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Immunocontraception of Captive Exotic Species. III. Contraception and Population Management of Fallow Deer (*Cervus dama*)

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Immunocontraception has become an increasingly valuable tool in the population management of captive exotic ungulates. Although porcine zona pellucida vaccine (PZP) was used successfully in other cervids, a previous study with fallow deer (*Cervus dama*) suggested that the vaccine did not work in this species. In the current study, PZP was tested in two captive herds of fallow deer. Antibody titers were monitored over a 3-year period to evaluate three different adjuvant protocols, and the vaccine was applied to an entire herd to determine the impact on fawning rates. In a semi-free-ranging herd, antibody titers rose from preimmunization levels of 2.6% of positive control serum to 56.5% 4 weeks after initial inoculations, to 65.1% at 1 year, and to 81.3% at 2 years, after a single annual booster was applied. Fawn production in this herd was reduced significantly over 3 years. The adjuvant protocol of Freund's Modified Adjuvant® (FMA) for the initial inoculation followed by a booster with Freund's Incomplete Adjuvant® (FIA), and the protocol of FMA for the initial inoculation followed in 3 weeks by a booster with FMA both produced significantly higher antibody titers than the 3 × FIA (3 weeks apart) protocol after year 1. The FMA + FMA protocol produced significantly higher titers than the 3 × FIA protocol at year 2, but was not different from the titers produced by the FMA + FIA protocol at year 2. Zoo Biol 22:261–268, 2003. © 2003 Wiley-Liss, Inc.

Key words: contraception; fallow deer; fertility control; porcine zona pellucida

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INTRODUCTION

Porcine zona pellucida (PZP) vaccine has been used successfully to inhibit fertility in numerous mammalian species [Kirkpatrick et al., 1996a], including at least 40 captive exotic species [Frisbie and Kirkpatrick, 1998; Kirkpatrick et al., 1995, 1996b]. The vaccine has been exceptionally successful in reducing fertility in ungulates; however, a prior attempt at PZP vaccination of fallow deer (*Cervus dama*) was reported as unsuccessful. In a study of PZP contraception in 10 fallow deer at Fossil Rim Conservation Center, there was no significant fertility inhibition (E. Blumer, DVM, personal communication).

The findings in the previous study in fallow deer were in contrast to success with the PZP contraceptive vaccine in several other species and subspecies within the family Cervidae. Contraceptive success has been reported in white-tailed deer (*Odocoileus virginianus*) [Turner et al., 1992, 1996; McShea et al., 1997; Naugle et al., 2002], Formosan sika deer (*Cervus nippon taiouanus*), axis deer (*Cervus axis*), Roosevelt elk (*Cervus elaphus roosevelti*), sambar deer (*Cervus unicolor*) [Kirkpatrick et al., 1996b], Rocky Mountain elk (*Cervus elaphus nelsoni*) [Garrott et al., 1998], tule elk (*Cervus elaphus nannodes*) [Shideler et al., 2002], and Mandarin sika deer (*Cervus nippon mandarinus*) (K. Frank, personal communication).

The evolutionary conservation of the zona pellucida sperm receptor has resulted in broad efficacy of the PZP vaccine not only in the cervids, but in the majority of mammalian species studied. Thus, there was no logical biological explanation for the failure of the PZP vaccine in fallow deer.

Another issue regarding immunocontraception in cervids is the adjuvant of choice. Because of the evolutionary conservation of the sperm receptor, PZP is a poor immunogen, and successful contraception relies on an effective adjuvant to enhance the vaccine's immunogenicity. A great deal of the original application of PZP in wildlife has utilized Freund's Complete Adjuvant® (FCA) [Kirkpatrick et al., 1996a], which is regarded as the "gold standard" among adjuvants [Bennet et al., 1992]. In those species for which reliable tuberculosis tests exist, FCA carries with it the possibility of causing TB + tests following its use. Also, there is historical data derived almost exclusively from laboratory animals indicating that the use of FCA can lead to abscesses [Broderson, 1989]. Thus, there is a clear need to identify and test alternate adjuvant systems.

The present studies were undertaken to 1) reevaluate the contraceptive efficacy of the PZP vaccine in fallow deer, 2) examine the B-cell-mediated humoral immune response in this species in response to PZP treatment, 3) test three different adjuvant protocols, and 4) attempt to limit fawn production in a captive herd of fallow deer.

METHODS AND MATERIALS

Animals

Two different populations of fallow deer were used in this study. One population was located in Cooperstown, New York, under the supervision of the Clark Foundation, and was housed exclusively outdoors in a 49-ha pasture. Each animal was identified with a uniquely numbered ear tag. These numbers were arbitrary and used only for TB-testing records, and there were no age records associated with the numbers. This herd was essentially managed as a free-ranging

herd, although there was a perimeter fence. A number of animals were sampled annually for tuberculosis testing and each animal was given an intramuscular injection of ivermectin (Ivomec; Merck, West Point, PA). The entire herd fed on pasture grasses and was given grain at a rate of 0.68 kg/deer/day, and a single bale of grass hay once per day in the summer and two bales per day in the winter. During the course of this study, the population ranged from 58 to 84 animals. Prior to the start of this project, managers did not collect fawning data on individual does, and time constraints did not permit pairing individual fawns with mothers after the project began. The second population of nine animals was displayed at Belle Isle Zoo, a component of the Detroit Zoological Institute. These nine animals (all nonbreeding adult females) were derived from a wild herd inhabiting Belle Island, and the age of only one animal was known (7297, born in 1999). These nine does were used for antibody titer analysis and a comparison of adjuvants. Each animal was identified with a uniquely numbered ear tag.

PZP Vaccine Preparation

The native PZP antigen was prepared at the Science and Conservation Center (SCC; ZooMontana, Billings, MT) from porcine ovaries by the modified methods of Dunbar et al. [1980]. The PZP antigen was screened for porcine viruses by the USDA laboratory in Ames, Iowa, and for pathogenic bacteria at the SCC. The antigen was titrated to 65 μg doses, stored at–44°C, and transported frozen to the site of use.

Treatment Protocol

Over the course of the 3-year study, 65 deer from the Cooperstown herd were treated in August/September. In 1998, 10 deer (20.8% of the herd's does) were treated with either (1) three inoculations of 65 μg PZP in 0.5 phosphate-buffered saline (PBS) emulsified with 0.5 ml Freund's Incomplete Adjuvant® (FIA) (Sigma, St. Louis, MO), as described by Kirkpatrick et al. [1996b], spaced approximately 2 weeks apart; or 2) with an initial injection of 65 μg PZP in 0.5 ml PBS + 0.5 ml Freund's Modified Adjuvant® (FMA; Calbiochem, Inc., La Jolla, CA) followed by a single inoculation of PZP + FIA 2 weeks later. Treated animals were given booster inoculations of 65 μg PZP + FIA in August/September 1999, 2000, and 2001, and new animals were started on treatment in each of those years as well. All inoculations were given on the right side. Blood was collected during August/September of each year. Deer were restrained in a drop chute and bled by jugular venipuncture in 5-cc tubes. Blood was collected at the time of the initial PZP inoculation and then at 12-month intervals.

Inoculation of the nine Belle Island deer was carried out with an initial inoculation of 65 μg PZP + FMA, followed by a second inoculation approximately 2 weeks later, using either PZP + FMA (n=5) or PZP + FIA (n=4). All annual booster inoculations in subsequent years were carried out with PZP + FIA. Thus, across the two populations comparisons were made between three adjuvant protocols: 3 × PZP + FIA; 1 × PZP + FMA followed by 1 × FIA; and 2 × FMA. All inoculations were given i.m., on the left side, in the semitendinosis or gluteal muscles, and 5-ml blood samples were taken during restraint in a drop chute.

Anti-PZP Antibody Analysis

A total of 5 ml of blood was collected in a serum separator tube and serum was stored frozen until assay. Antibody titers were measured by the method described by Bynum [2001], with modifications for fallow deer. Heat-solubilized PZP protein was diluted to a concentration of 130 μg/ml in PBS and then further diluted in coating buffer (0.1 M Na_2CO_3, pH 9.6) using 138 μl of the diluted PZP solution and 22.5 ml coating buffer. Then 200 μl of this dilution was placed in each well of a 96-well Nunc plate and incubated overnight at 4°C. Blank wells contained only coating buffer. After incubation, the plates were washed three times with PBS-0.05% Tween buffer. A blocking solution consisting of PBS-0.05% Tween and 1.0% gelatin was placed into each well and incubated 1 hr at 37°C.

The plates were washed five times with 200 μl of PBS-Tween buffer each time, followed by the addition of the primary antibody. Initial dilutions of reference, control, and test sera were made with PBS-0.05% Tween-0.1% gelatin and incubated 1.5 hr at 37°C. After the plates were washed five times with PBS-0.05% Tween buffer, a 1:400 solution of secondary antibody, consisting of anti-deer IgG conjugated to alkaline phosphatase enzyme (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted in PBS-0.05% Tween-0.1% gelatin was added to each well (200 μl/well) and incubated 1.5 hr at 37°C. Following incubation, plates were washed three times with PBS-0.05% Tween buffer and then twice with PBS.

Finally, an enzyme substrate solution (200 μl/well) of 0.1 M Na_2CO_3, $MgCl_2$, H_2O and p-nitrophenyl phosphate (Sigma Chemical, St. Louis, MO), was added to each well and allowed to react for 30–60 min at room temperature with gentle shaking until absorbance of the positive reference serum reached an optical density of approximately 1.5. After color development the plates were read at 405 nm on a Molecular Devices Model Emax® spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA) using noncoated wells as reagent blanks.

All test sera were assayed in duplicate and expressed as a percent of the positive reference sera, which consisted of a pool of sera from white-tailed deer that had demonstrated anti-PZP titers in the high-positive range (mean of experimental serum absorbance/mean of reference serum absorbance), and had not become pregnant following treatment. The dilutions used in these determinations correspond to the dilution of the reference sera giving 50% maximum binding. Differences in antibody titers were tested for significance by Fisher's exact test for contingency tables [Motulsky et al., 2001].

RESULTS

Among the does of the Cooperstown herd, fawning rates dropped from 63.4% in 1998, after only 10 of 48 adult females (20.8%) were treated, to 0% in 2001, after 59 of 61 adult females (96.7%) were treated (Table 1). Linear regression analysis showed an $r=0.9968$ correlation between percent of does treated and fawns produced over the 3-year period of the study. Preimmunization antibody titers were 2.5%±0.5% (SEM) (n=15) of positive reference standards. They rose to 56.5%±6.0% (n=10) at 4 weeks postimmunization (or 2 weeks following the first booster inoculation), and continued to rise to 66.9%±3.1% (n=47) at 12 months, and to 85.8%±6.2% (n=21) at 24 months (Fig. 1).

TABLE 1. Fawn production in the Cooperstown herd between 1998 and 2000*

Treatment year	Total females	PZP treated females	Post-treatment fawns (%)
1998	48	10	–
1999	59	49	26 (54.1)
2000	61	59	3 (4.9)
2001	58	58	0 (0.0)

*The results of contraception occur during the year followin treatment.

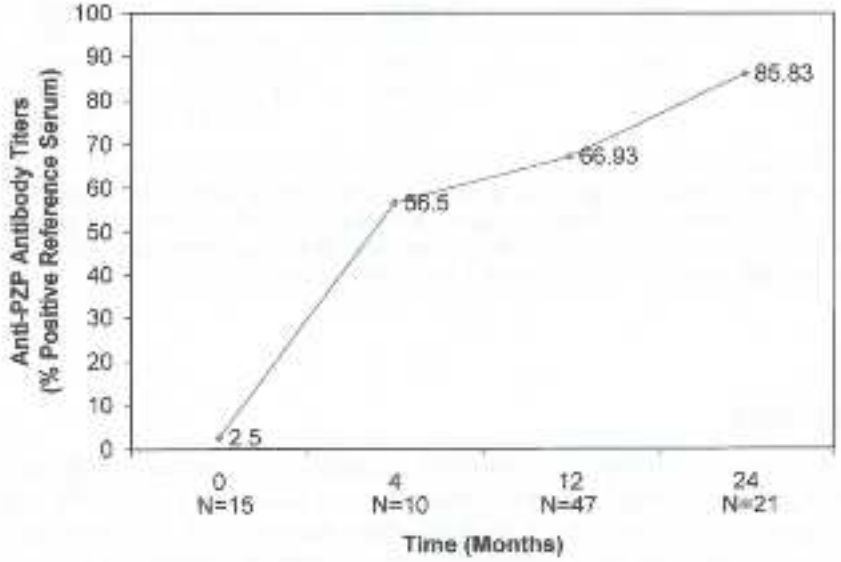

Fig. 1. Anti-PZP antibody titers in the Cooperstown fallow deer over 24 months. Titers are given as a percent of a reference standard for PZP-treated white-tailed deer that did not fawn after treatment.

At 12 months postimmunization, antibody titers for deer immunized with 3 × FIA (n=8) were 52.6% ± 6.9% (Table 2). They were significantly less than the four Belle Island deer immunized with two inoculations of FMA + FIA (114.8% ± 11.7%; P=0.0006, t=4.90), and also significantly less than the four Belle Island deer immunized with FMA + FMA (113.0 ± 11.4; P=0.0007, t=4.80). After all animals had been given a single booster of PZP + FIA at year 1, the difference between titers in the 3 × FIA group (75.1% ± 7.0%) and the FMA + FIA (89.0% ± 17.4%) group was no longer significant (P=0.40; t=0.88). In contrast, the second-year titers for the FMA + FMA group (113.0 ± 11.4) were significantly higher than the second-year titers for the 3 × FIA group (75.1% ± 7.0%; P=0.01, t=3.0). By the second year there was no significant difference between the FMA + FIA group and the FMA + FMA group (P=0.67, t=0.44).

There were no health problems associated with treatment during the study period, and no abscesses or granulomas at the injection sites in any animals, regardless of the adjuvant protocol used.

TABLE 2. Antibody titers for Belle Island fallow deer*

Deer no.	Booster adjuvant type	Pretreatment antibody titers % of reference standard (O.D.)	Posttreatment antibody titers % of reference standard (O.D.)	
			Year 1	Year 2
7111	FMA	10 (0.13)	112 (1.56)	115 (1.61)
7114	FMA	11 (0.14)	138 (1.93)	91 (1.30)
7115	FMA	12 (0.16)	83 (1.16)	30 (0.42)
7117	FMA	10 (0.13)		
7118	FMA	6 (0.07)	119 (1.67)	76 (1.07)
7119	FIA	8 (0.10)	99 (1.38)	51 (0.71)
7297	FIA	3 (0.04)	148 (2.08)	128 (1.79)
7112	FIA	4 (0.06)	98 (1.38)	70 (0.99)
7116	FIA	6 (0.08)	114 (1.60)	107 (1.50)
Reference serum	(1.31)	(1.31)	(1.40)	(1.40)

*Antibody titer values are given as a percent of the optical density for reference serum standard for PZP-treated white-tailed deer which did not produce fawns. All animals were given FMA for the initial inoculation and booster inoculation adjuvants were either Freund's Incomplete Adjuvant[R] (FIA) or Freund's Modified Adjuvant[R] (FMA).

DISCUSSION

All inoculated fallow deer in both populations, regardless of the adjuvant protocol used, produced anti-PZP antibodies in response to the PZP-adjuvant inoculations. The production of antibody titers occurred with the same vigor and over approximately the same time frame as was previously observed in other cervids, including white-tailed deer [Turner et al., 1996; McShea et al., 1997; Miller et al., 2000], wapiti [Garrott et al., 1998], Formosan sika deer, axis deer, muntjac deer, Roosevelt Elk, and sambar deer [Kirkpatrick et al., 1996b]. Titers generated at 4 weeks postinoculation, after 3 × PZP + FIA, reveal a statistically significant response after the initial priming inoculation and a single booster 2 weeks later. At this time, however, no "contraceptive threshold value" has been determined for antibody titers for fallow deer, as was done previously for horses [Liu et al., 1989]. Thus, the second booster at 4 weeks is probably necessary for some assurance that animals will remain infertile for a year. The 4-week time frame for significant antibody titers to appear also indicates that treated females should not be placed back with males for at least 6 weeks, if the breeding season is already under way.

However, the presence of anti-PZP antibodies alone does not ensure interference with fertilization and infertility [Paterson et al., 1996; Prasad et al., 1996]. That these antibodies can cause infertility in fallow deer is demonstrated by the dramatic decrease in fawning rates among the Cooperstown herd. Thus, it may be concluded that well-elevated anti-PZP antibodies in fallow deer are associated with infertility, and, based on other studies of the PZP vaccine [Nakano et al., 1996], it can also be inferred that the infertility results from interference with fertilization.

One of the major difficulties with the application of the PZP vaccine in wildlife, captive or otherwise, is the need for multiple inoculations during the first year of treatment. In previous experiments with horses, FCA was used for the initial inoculation. This adjuvant was sufficiently effective to permit 1 year of effectiveness with a single booster at 1 month [Kirkpatrick et al., 1990]. However, in species other than equids FCA may cause false-positive tuberculosis tests, because of the incorporation of dried cell fragments of *Mycobacterium tuberculosis* in the adjuvant. This is unacceptable in captive wildlife populations. To overcome this problem, the application of PZP in captive wildlife has most often utilized three inoculations using FIA [Kirkpatrick et al., 1996b], which does not cause false-positive TB tests. However, FIA is such a weak adjuvant that three inoculations are necessary the first year.

The use of FCA has been associated with abscess formation in laboratory animals [Smith et al., 1992] and in horses when given in the neck (I.K. Liu, personal communication). When given in the hip, gluteal, or semitendinosis muscles, this has not been a problem in wild horses [Kirkpatrick et al., 1990] or other captive [Frisbie and Kirkpatrick, 1998; Kirkpatrick et al., 1996b] or free-roaming [Turner et al., 1996] wildlife species. Nevertheless, some concern remains regarding abscess formation. In the present study, no abscesses occurred, which demonstrates the safety of FMA and FIA in this regard when administered in the semitendinosis or gluteal muscles.

The use of FMA in the initial inoculation, and a single booster inoculation containing FIA in the first year produced high antibody titers and necessitated only two inoculations. The titers observed following this protocol were statistically significantly higher than those generated with 3 × FIA at the end of the first year, and there was no danger of false-positive TB tests because FMA contains the dried cell walls of *M. butyricum* rather than *M. tuberculosis*. When the protocol of 2 × FMA was used, antibody titers were higher than the 3 × FIA in both years 1 and 2.

Although the causes for the earlier contraceptive failure with fallow deer remain undetermined, it is unlikely that they were biological in nature. Reversibility data for treatment effect are currently inadequate, but based on previous studies with white-tailed deer and elk [Turner et al., 1996, McShea et al., 1997; Shideler et al., 2002], fertility is expected to return as antibody titers fall over time.

CONCLUSIONS

1. PZP vaccine delivered to fallow deer i.m. causes a significant antibody response that follows a time course similar to that in other PZP-treated cervids.

2. PZP vaccine delivered to fallow deer is associated with a decline in fertility, as evidenced by declining fawning rates.

3. Contraceptive PZP treatment of fallow deer requires either three inoculations using FIA or two inoculations using 1) FMA for the initial inoculation and FIA for the booster, or 2) FMA for both the initial and booster inoculation during the first year of treatment.

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