in lion 1 and 1:1350 in lion 2.

**Immunohistology**

Immediately after excision, ovaries from pigs and domestic cats were fixed with 3% (v/v) glutaraldehyde for at least 24 h, dehydrated and embedded in paraffin wax. Serial sections (5 μm) of ovarian tissue were cut, mounted on glass slides and air dried. Xylene was used to remove the paraffin wax from the sections and they were rehydrated in graded alcohol. After washing, the sections were exposed to 1.5% (v/v) hydrogen peroxide in PBS for 30 min to inhibit the endogenous peroxidase activity. The slides were treated with one of the anti-ZP sera (see above) by increasing dilutions for 2 h at 37°C followed by incubation with anti-rabbit IgG (whole molecule, developed in goat, 1:100; 2 h at 37°C) and peroxidase–anti-peroxidase developed in rabbit (1:100; 1 h at...
37°C). Repeated washing was performed after each incubation step. The specific anti-ZP binding sites were visualized by incubation with enzyme-substrate solution (0.8 mmol 3,3′diaminobenzidine 1−, 50 mmol Tris–HCl 1−, 0.15% (v/v) hydrogen peroxide) for 5 min at room temperature. All sections were counterstained in haematoxylin, dehydrated and embedded in Histokit (Carl Roth GmbH, Karlsruhe).

Crossed immunoelectrophoresis and ELISA

Crossed immunoelectrophoresis (Weeke, 1973) was performed in 1% (w/v) agarose (Tris-veronal buffer; pH 8.5, Biomidi, Toulouse). In the first dimension, 1 µg of either pig or cat zona pellucida protein (10 µl of 0.1 mg ml−1 stock solution) was separated followed by electrophoresis into second dimension against 200 µl of either anti-pZP or anti-fZP serum per 10 ml gel. The immunoprecipitation was stained with Coomassie blue (Serva, Heidelberg).

The ELISA was developed according to Meyer (1989) using anti-ZP serum as primary antibody and both pZP and fZP as standards. Briefly, 96-well microtitre plates (Nunc) were coated with anti-rabbit IgG. Forty microlitres of either biotinylated pZP or biotinylated fZP (1:10 000), together with 40 µl of either pZP or fZP (standard dilution of 2–2000 ng ml−1) and either 100 µl anti-pZP or anti-fZP serum per 10 ml gel was added to each well and incubated overnight at 4°C. After washing, 100 µl streptavidin–horseradish peroxidase conjugate was added to each well followed by washing, substrate reaction (TMB, 40 min, room temperature) and photometric measurement at 450 nm.

In vitro test for sperm binding and zona penetration of cat oocytes

Immediately after excision, ovaries of hysterectomized domestic cats were placed into DPBS containing 1% (v/v) antibiotic–antimycotic solution and stored for no longer than 24 h at 4°C. Immature cumulus–oocytes complexes (COCs) were liberated by mincing the ovaries with scalpel blades in Hepes minimal essential medium (MEM) with glutamine and 0.4% (w/v) BSA. The COCs were washed and transferred into 50 µl drops of culture medium, consisting of MEM containing Earle’s salts, bicarbonate and L-glutamine supplemented with 0.4% (w/v) BSA, 1.0 mmol pyruvate 1−, 10 µg LH ml−1, 1 µg FSH ml−1 and 1 µg oestradiol ml−1. For maturation, COCs were cultured in groups of 10–25 per drop covered with light mineral oil at 37.5°C in air for 24 h. After washing with culture medium, the mature oocytes were transferred into new drops and co-incubated with frozen–thawed cat spermatozoa (1 × 106 sperm cells ml−1) for 24–26 h. Half of the BSA from the culture medium (0.2% w/v) was replaced by 5% (v/v) rabbit serum (pre-immune serum or anti-ZP serum) to test the influence of ZP antiserum on sperm binding and fertilizing capacity. The rabbit sera were heat-inactivated by incubation at 56°C for 30 min.

Cat spermatozoa were obtained from cauda epididymides that were collected during castration in local animal clinics. The caudae epididymides were dissected from the testes, washed in DPBS and transferred into a culture disk (3.5 mm diameter, Nunc) containing 2 ml sperm medium (M199 with Hanks salts, with 0.4% (w/v) BSA, 10 mmol sodium lactate 1−, 1.0 mmol pyruvate 1− and 1% (v/v) antibiotic–antimycotic solution). After determination of motility, sperm suspensions with more than 80% motile spermatozoa were pooled and centrifuged (700 g, 7 min, room temperature). The pellet was resuspended in sperm medium to achieve a concentration of 5 × 106 spermatozoa 1−. A fourfold volume of cryopreservation medium (TEST-yolk extender with 7.5% (v/v) glycerin; Lengwinat and Blottner, 1994) was added slowly and the sperm samples were transferred into 0.3 ml cryo-vials (Greiner Labortechnik, Frickenhausen) and chilled for 1 h at 4°C before cooling in a programmable freezer (Nicoool 10, Air Liquide, Düsseldorf) at −1°C min−1. Seeding was performed at −7°C and at −70°C the vials were plunged into liquid nitrogen. Frozen cat spermatozoa were thawed, diluted at 1:4 with sperm medium and incubated for 10 min at 38°C. After centrifugation (900 g, 5 min, room temperature), the supernatant was discarded and the remaining pellet was carefully overlaid with culture medium allowing motile spermatozoa to swim up in a waterbath at 38°C. After 30 min incubation, the highly motile sperm population was removed, adjusted to 1 × 106 spermatozoa ml−1 and transferred to fertilization drops (5 µl per drop).

After fertilization, the oocytes were washed three times in DPBS with 0.4% (w/v) BSA to remove all loosely attached spermatozoa from the zona pellucida. The oocytes were fixed in DPBS with 4% (w/v) paraformaldehyde and 0.02% (v/v) Triton-X100 for 45 min at 37.5°C and washed again. Oocytes were stained by placing them into 10 µg Hoechst 33258 ml−1 for 45 min at 37.5°C and were washed three times before mounting on slides. Each oocyte was evaluated under fluorescence excitation using a Jenalum microscope (Carl Zeiss Jena, Jena). The number of attached sperm heads and the structure of intracytoplasmic DNA (germinal vesicle, stages of metaphase, male and female pronuclei) were estimated.

Statistical analysis

Values of bound spermatozoa are presented as means ± SEM. Data were analysed using a multi-way analysis of variance (MANOVA). For calculating significant differences among test groups, the LCD test for planned comparison (t test for dependent samples) was applied. The fertilization rate was analysed by the chi-squared test. All statistical procedures were performed with the software program Statistica for Windows (Release 4.5, copyright StatSoft Inc., 1993).

Results

A total of 2680 pig ovaries and 990 cat ovaries were processed for zona pellucida protein preparation; 2.5 µg protein per pig ovary and 9.8 µg protein per cat ovary was isolated. The immunization of rabbits with zona pellucida protein of pigs (pZP proteins) and cats (fZP proteins) resulted in a high
antibody titre after five to six booster injections. The anti-pZP sera were 1:100 000 and 1:160 000, whereas the antibody titre against fZP reached 1:160 000 in one rabbit and 1:80 000 in the other rabbit.

Immunological crossreactivity of anti-pZP antiserum to pZP and fZP

The immunological crossreactivity of the anti-pZP antibody against pig and cat oocytes was determined using several methods. In sections of pig ovary, a specific immune reaction was located at the zona pellucida and inside fully grown oocytes (Table 1; Fig. 1a), whereas in sections of cat ovary specific immunostaining could not be detected (Fig. 1b). Cross-immunoelectrophoresis showed that the antiserum precipitated exclusively with pZP (Fig. 2a,b). In the ELISA, the pZP standards (dilution from 2–2000 ng ml⁻¹) showed a typical displacement curve and 50% binding \( B_{50} \) at 660 ng ml⁻¹, whereas the fZP standards did not displace the biotinylated pZP from the antibody binding (Fig. 3a).

Immunological crossreactivity of anti-fZP antiserum to pZP and fZP

In histological sections of pig ovaries, only undiluted anti-fZP antiserum crossreacted with the pig zona pellucida (Table 1; Fig. 1c). In sections of cat ovary, antibody–antigen reactions were detectable at dilutions up to 1:1000 only on the zona pellucida outside the oocytes (Fig. 1d). In the cross-immunoelectrophoresis, both zona pellucida proteins reacted with the anti-fZP antibody (Fig. 2c,d). In the ELISA, the fZP showed a displacement curve \( B_{50} = 220 \text{ ng ml}^{-1} \), but no displacement of biotinylated fZP by increasing concentration of pZP (Fig. 3b).

Inhibition of sperm binding and penetration of cat oocytes

The effect of different schemes of protein supplementation of the culture medium on cat sperm–oocyte interaction \textit{in vitro} is shown (Table 2). The addition of rabbit pre-immune serum (5%) and anti-pZP serum at 1% and 5% resulted in a significant reduction in sperm binding and penetration rate (75% of BSA control). A specific dose-dependent inhibition was found only for the anti-fZP antiserum. Supplementation with 1% anti-fZP serum induced a highly significant \( P < 0.001 \) reduction in sperm binding and penetration rate, and 5% of this serum completely inhibited the binding and fertilizing ability of cat spermatozoa.

In a second experiment, serum samples of two lions were added to the \textit{in vitro} culture drops (Table 2). Although the lions were treated with pZP and expressed a reasonable antibody titre, the sperm binding and penetration rate were not significantly decreased.

\textbf{Discussion}

This study reveals that pig zona pellucida antigenic determinants are different from cat zona pellucida. This is in accordance with previous results which showed that zona pellucida proteins of different species express both unique and shared antigenic determinants (Drell and Dunbar, 1984; Timmons \textit{et al.}, 1987). The immunogenity of zona pellucida proteins depends not only on the type and purity of the protein administered, but is also species-specific. This species specificity is important for the design of effective zona pellucida vaccines for immunocontraception.

The zona pellucida of all eutherian mammals consists of three major glycoproteins. The primary amino acid sequences of some species, including cats, have been deduced from the nucleotide sequences of their cognate cDNAs (Harris \textit{et al.}, 1994). Although there is no overall similarity in the amino acid sequence (for example cat and pig: 72.8%), they share certain common structural motifs and even specific protein domains have been conserved among widely divergent species (Castle and Dean, 1996). Antibodies against zona pellucida protein sequences express a high cross-species specificity (Jones \textit{et al.}, 1992; Paterson \textit{et al.}, 1996; Hinsch \textit{et al.}, 1999) independent of the type of peptides used (deglycosylated, recombinant or fully synthetic). The species specificity of zona pellucida proteins is mainly

\textbf{Table 1. Detection of zona pellucida determinants by immunohistology of pig and cat ovaries using rabbit anti-pZP and rabbit anti-fZP antibodies}

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Pig ovaries</th>
<th>Cat ovaries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Granulosa cells</td>
<td>Zona pellucida</td>
</tr>
<tr>
<td>α-pZP</td>
<td></td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>–</td>
<td>+</td>
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<tr>
<td></td>
<td>1:100</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>α-fZP</td>
<td></td>
<td>–</td>
<td>+</td>
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<td></td>
<td>1:10</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>1:100</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

fZP: cat zona pellucida protein; pZP: pig zona pellucida protein.
caused by post-translational modifications, especially the N- and O-linked glycosylation. As a consequence, native zona pellucida proteins from different species are heterogeneous with respect to their immunological properties (Maresh and Dunbar, 1987).

Currently, little is known about the immunogenicity of pig zona pellucida proteins in cats or other felid species. Kirkpatrick et al. (1993) reported on the treatment of African lion and tiger with pig zona pellucida vaccine. They found high antibody titres, but no data about contraceptive efficiency were published. Contraception was not effective as one lion had one cub (the other lion was also treated with the contraceptive megestrol acetate; W. Schaftenaar, personal communication) and the tiger had three cubs (L. Kolter, personal communication). Ivanova et al. (1995) studied the contraceptive potential of pig zona pellucida in cats and reported the suitability of pZP vaccination for cat contraception. However, the vaccination dose exceeded the ‘standard’ dose of pZP vaccine (65 μg, Kirkpatrick et al., 1993) five times and four booster injections were necessary to reach an effective contraceptive antibody titre. In the study of Ivanova et al. (1995), the indirect immunofluorescence of feline anti-pZP serum to pig oocytes was much more intensive than the reaction with cat oocytes. These studies on pZP contraception in cats support the findings of the present study that pig and cat zona pellucida proteins are

**Fig. 1.** Detection of zona pellucida determinants by immunohistology of pig (a,c) and cat (b,d) ovaries using rabbit anti-pig zona pellucida protein (pZP) (a,b) and rabbit anti-cat ZP (fZP) (c,d) antibodies. For each combination, the highest antibody dilution showing an immune reaction is presented. (a) Immunostaining of pig zona pellucida and oocytes using an anti-pZP antibody diluted 1:100. (b) No detection of crossreaction of anti-pZP antibody (undiluted) with cat oocytes. (c) Immune reaction of anti-fZP antibody (undiluted) with zona pellucida and ooplasm of pig oocytes. (d) Immunostaining of cat zona pellucida using a highly diluted (1:1000) anti-fZP antibody. Scale bars represent 20 μm.
characterized by different immunogenic epitopes and cast doubt on the contraceptive effectiveness of pZP vaccines.

The high antibody titres in lion and tiger after vaccination with pZP demonstrate the immunogenity of the vaccine. However, this finding does not imply that antibodies that react specifically with pig zona pellucida proteins inhibit sperm–zona pellucida binding in the target species (Bagavant et al., 1993; Hinsch et al., 1999). Thus, in addition to the screening of antibody titres, the contraceptive potential of anti-pZP antibodies should be examined. In vitro systems are very suitable for this purpose. The results of the present study show that in vitro binding to, and penetration of, homologous
oocytes by cat spermatozoa is an appropriate prerequisite for analysing contraceptive potential of anti-ZP sera. In the case of the two lion sera from Rotterdam Zoo, it was possible to show that no inhibition of cat fertilization was achieved despite a reasonable antibody titre (1:850 and 1:1350).

The application of pig zona pellucida protein vaccines for the contraception of wildlife and feral animals has been promoted, especially since it was demonstrated to be effective in inhibiting fertility in free-roaming feral mares on Assateague Island National Seashore (Kirkpatrick et al., 1990). More than 60 species in four orders have been treated with pZP vaccine with varying success. Fertility suppression has been effective in a variety of ungulates, including feral (Equus caballus) and Przewalski’s (E. przewalski) horse, Himalayan tahr (Hemitragus jemlahicus), sika (Cervus nippon), and white-tailed deer (Odocoileus virginianus), but little or no contraception has been achieved in fallow (C. dama dama), axis (C. axis), sambir (C. unicolor) and muntjac deer (Muntiacus reevesi) (Kirkpatrick et al., 1993, 1996), felids and African elephants ((Loxodonta africana) (Kirkpatrick et al., 1998). In equids, there were no changes in social organization and behaviour, whereas in some cervids, the rutting period was prolonged (McShea et al., 1997). The efficiency of immunocontraception may vary significantly among individuals of the same species (Asa et al., 1996).

The most severe side-effect of pZP immunization is ovarian failure. This has been observed in rodents, canids and primates (Sacco et al., 1987; Mahi-Brown et al., 1988; Sacco et al., 1989; Jones et al., 1992). Immunization with zona pellucida antigens that appear early in folliculogenesis can induce inflammatory processes in the ovary that can lead to sterility. Conversely, antibodies to zona pellucida antigens that appear late in oocyte growth apparently do not interfere with ovarian function (Timmons et al., 1990). Reversibility of contraception is important when the purpose is to slow down the reproductive rate in small valuable populations (for example, zoo animals). However, in feral domestic cats, irreversibility of contraception would be tolerable if not desirable.

As the application of pZP vaccine for contraception is inefficient in cats, the decision was made to develop a feline zona pellucida vaccine. Antibodies against feline zona pellucida protein raised in rabbits (this study) and in guinea pigs (Jewgenow et al., 1994) are very effective in blocking sperm–egg interactions in cats. The immune reaction of cats to fZP vaccination is currently still insufficient (I. Wegner, unpublished) and requires new approaches to fZP vaccine production. Since feline ZP1 protein is synthesized very early during oogenesis in cats (Jewgenow and Fickel, 1999), recombinant or synthetic feline ZP1 peptide sequences, which include immunogenic epitopes, should be identified. The highly specific anti-fZP antibody reported in the present study can be used to define immunogenic epitopes by screening libraries made from randomly fragmented DNAs encoding feline ZP1 (Castle and Dean, 1996). ZP peptides in mice, which were identified by epitope mapping and coupled to a carrier protein, not only elicited antibodies that reacted to native zonae pellucidae, but also induced long-term infertility (Paterson et al., 1998).

In conclusion, the low degree of common immunological determinants between cat and pig zonae pellucidae indicate that the pZP vaccine is inefficient for feral cat contraception. Thus, it is necessary to develop a specific fZP-based vaccine which has sufficient immunogenity in cats.

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Table 2. Examination of contraceptive efficiency of anti-zona pellucida protein (ZP) antibodies using a feline in vitro test for sperm binding and zona penetration

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Protein supplement</th>
<th>Number of oocytes analysed</th>
<th>Number of spermatozoa per oocyte</th>
<th>Penetrated oocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-control</td>
<td>0.3% (w/v) BSA</td>
<td>74</td>
<td>23.2 ± 3.4*</td>
<td>45.5*</td>
</tr>
<tr>
<td>Control I</td>
<td>5% rabbit pre-immune serum</td>
<td>98</td>
<td>13.5 ± 2.3*</td>
<td>28.9*</td>
</tr>
<tr>
<td>Anti-pZP serum</td>
<td>1% serum + 4% pre-immune serum</td>
<td>114</td>
<td>12.4 ± 1.7*</td>
<td>34.6*</td>
</tr>
<tr>
<td>Anti-ZP serum</td>
<td>1% serum + 4% pre-immune serum</td>
<td>107</td>
<td>15.8 ± 2.2*</td>
<td>35.5*</td>
</tr>
<tr>
<td>Control II</td>
<td>5% lion pre-immune serum</td>
<td>76</td>
<td>20.9 ± 6.2</td>
<td>35.7*</td>
</tr>
<tr>
<td>Lion 1</td>
<td>1% lion serum + 0.24% (w/v) BSA</td>
<td>22</td>
<td>29.9 ± 6.2</td>
<td>45.5*</td>
</tr>
<tr>
<td>Lion 2</td>
<td>1% lion serum + 0.24% (w/v) BSA</td>
<td>35</td>
<td>15.9 ± 2.5</td>
<td>28.6*</td>
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<tr>
<th>Experimental group</th>
<th>Protein supplement</th>
<th>Number of oocytes analysed</th>
<th>Number of spermatozoa per oocyte</th>
<th>Penetrated oocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control II</td>
<td>5% lion pre-immune serum</td>
<td>76</td>
<td>20.9 ± 6.2</td>
<td>35.7*</td>
</tr>
<tr>
<td>Lion 1</td>
<td>1% lion serum + 0.24% (w/v) BSA</td>
<td>22</td>
<td>29.9 ± 6.2</td>
<td>45.5*</td>
</tr>
<tr>
<td>Lion 2</td>
<td>1% lion serum + 0.24% (w/v) BSA</td>
<td>35</td>
<td>15.9 ± 2.5</td>
<td>28.6*</td>
</tr>
</tbody>
</table>

a,b,c,d Different superscripts show significant differences (P < 0.05) between the mean number of spermatozoa per oocyte.

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