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Immunologic responses and adverse reactions to Freund's-adjuvanted porcine zona pellucida immunocontraceptives in domestic cats

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Abstract

A vaccine of native PZP with Freund's adjuvant has been widely used in zoo and wild ungulates, but safety in felids has not been evaluated. General health, immune response, and ovarian histology were assessed in five domestic cats vaccinated with PZP-Freund's and five cats given Freund's adjuvant alone. Peak antibody titers occurred 3 weeks after the third vaccination, and no ovarian lesions were present 6 months after vaccination. Seven cats developed extensive granulomatous reactions at injection sites, lymph nodes, and multiple visceral organs including lungs and brain. Persistent hypercalcemia and compromised renal function occurred in three cats with elevated serum calcitriol of probable macrophage origin. One cat died from an injection site sarcoma. Because of these severe adverse reactions, Freund's adjuvant is contraindicated in cats, and other adjuvants for PZP vaccines should be tested in cats for adverse reactions before use.

Keywords: Freund's adjuvant; Hypercalcemia; Porcine zona pellucida

1. Introduction

Controlling reproduction in both genetically-valuable and overabundant zoo felids is essential for ex situ conservation programs. Reproductive planning requires effective contraception so that captive breeding programs can be implemented without disrupting established social groups. Effective and reversible contraceptives also would be valuable for some domestic cat breeding programs in catteries and might be an effective tool for controlling feral cats.

Immunocontraception with native porcine zona pellucida (PZP) antigens has been widely applied across many species, because these heterologous antigens incite antibodies that prevent fertilization, presumably by blocking the sperm attachment site on the zona [1,2]. Varying degrees of efficacy have been demonstrated for PZP immunocontraceptives in hamsters [3], rabbits [4,5], mice [6], dogs [7], seals [8,9], non-human primates [10], elephants [11,12], a variety of cervids [13–19], horses [20,21], and exotic equids and bovids [22]. Two previous studies have used PZP immunocontraceptives in domestic cats [23,24]. In one study, five cats were given 50 μg PZP administered intramuscularly with Freund's complete adjuvant (FCA; 0.5 ml), revaccinated at 2, 4, and 6 weeks with 50 μg PZP in Freund's incomplete adjuvant (FIA), and then revaccinated with 200 μg PZP in FIA at 92 days [23]. After 90 days with a fertile male, one of five cats became pregnant, suggesting the vaccine provided some contraceptive efficacy. However, in a second study in which 30 8–12-week-old kittens were given a single dose of either 125 μg PZP in liposomes (Spay Vac™, Immuno-vaccine Technologies, Inc., Halifax, NS, Canada) emulsified with 0.25 ml FCA or 200 μg Spay VacJ emulsified with an alum adjuvant, all vaccinated cats became pregnant when placed

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with a fertile male after they were 5–6 months old despite high anti-PZP titers [24]. These results indicate that a single vaccination with this formulation at this age did not achieve contraception. Differences in efficacy between these trials, as well as across many other clinical trials, suggest that PZP dose, adjuvant, and vaccination schedule influence efficacy and that there are notable species differences in response.

One concern of using immunocontraceptives in genetically valuable animals has been the potential for permanent ovarian damage that could affect reversibility. As with the efficacy trials, there has been considerable disparity in ovarian pathology results among PZP contraceptive studies and species. The most common lesions have included ovarian atrophy from depletion of oocytes, reduced folliculogenesis, and follicular degeneration, noted in mice, rabbits, baboons, bonnet monkeys (*Macaca radiata*), and marmosets (*Callithrix jacchus*) [4,25–34]. Increased follicular atresia and granulosa cell nests also have been noted [3,26,29,33]. Inflammatory cell infiltrates have been observed in some cynomolgus macaques (*Macaca fascicularis*) and mice [6,35,36], but oophoritis did not occur in other studies with these species. These conflicting results can be explained in part by different formulations of PZP (whether native, purified, or recombinant) and by use of different adjuvants. Several studies strongly support an adjuvant influence on the development of oophoritis [36]. In one trial, mice given ZP peptides bound to keyhole limpet haemocyanin had no ovarian lesions, whereas mice given these ZP peptides with FCA developed oophoritis [6,36]. Another study using PZP with FCA or a lipopolysaccaride adjuvant in bonnet monkeys found follicular atrophy only in FCA treated animals [29]. Although Freund's adjuvants are widely used for PZP immunocontraceptives in deer populations, ovarian damage was not observed [19].

Freund's adjuvant is commonly used with PZP vaccines because the resulting inflammatory reactions incite an excellent antibody response [37]. However, potential adverse effects of FCA/FIA on ovarian function in felids need to be assessed before PZP immunocontraceptives are widely applied to zoo and cattery populations. Reactions to FCA/FIA are of particular concern in cats, because of recent links between vaccine site reactions and the development of sarcomas [38]. The aim of this study was to assess the safety in domestic cats of the PZP-Freund's adjuvanted vaccine currently used for zoo and wildlife felids and to distinguish the effects of Freund's adjuvant from those of PZP. The study monitored physical and clinical pathologic parameters and evaluated ovarian health after vaccination.

2. Materials and methods

2.1. Study animals

Ten 1–1.5 year-old specific pathogen free female domestic cats were obtained from Cornell University. The cats received a preliminary health evaluation including a complete physical examination, hematology and serum chemistry analyses, and were given 30 days to acclimate to their surroundings. All cats were vaccinated with FVRCP (BioVac Inc. Sioux City, SD) on Day-25 and Day-7 of the study, rabies (ImRab3, Rhone Merieux, Athens, GA, USA) on Day-25, and feline leukemia (Fel-O-Vax Lv-K, Fort Dodge Laboratories, Ft. Dodge, IA, USA) on Day-7 and Day 21 of the study. The study animals were housed as a group at the University of Tennessee Laboratory Animal Facility with a 12 h light/dark cycle. They had free access to commercial dry food and water.

2.2. Vaccine and adjuvant preparation

Porcine ZP were isolated using techniques described previously [39,40]. Briefly, frozen thawed porcine ovaries were minced in cold phosphate-buffered saline (PBS) using a razor-blade apparatus. The oocytes were separated from other tissues, including granulosa cells, by screen filtration, counted, and homogenized in a Potter-Elvehjen homogenizer. The zonae were isolated on a 48 μm screen, heat solubilized at 70 °C, and frozen in doses of 20 μg protein (approximately 1500 zonae) in 0.5 ml PBS until shipment. Freund's complete adjuvant and FIA were purchased commercially (Sigma–Aldrich Co., St. Louis, Missouri 63178, USA). Freund's complete adjuvant consisted of paraffin oil, mannide monooleate, and heat-killed *Mycobacterium tuberculosis*; FIA consisted of paraffin oil and mannide monooleate alone. Individual vaccines were prepared using 0.5 ml PZP and 0.5 ml adjuvant, emulsified together immediately prior to injection.

2.3. Study schedule

On Day 0, five cats received 0.5 ml of PZP with 0.5 ml of FCA and five cats received 0.5 ml of FCA mixed with 0.5 ml of sterile saline (control). On Days 21 and 43, PZP-treated animals received a second and third vaccine consisting of PZP in FIA, and control animals received FIA alone. For all vaccinations, the 1 ml vaccine volume was divided and administered intramuscularly in two sites in the lumbar musculature by hand injection. Cats were given butorphanol (Fort Dodge Laboratories, Fort Dodge, Kansas 50501, USA; 0.2 mg/kg, SQ) concurrent with each vaccination for pain management.

Cats were observed daily for appetite, signs of estrus, or abnormal behavior. Cats received weekly physical examinations by a veterinarian, including body temperature, auscultation, and palpation of the injection site. Blood was taken on Day 0, 21, 43, 64, 108, 134 and 219 for hematology, serum chemistries, and PZP titers. Additional blood samples were taken as needed to monitor health, when abnormal results were detected during scheduled sampling.

At 6 months after the third vaccination, all cats were ovariohysterectomized using standard surgical procedures.

The cats were anesthetized with ketamine (25 mg/kg IM; Fort Dodge Laboratory, Fort Dodge, Kansas 50501, USA), xylazine (1 mg/kg IM; Rompun, Miles, Shawnee Mission, Kansas 64506, USA) and atropine (Miles; 0.04 mg/kg IM), then intubated and maintained on isoflurane gas anesthesia. Because three cats developed persistent hypercalcemia and abnormal renal parameters during the trial, kidney biopsies were taken from all three hypercalcemic cats and three normocalcemic cats at the time of ovariohysterectomy. For renal biopsies, the capsule of the left kidney was incised and a 5 mm diameter wedge biopsy including cortex and medulla was removed from the caudal pole. The capsule was closed with 3-.0 PDS (Ethicon Inc., Somerville, NJ, USA) using a single horizontal mattress suture. All surgical sites were checked for hemostasis before the abdominal incision was closed. The cats were recovered with 0.13 mg/kg yohimbine (Lloyd Laboratories, Shenandoah, IA, USA), 3 mg/kg doxapram (Fort Dodge Laboratories), and 0.2 mg/kg butorphanol.

At the termination of the study, six habituated cats were placed in homes. The other four cats (Cat B, Cat F, Cat I and Cat J) were transferred into another study. After the termination of that study on post-PZP vaccination day 360, the four cats were humanely euthanized and complete necropsies were performed.

2.4. Histological analyses

All tissue samples were fixed in 10% neutral buffered formalin. Ovaries were measured then sectioned along the sagittal plane and one half of each ovary cut at 5 mm transverse sections. All ovarian sections were examined histologically. Two cross sections of each uterine horn were sampled for histopathology, and all renal biopsy tissue was processed. All major organs were sampled from necropsied animals, as well as lumbar musculature at injection sites and regional lymph nodes. All tissues were processed routinely, sectioned at 7 μm, and stained with hematoxylin and eosin.

One sagittal section and one midline transverse section from each ovary were randomly selected for quantitative analyses. Each selected tissue section was examined at least twice to minimize intraobserver error rates. The following structures were quantified by light microscopy: secondary follicles without zona, secondary follicles with developing zona pellucida, tertiary follicles, total number of intact zona pellucidas, corpora lutea, granulosa cell nests, atretic secondary follicles, and atretic tertiary follicles. For the purpose of this study, secondary follicle atresia was defined by the presence of a shrunken oocyte, at least 5% pyknotic or fragmented granulosa cell nuclei, or a fragmented, deformed, or collapsed zona. Tertiary follicle atresia included the same criteria as for secondary atresia and the presence of fibroplasia in the antrum. Granulosa cell nests were solid clusters of granulosa cells without an oocyte or zona pellucida present in cut section. All ovarian sections were evaluated for the presence of primordial and primary follicle, mineralization, inflammatory cell infiltrates, and abnormal ovarian structures such as cystic rete ovarii. Uterine and kidney samples were evaluated for any abnormal histologic findings.

2.5. Hematology and clinical chemistries

Blood taken at Day 0, 21, 43, 64, 108, 134 and 219 was submitted for hematology and serum chemistries to the clinical pathology laboratory of the University of Tennessee College of Veterinary Medicine. Laboratory reference intervals (RI) for healthy cats were used for comparisons.

2.6. Calcitriol and PTH assays

1-25-dihydrocholecalciferol (calcitriol) was measured using a commercial competitive radioimmunoassay with a column extraction procedure (DiaSorin Inc., Stillwater, MN, USA). The assay used a polyclonal antibody specific for both 1,25-$(OH)_2$ D_3 and 1,25-$(OH)_2$ D_2. Immunoreactive PTH was measured from a subset of cats for which adequate serum was available, using a commercial IRMA immunoradiometric assay from Diagnostic Products Corporation (Los Angeles, CA, USA).

2.7. Analyses

Mean values for hematological and clinical chemistry parameters were calculated for: (1) all cats before vaccination, (2) Freund's adjuvanted PZP-vaccinated cats (PZP-F), and (3) cats vaccinated with Freund's adjuvant alone (controls). Differences among groups were evaluated by non-parametric methods (Kruskal-Wallace test). Differences between PZP-F and control groups were analyzed by Dunn's Multiple Comparison Test. Relationships between creatinine and calcium levels and between calcium and calcitriol levels were analyzed by Spearman regression coefficients. Concentrations of calcitriol were compared between treated and control animals and between study animals before and after vaccination by Mann-Whitney tests. Quantified ovarian features in sagittal and transverse sections were added for a total count for each cat, then values for each feature were compared between PZP-treated and control animals by nonparametric tests (Mann-Whitney). Significance for all tests was set at $p < 0.05$.

3. Results

3.1. PZP titers

All vaccinated cats developed antibody responses to PZP (Fig. 1). Maximum titers were measured on day 63 of the study, 3 weeks after the third vaccination.

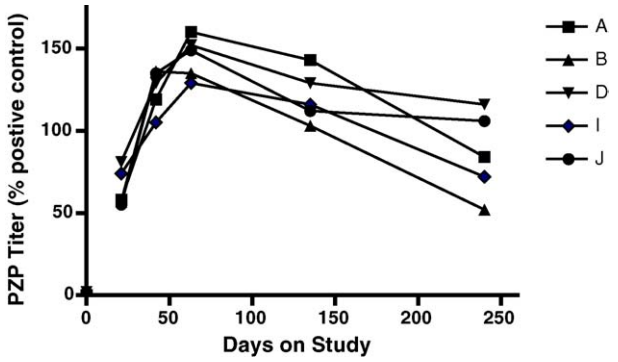

Fig. 1. Antibody responses to porcine zona pellucida (PZP) vaccination in domestic cats.

3.2. Injection site reactions

Seven of 10 cats developed painful swellings in the lumbar musculature at the site of vaccination and in five of these cats, the swelling was extensive and accompanied by regional lymphadenopathy (Table 1). The injection site reactions were first detected between day 35 and 50 of the study, and regional lymphadenopathy was noted by Day 85. Muscle lesions were soft and focal at first, but later became firm and extended proximally and distally from the injection site. The swellings did not resolve with time and were present up to 1 year after vaccination. Three PZP-treated cats (Cat A, Cat I, and Cat J) and two control cats (Cat E and Cat H) had moderate to severe reactions. One additional treated and two control cats (Cat B, and Cats C and F, respectively) had mild injection site reactions. Cat G died from a fibrosarcoma that originated in the axial muscles above the kidneys at the site of vaccination. In general, PZP-treated animals had more severe reactions with bilateral lumbar musculature and lymph node involvement.

Cats gained weight during the study, except for a transient weight loss in two cats that developed persistent hypercalcemia after the third vaccination (Cat A and Cat I). No animals became febrile after treatment. Behavioral signs of estrus were not noted until January (Day 160), although behavioral observations were hampered by the fractious nature of the animals.

3.3. Abnormal clinical pathologic parameters

After PZP vaccination, several blood parameters were significantly different from pre-vaccination levels (Table 2). Calcium was elevated in 9 cats with 31 of 94 post-vaccination blood samples >11 mg/dl (range of values outside of RI = 11.1–16.2). Five cats had only a transient elevation, but two cats had prolonged (4–6 months) hypercalcemia.

Table 1
Local injection site reactions and maximum calcium levels in 10 domestic cats vaccinated with Freund's adjuvant (F) with or without porcine zona pellucida antigens (PZP)

Cat ID	Treatment	Injection site reaction[a]	Highest serum calcium (mg/dl)	Outcome
A	PZP + F	+++	16.2	Hypertensive at 10 years
B	PZP + F	+/–	11.1	Euthanasia at 2 years
C	F	+/–	10.9	Healthy until killed by car at 8 years
D	PZP + F	–	11.3	Healthy at 10 years
E	F	++	11.6	Healthy until lost to follow up at 9 years
F	F	+/–	11.2	Euthanasia at 2 years
G	F	–	11.9	Died from fibrosarcoma at vaccination site at 11 years
H	F	++	13.3	Died of cardiomyopathy at 4 years
I	PZP + F	+++	14.8	Euthanasia at 2 years
J	PZP + F	+++	11.4	Euthanasia at 2 years

[a] Injection site reactions were defined as firm swellings detected during physical examination.

Table 2
Mean values for selected serum chemistry and hematology parameters in domestic cats vaccinated with porcine zona pellucida (PZP) in Freund's adjuvant ($n = 5$) or with Freund's adjuvant alone ($n = 5$)

Blood parameter	Reference intervals for domestic cats	Pre-vaccination	PZP-vaccinated cats	Adjuvant-vaccinated cats
Calcium (mg/dl)	9–11	10.5 ± 0.57	11.6 ± 1.9 a	10.4 ± 1 b
Phosphorous (mg/dl)	3–7	6.2 ± 0.9 a	5.3 ± 1.2 b	5.5 ± 1
Creatinine (mg/dl)	0.8–1.8	1.1 ± 0.16 a	1.5 ± 0.46 b	1.5 ± 0.36 b
Urea nitrogen (mg/dl)	15–30	26.4 ± 5.3	29.1 ± 7.4	29.9 ± 3.3
Albumin (g/dl)	2.5–4.5	4.0 ± 0.2 a	3.4 ± 0.4 b	3.5 ± 0.3 b
Globulin (g/dl)	2.9–5.1	3.2 ± 0.27	3.5 ± 0.49	3.7 ± 0.64
WBC ($10^3/\mu l$)	5.5–19.5	10.6 ± 3	11.6 ± 3.7 a	8.6 ± 3.1 b
Neutrophils ($10^3/\mu l$)	2.5–12.5	5.8 ± 2.6	6.3 ± 2.8 a	4.6 ± 2.9 b
Band neutrophils ($10^3/\mu l$)	0–0.3	0.04 ± 0.06	0.13 ± 0.22	0.06 ± 0.09
Lymphocytes ($10^3/\mu l$)	1.5–7	3.9 ± 1.0	4.2 ± 2.3	3.4 ± 1.4
Monocytes ($10^3/\mu l$)	0–.85	0.16 ± 0.18	0.25 ± 0.24	0.14 ± 0.14

Within rows, values with different letters (a, b) are significantly different ($p < 0.05$) by Kruskal-Wallace nonparametric tests.

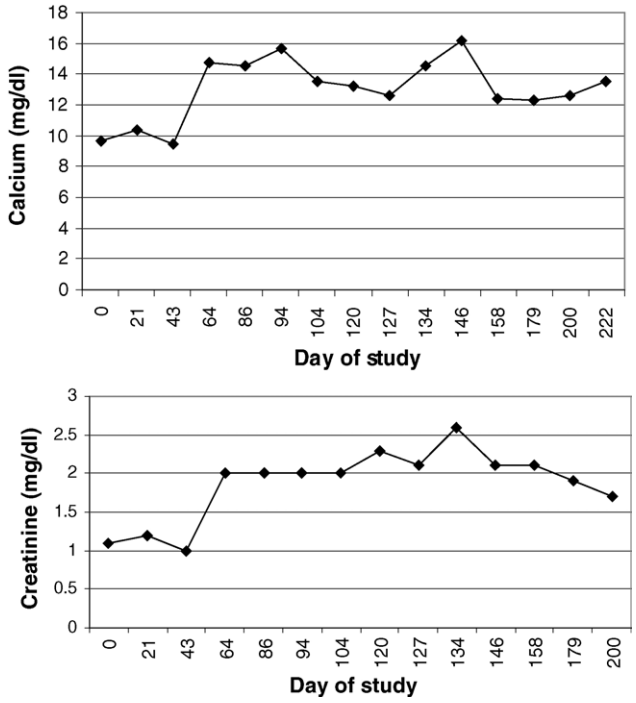

Fig. 2. A domestic cat vaccinated with porcine zona pellucida in Freund's adjuvant. Persistent hypercalcemia paralleled elevations in serum creatinine and reduced glomerular filtration rate. The affected cat lost 25% of pre-vaccination body weight during the period of hypercalcemia.

Elevated serum calcium concentrations for PZP-vaccinated animals were first detected on Day 43, and the highest calcium concentration was 16.2 mg/dl in Cat A on Day 143 (Fig. 2). The earliest and highest serum calcium value for control vaccinated cats was Day 108 and 13.3 mg/dl respectively. Mean calcium concentration in PZP-treated cats was significantly higher ($p < 0.01$) than controls. Phosphorous concentrations were elevated in four cats post-vaccination (range of values outside of the RI = 7.1–9.4 mg/dl). The two highest phosphorous concentrations occurred in Cat A during periods of compromised renal function (Day 86 and Day 157; see case summary). However, mean phosphorous concentration for the PZP- vaccinated group was significantly lower ($p < 0.05$) than pretreatment concentrations.

BUN was mildly elevated in nine cats (approximately one third of all samples). The range of values outside of RIs were 31 to 161 mg/dl with all values >90 mg/dl in Cat A). Creatinine concentrations were outside of normal RIs in seven cats (21 of 108 samples; range of values outside of RI were 1.9–2.7 mg/dl), but only one cat had prolonged elevations. The earliest elevated creatinine concentration for PZP-vaccinated animals was noted on Day 64 and the highest value was 2.6 mg/dl. Earliest and highest value for control vaccinated cats were Day 108 and 2.5 mg/dl, respectively. Mean creatinine concentrations for PZP- vaccinated and control-vaccinated animals were significantly higher ($p < 0.05$) than pretreatment concentrations. Alanine aminotransferase (ALT) was mildly elevated in nine cats (approximately 25% of samples; range of 76–289 IU/L), although most values above normal RIs were in pre-vaccination samples. The mean ALT in prevaccination samples was significantly higher ($p < 0.05$) than control-vaccinated and PZP-vaccinated concentrations, although there were no differences between mean ALT levels in PZP and controls. For aspartate aminotransferase (AST), all except one sample were within normal RI. This one sample was only slightly elevated (102 IU/L) and was from an animal being treated for hypercalcemia (Cat A).

WBC levels in PZP vaccinated cats were significantly higher ($p < 0.01$) than controls. One PZP-vaccinated cat had marked lymphocytosis (>10,000 cells/μl) at two sampling periods (Day 108 and Day 134). Values for all other parameters were within normal RI for cats.

3.4. Hypercalcemia

Three cats (2 PZP-vaccinated and 1 control vaccinated cat) developed a persistent hypercalcemia that was first detected in one cat 3 weeks after the second vaccination and in two other cats 3 weeks after the third vaccination. Four additional cats (2 PZP and 2 control) developed a transient hypercalcemia detected on Day 64 or Day108. In two persistently hypercalcemic cats, phosphorous also was elevated. Ca*Phos product in hypercalcemic cats (PZP vaccinated = 75.9; control vaccinated = 71) increased above mean pre-vaccination values (65.1) by Day 64 and remained greater than normal until the end of the study.

Calcitriol concentrations (293.9 ± 121.6 pmol/ml) were significantly higher ($p < 0.05$) in cats with serum calcium concentrations ≥11.5 mg/dl than in cats with serum calcium concentrations ≤11 mg/dl (197.6 ± 62.4 pmol/ml)(Table 3). Calcitriol increased over pre-vaccination concentrations in all three hypercalcemic cats, but not in normocalcemic cats. Calcitriol concentrations correlated with calcium levels ($p = 0.02$; $r = 0.58$). Immunoreactive parathyroid hormone (IPTH) was significantly lower ($p = 0.05$) in hypercalcemic

Table 3
Serum concentrations of calcium, 1–25 dihydrocholecalciferol (calcitriol) and immunoreactive parathyroid hormone (PTH) in domestic cats vaccinated with porcine zona pellucida (PZP + F) in Freund's adjuvant ($n = 5$) or with Freund's adjuvant (F) alone ($n = 5$)

Cat ID	Vaccination	Serum calcium (mg/dl)	Calcitriol (pmol/l)	PTH[a] (pmol/l)
A	PZP + F	14.5	514.8	
B	PZP + F	11.1	186.4	
C	F	10.5	254.1	
D	PZP + F	9.2	196.1	2.3
E	F	11.6	196.1	
F	F	9.2	164.1	
G	F	9.9	170.3	3.1
H	F	13.8	224.3	1.7
I	PZP + F	14.6	248	2
J	PZP + F	11.5	349.7	

[a] PTH concentrations were only determined in a subset of cats from which sufficient serum was available.

cats (1.6 ± 0.3 pg/ml; $n = 3$) than normocalcemic cats (2.5 ± 0.2 pg/ml; $n = 3$).

For all cats in the study, creatinine concentrations correlated with calcium concentrations ($p < 0.01$; $r = 0.296$). For vaccinated cats, this relationship was stronger ($p < 0.002$; $r = 0.325$) and for PZP treated animals alone the correlation was strongest ($p = 0.0003$; $r = 0.489$).

3.5. Summary of the most severely affected case

Cat A developed a firm mass at the PZP vaccine injection site 2 weeks after the third vaccination (Day 57). One week later, serum calcium was 14.7 mg/dl and creatinine was 2 mg/dl (Fig. 2). The cat began to lose weight and by Day 85 had lost 24% of the pre-vaccination body weight. Serum calcium continued to rise (Fig. 2) and serum phosphorous increased. Ca*Phos product on Day 86 was 136 and on Day 142 was 110. On Day 64, serum creatinine was elevated above RI and remained elevated for the remainder of the study. Because of the persistent azotemia, glomerular function was measured by exogenous creatinine clearance and determined to be 1.96 (normal rate for domestic cats = 2.5). The cat became anorectic, had intermittent vomiting, and was hospitalized when serum calcium became >16 mg/dl. The cat was treated with subcutaneous saline solution, placed on a low calcium diet, and returned to the study group when serum calcium was <13 mg/dl. Glomerular function was again measured at the termination of the study (Day 219) and determined to be only 45% of normal. At the termination of the study, the cat was placed in a home. Eight years after the study, the cat developed hypertension (systolic blood pressure = 240 mm), was euthyroid, but had a creatinine of 2.0 mg/dl. Renal ultrasound revealed small, irregular kidneys.

3.6. Histopathologic findings

Mean counts for each ovarian structure in PZP-treated and control groups are listed in Table 4. The only significant difference between groups was the greater number of atretic secondary follicles in control cats. This difference was largely due to a single cat with abundant folliculogenesis and atresia. Although this cat was one of the persistently hypercalcemic cats, increased atresia was not noted in other hypercalcemic cats. Small numbers of lymphocytes were noted in the ovarian stroma of most cats, but no lymphocytes were spatially associated with follicles or zona pellucidas. Eight of 10 cats (four PZP-treated and four controls) had mild endometrial hyperplasia, and all cats had mild lymphocytic infiltrates in the endometrium.

The three cats with persistent hypercalcemia had multifocal pyogranulomatous nephritis that was most marked in the interstitium surrounding blood vessels, but also encompassed and obliterated some tubules. Clear vacuoles (presumed paraffin oil) or necrotic debris were present in the center of some inflammatory foci. Additionally, some renal tubules were ectatic, but glomeruli appeared normal. Renal biopsies in normocalcemic cats were within normal limits.

Table 4
Quantified ovarian features in cats vaccinated with Freund's adjuvanted porcine zona pellucida (PZP) antigens

Ovarian feature	PZP mean ± S.D.	Control mean ± S.D.	*P* value[a]
Ovarian volume (mm^3)	518 ± 725.8[b]	314.2 ± 173.4	0.99
Primordial and primary follicles	Plentiful	Plentiful	ND
Secondary follicle with oocyte	11 ± 6.7	8.2 ± 7.4	0.46
Secondary follicle with zona	5.8 ± 4.8	7 ± 7.1	0.92
Tertiary and preovulatory follicle	11.4 ± 5	13.2 ± 6.3	0.92
Total healthy zona pellucidas	18.4 ± 8.1	21.2 ± 14.7	0.92
Corpora lutea	2 ± 2.1	1.4 ± 3.1	0.52
Granulosa cell nests	6.2 ± 3	6.4 ± 6.5	0.69
Atretic secondary follicles	4.2 ± 3.1	24.6 ± 17.4	0.008
Atretic tertiary follicles	12.2 ± 6.4	24.2 ± 11.4	0.1

Control cats were vaccinated with Freund's adjuvant alone.
[a] Based on Mann-Whitney nonparametric tests.
[b] One PZP treated cat had several cystic rete ovarii.

The four cats that were euthanized had severe locally extensive granulomatous myositis with fibrosis that was most severe at the injections sites, but also extended for several centimeters proximally and distally (Fig. 3). Sublumbar lymph nodes were markedly enlarged (4–5 times normal) and firm. Histologically, normal muscle tissue was effaced by extensive coalescing pyogranulomas surrounding necrotic debris and lipid (Fig. 4). The architecture of the sublumbar lymph nodes was also effaced and replaced by pyogranulomas. Additionally, the cats had disseminated pyogranulomas with central necrosis in the lungs (Figs. 5 and 6), kidneys, distant lymph nodes, spleen, liver, lamina propria of the esophagus and intestines, bladder, foot pads, choroid plexus, and meninges. Acid fast bacteria were not identified in any lesions, but lipid vacuoles were present in some of the disseminated granulomas.

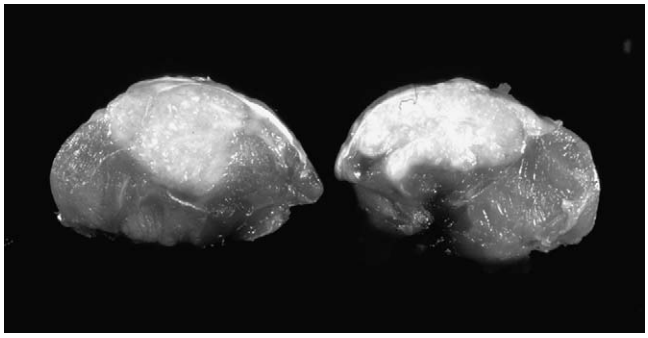

Fig. 3. Gross appearance of the lumbar musculature of a domestic cat with chronic myositis at vaccination sites of Freund's adjuvanted porcine zona pellucida.

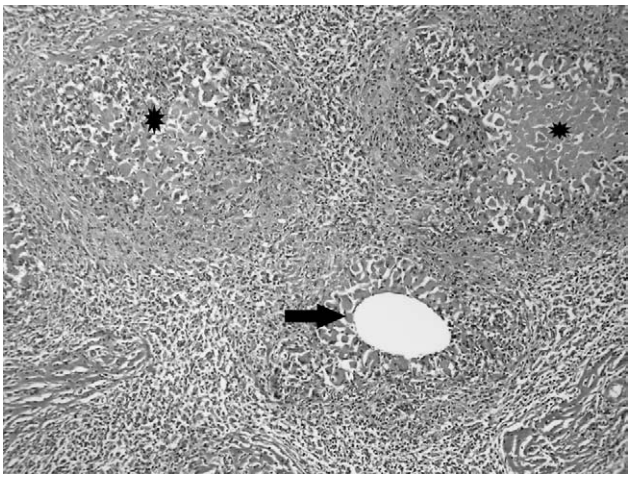

Fig. 4. Granulomatous myositis in a cat vaccinated with Freund's adjuvanted porcine zona pellucida. Note the multiple granulomas (asterisks) associated with adjuvant lipid (arrow).

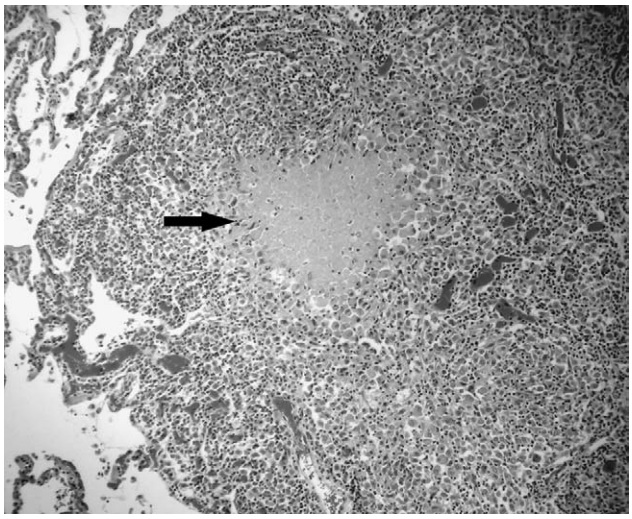

Fig. 6. Granulomatous reaction in the lung of a domestic cat vaccinated with Freund's adjuvanted porcine zona pellucida. The reaction surrounds a central zone of necrosis (arrow).

4. Discussion

This study demonstrated that PZP vaccines when given with Freund's adjuvant stimulate a robust antibody response without evidence of ovarian atrophy or oophoritis. Regardless, use of this vaccine with Freund's adjuvant should be contraindicated for cats because of the hypercalcemia that occurred in some vaccinated animals. This hypercalcemia occurred in both PZP- and adjuvant-vaccinated animals, demonstrating a clear association with the adjuvant. However, the two most severely affected animals were PZP vaccinated, suggesting that PZP antigens may enhance the negative effect. PZP-vaccinated animals also developed hypercalcemia and azotemia earlier than control animals. Hypercalcemia has been noted in other domestic cats several months after receiving Freund's adjuvant (S VandeWoude, pers. com.), so this reaction was not unique to this study.

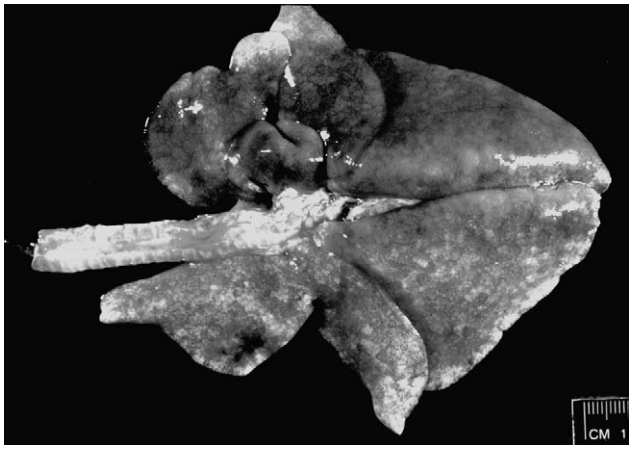

Fig. 5. Gross appearance of the widely disseminated granulomatous pneumonia in a domestic cat vaccinated with Freund's adjuvanted porcine zona pellucida.

The elevated serum calcitriol concentrations in the hypercalcemic cats indicates unregulated endogenous production leading to increased intestinal uptake and reduced renal excretion of calcium. The source of calcitriol in these cats was most likely macrophages that have 1α hydroxylase capable of metabolizing 25-hydroxycholecalciferol to 1,25-OH D_3 [41–45]. The effectiveness of Freund's adjuvant is largely due to the extensive monocytic infiltration it incites, and adding an immunizing antigen enhances this effect [37,46]. The extensive macrophage infiltrates noted in the injection site lesions, as well as systemically in the cats, would provide a potential source of unregulated calcitriol production. It is interesting to note that PZP-vaccinated cats appeared to be affected more severely and earlier than animals vaccinated by adjuvant alone, corroborating the synergistic effect noted by Freund [37].

The cause of the azotemia and loss of glomerular function in the three cats with persistent hypercalcemia was not apparent from the renal biopsies. Although interstitial microgranulomas damaged some tubules, the extent of renal parenchymal loss was not sufficient to account for the azotemia. Also, renal parenchymal calcification was minimal in biopsies, although this negative finding could be due to fact that biopsies were taken at the end of the study when calcium levels were not maximal. Hypercalcemia affects glomerular function in other species without a light microscopic lesion [47]. A similar mechanism likely occurred in the study cats because serum creatinine levels paralleled those of calcium. A direct effect of Freund's adjuvant on glomerular function also is possible, as has been proposed in rats, although serum calcium was not measured in these studies [48]. The specific cause of the remarkable weight loss in the cats with hypercalcemia also is unknown, although weight loss also occurred in other cats with

Freund's adjuvant-associated hypercalcemia (S Vande-Woude, pers. com.) and has occurred in black rhinoceros (*Diceros bicornis*) with hypercalcemia (Munson and Citino, unpublished).

The extensive tissue damage at the injection sites was similar to, but more severe than, lesions reported in other species [49–51]. Also, the disseminated granulomatous lesions distant from the vaccination site was more notable in extent in cats. Whether the fibrosarcoma at the injection site of one cat was associated with vaccination cannot be confirmed, although this is an atypical site for this cancer in cats and the tumor surrounded the chronic granulomas associated with the adjuvant. Granulomatous lesions in the lungs and lymph nodes have been reported in rabbits, guinea pigs, monkeys, and mice after Freund's administration [50,52–55], but no other visceral lesions nor meningeal or choroid plexus involvement was reported. In these reports as well as in the study cats, the granulomatous response surrounded adjuvant lipid and not *Mycobacteria*, unless they were too scarce to be detected by acid fast stains.

Most cats appeared to have no long term health problems from the vaccine-site reactions or hypercalcemia. One study cat died from cardiomyopathy and another has hypertension and azotemia, but the link between these conditions and hypercalcemia cannot be proven. Certainly myocyte function would have been compromised by the levels of calcium that occurred in some cats, but these levels did not persist once vaccination was discontinued.

Freund's adjuvant has been used in other PZP contraceptive trials in cats [23,24], but extensive tissue damage and hypercalcemia were not reported. Differences in adjuvant volume as well as number of vaccinations could account for this discrepancy with one study [24]. The other study used the same volume of adjuvant and multiple doses, but serum chemistries were not evaluated, so subclinical effects may have been overlooked [23].

The current study focused on vaccine safety, so efficacy was not assessed. Although measurable titers were achieved with this vaccine formulation, PZP antibody titers of less magnitude were not sufficient to prevent fertilization in another group of domestic cats [24]. It was presumed that the absence of estrus behavior during the first segment of the study was attributable to the late summer/autumn scheduling of the study (when normal seasonal anestrus occurs) and not to ovarian damage. The reappearance of estrus cycles in December and January when antibody titers were at their highest supports a seasonal and not vaccine-induced anestrus.

Despite the widespread inflammation associated with adjuvant oil that occurred in the PZP-Freund's vaccinated cats, no inflammation was seen in the ovaries in contrast to studies in Freund's treated mice [6,35]. Why some formulations of ZP antigens and adjuvants incite oophoritis in several species [35,36] is not clear, but warrants further investigation before these vaccines are to be used in genetically valuable zoo animals.

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