IMMUNOCONTRACEPTION IN FERAL HORSES: ONE INOCULATION PROVIDES ONE YEAR OF INFERTILITY

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Abstract: We determined contraceptive effectiveness of a 1-inoculation, 1-year porcine zona pellucida (PZP) vaccine in free-roaming feral horses (*Equus caballus*) in Nevada. We captured, freeze-branded, treated, and subsequently released 267 adult feral mares given (1) 2 inoculations (13–17 days apart) of vaccine emulsion consisting of aqueous PZP and Freund's Complete Adjuvant (FCA; injection 1) or Freund's Incomplete Adjuvant (FIA; injection 2), (2) same 2 inoculations as in (1) except also containing a carbomer adjuvant in inoculation 1, or (3) a single inoculation of an emulsion of PZP and FCA containing a second dose of PZP and carbomer adjuvant in controlled release polymer microspheres. We administered inoculations in January 1996, and monitored the mares via fecal analysis for pregnancy and via ground survey for foal production through October 1997. We determined pregnancy via measurement of estrone sulfate and progesterone metabolites in fresh feces collected from the ground. Among 2-inoculation mares, reproductive success across 1 year was 12.8% (carbomer adjuvant absent) and 10.6% (carbomer adjuvant present). In mares given 1 injection containing microspheres, reproductive success was 11.3%. The concurrent rate in 72 untreated mares was 62.5%. This study revealed marked and equivalent 1-year contraceptive efficacy in 1- and 2-inoculation PZP vaccine, indicating that controlled-release technology can replace a second inoculation and thereby increase PZP vaccine cost effectiveness and potential for use in feral horse management.

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The potential use of contraception to provide a safe and cost-effective means of regulating feral horse populations has been under investigation for 25 years (Turner and Kirkpatrick 1991). The strongest candidate from these investigations is a PZP vaccine first tested in the horse by Liu et al. (1989). The vaccine appears to act by stimulating anti-PZP antibodies that bind to the surface of the ovulated egg, preventing sperm attachment (Liu et al. 1989).

In initial studies, a vaccination protocol of 2 separate inoculations about a month apart has yielded reversible infertility for 1 breeding season in a number of species, including horses (Liu et al. 1989, Kirkpatrick et al. 1990), burros (*E. asinus*; Turner et al. 1996), white-tailed deer (*Odocoileus virginianus*; Turner et al. 1992), and African elephants (*Loxodonta africana*; Fayrer-Hosken et al. 1997). However, it is impractical economically and strategically in wildlife management to access free-roaming species for 2 separate inoculations in 1 month. Thus, a 1-injection vaccine effective for a minimum of 1 year is needed.

Toward that end we have pursued the use of polymers to delay the release of a portion of the vaccine as a mimic of the second injection. This methodology involves forming a homogenous mixture of bioactive ingredients with a biodegradable, nontoxic lactide-glycolide polymer in the form of microspheres (Eldridge et al. 1989, Wang et al. 1990). Upon intramuscular injection and contact with tissue fluids, the polymer material erodes and releases the bioactive contents (Wang et al. 1991).

Preliminary assessment of several controlled-release preparations in vitro revealed continuous release with greater amounts released at the beginning and end of the release period (D. Flanagan, University of Iowa, personal communication). We subsequently tested 1 of these controlled-release preparations of lactide-glycolide microspheres in a 1-injection PZP vaccine in free-roaming feral horses (Turner et al. 1997). Although a single injection containing PZP microspheres was associated with reduced fertility,

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it was less effective than 2 separate inoculations and was not more effective than a single injection without microspheres.

The microspheres used in this study did not contain adjuvant. Incorporation of adjuvant into polymer microspheres requires that the adjuvant be water-soluble. A carbomer (Carbopol 934, B. F. Goodrich, Cleveland, Ohio, USA) has previously been used as a water-soluble adjuvant in pigs (*Sus* sp.; Gualandi et al. 1988) and was effective as an adjuvant for PZP in raising serum anti-PZP titers in laboratory studies with rabbits (*Oryctolagus cuniculus*; Fritz 1997).

We report the field test of a 1-inoculation contraceptive vaccine on feral horses. Specifically, we tested (1) the effectiveness of microspheres formulated for bolus-release (rather than continuous-release) characteristics and (2) the effect of combining PZP microspheres and Carbopol® adjuvant microspheres in a single injection. We hypothesized that these conditions would closely resemble a second injection of vaccine and would yield infertility similar to that obtained with 2 separate inoculations.

STUDY AREA

The study area, Nevada Wild Horse Range, encompasses about 2,880 km^2 in south-central Nevada centered at 37° 40′ latitude and 116° 40′ longitude inside the Nellis Air Force Base complex. Elevations ranged from 1,561 to 2,493 m. Vegetation on the portion of the range most used by experimental horses was composed largely of black brush (*Coleogyne ramosissima*) and sagebrush (*Artemisia* sp.) communities. Grasses commonly used by horses in these communities are Indian rice grass (*Oryzopsis hymenoides*), galleta (*Hilaria jamesii*), and desert needlegrass (*Stipa speciosa*). Average annual precipitation (1992–98) was 74.4 cm at 2,000 m elevation. Water was available from several permanent springs in the study area. During the study period >90% of the observations were made in an area <1,000 km^2, because drought had limited water-site availability. The study area was administered by the Las Vegas district of the Bureau of Land Management (BLM), United States Department of the Interior.

METHODS

Horse Population

The study area was inhabited by about 1,700 horses with a harem band social structure (Turner et al. 1981, Berger 1986). We subjectively estimated the physical condition of mares included in the study in the corral prior to inoculation and in the field during subsequent observations according to the condition scale (1–10) developed by Henneke et al. (1983). Initial mare condition ranged from poor (2) to good (6). Condition-2 mares comprised <20% of those treated, and no condition-1 mares were treated. None of the mares deteriorated in condition across the study, and many improved as a result of better range condition after a January 1996 removal of several hundred horses from the range.

PZP Vaccine Emulsion Preparation

We prepared PZP from porcine ovaries (Liu et al. 1989). The non-microsphere portion of the vaccine consisted of an emulsion of 0.5-cc FCA with 65 µg PZP (Group A) or 65 µg PZP + 10 mg CAR (Group B) in 0.5-cc phosphate buffer solution. We emulsified PZP with FIA for the second injection. We prepared the emulsion, 8 cc's at a time, within 24 hr of injection, using 2 10-cc glass syringes joined with a plastic connector. After 100 plunger strokes we loaded the emulsion into 3-cc plastic syringes for injection via an 18-gauge 3.7-cm needle. We prewashed the needle and injection site with 70% ethanol. The emulsion was kept chilled but was hand-warmed prior to injection.

Microsphere Preparation

The microspheres were 10–100 µm in diameter and composed of bioerodable lactide and glycolide polymers. They were prepared using a coacervation method (Wang et al. 1991). Projected release rates were to be characterized by some initial release, with most release after week 4 (Wang et al. 1990, 1991). In vitro testing of microsphere release characteristics was performed prior to bulk PZP–CAR incorporation using a Solid-Phase Protein Assay (SPA) (Sulter et al. 1993) and a modified enzyme-linked immunosorbent assay (ELISA) to measure PZP release into the incubation medium from microspheres. This SPA ELISA technique used affinity biotinylation of amino groups found in proteins with subsequent binding of enzyme-labeled avidin for detection. Quantification was based on the optical density of each sample upon reaction with a color substrate. A 60–80 µg dose of PZP incorporated into 15 mg of microspheres was placed into each of 3 molecular-sieve capsules (Millipore, Bedford, Massachusetts, USA), which permitted passage of released PZP but not

microspheres. The capsules were incubated separately with gentle shaking (2 back-forth motions/sec) in 3.0 ml bacteriostatic PBS at 37°C for 6 months. Every 3 days 100 µl of the solution was removed and assayed for PZP.

The ratio of mixed CAR–PZP microspheres in the medium for vaccine injection was 3:1. The average PZP content of PZP-containing microspheres was 0.5%, and the average CAR content of CAR-containing microspheres was 2.0%. The release of CAR from microspheres was not measured. However, release characteristics were expected to be similar to PZP, with the exception that the greater loading percentage of CAR microspheres was expected to yield some CAR release immediately after injection (Wang et al. 1990).

Accessing, Handling, and Inoculating Horses

The BLM gathered horses by helicopter, permitting injection by hand. We chose this method because access to the horses was possible as a part of scheduled roundup for the BLM horse adoption program. Between 3 and 9 January 1996, the BLM gathered about 800 horses by helicopter into portable corrals. With our assistance, the BLM (1) separated the gathered stallions and mares, (2) moved them singly through a chute, (3) estimated age through dentition and physical condition, (4) gave a 1-cc prophylactic Strepguard® (Miles Laboratories, Shawnee Mission, Kansas, USA) injection intramuscularly, and (5) permanently marked 267 healthy mares >10 years of age with consecutive numbers by freeze-branding. The brands were located on the upper left hip, were 10 cm in height, and were readable from a helicopter and through a spotting scope at >500 m distance on the ground.

In some feral horse herds reproduction is reduced in mares >20 years old. However, data for Nellis (NWHR) horses from prior years showed fertility rates in mares aged 10–20 were similar to those rates in mares >20 (Bureau of Land Management, unpublished data). Thus, we used mares aged >20 which also were in condition >3. We hand-injected intramuscularly 1 cc of freshly prepared emulsion of buffered PZP-adjuvant into the left gluteus muscle, with or without an accompanying injection of microspheres (in 2 cc of 1% aqueous CAR), and we photographed each marked mare. The BLM maintained mares not scheduled for a later injection in the portable corrals on grass-hay and water ad libitum ≤4 days and then released them into the range area from which they had been gathered. Before release, the BLM separated the mares and their foals and retained the foals for adoption. They separated mares scheduled for the month-later injection protocol from their foals and transported these mares by truck to the Palomino Valley BLM corral facility. The BLM maintained these mares, under observation, for 13–17 days on a grass-hay and water (ad libitum) diet until we gave a second PZP injection (PZP-FIA). The BLM then returned the mares to their home range area and released them. Members of the same harem band, previously marked with a same-color grease pencil, were released together. Within 24 hr of the release, representatives of the BLM and the research team surveyed the study area by helicopter to determine the well being and dispersal of the mares.

Pregnancy Testing by Fecal Steroid Analysis

In the study area, most foaling occurs in April and May, and breeding is uncommon after mid-June. Pregnancy can be detected reliably with fecal analysis within 80 days of conception (Kirkpatrick et al. 1991*b*). We sampled mares for pregnancy detection in fall 1996 to determine treatment efficacy. Between mid-September and mid-October 1996, we observed mares for defecation using binoculars and 20–40× spotting scopes. We ensured that each sample collected was from the desired mare by pairing observers such that 1 person maintained view of the sample through the scope while hand-signaling the other observer to the sample. In any case where the location or specificity of the sample was in question, none was collected. We collected 2 freshly dropped fecal balls in a sealable plastic bag, labeled it, and stored it on ice until it could be placed in a freezer (within 72 hr). We determined pregnancy in each sample via measurement of estrone sulfate (E1C) and immunoreactive progesterone metabolites (iPdG) by ELISA (Kirkpatrick et al. 1991*b*). The combined measures have proven >90% accurate in pregnancy diagnosis in several species (Lasley and Kirkpatrick 1991), including the feral horse. We considered a mare pregnant when values for a given sample were E1C >1,850 pg/g wet feces and iPdG >250 ng/g wet feces.

Foal Counts

The presence of a foal with a marked mare was determined by ground observations in May and October 1996 and 1997. We also counted foals among untreated, unmarked mares in October

1996 and 1997. Among unmarked mares we avoided double counting by restricting the number of mares included in a count to those that came to a single water site in 1 daylight period. Unusual drought conditions ensured large numbers of horses coming to water on a single day, and long distances from water to feed ensured that horses did not visit water more than once per day. We verified which foal was with which mare in ground surveys by observing the horses until the foal clearly associated with a given mare by its repeated proximity to her during grazing and travelling and/or by nursing from her.

Experimental Design and Analysis

The study examined the antifertility effectiveness across 1 breeding season of 2 separate inoculations of PZP vaccine vs. the single injection of PZP vaccine with controlled-release components. The first injection of vaccine always contained FCA, and the second injection contained FIA. We randomly assigned mares to 3 treatment groups consisting of (1) 2 separate inoculations, 13–17 days apart, of vaccine containing Freund's adjuvant and native PZP ($n = 95$); (2) 2 inoculations of vaccine, 13–17 days apart, containing combined Freund's and CAR adjuvants and native PZP ($n = 60$); and (3) 1 injection of vaccine consisting of Freund's adjuvant and native PZP, with coincident, same-site injection of a mix of controlled-release PZP microspheres and controlled-release CAR microspheres ($n = 112$). Contraceptive treatment was administered in January 1996. Baseline (no treatment effect) fertility rates were determined by counting foals in May and October 1996. Treatment effectiveness was determined by collecting fresh feces for pregnancy testing in October 1996 and by counting foals in May and October 1996 and 1997. In addition to comparing pretreatment with treatment in each group, we compared foal counts in treated and untreated mares in 1996 and 1997. We considered a mare to be successful reproductively if she tested positive for pregnancy and/or had a foal.

Where appropriate, we present data as $\bar{x} \pm SE$. We employed Student's *t*-test for statistical analysis of fecal data. We determined possible group differences among reproductive success rates using a Tukey-type multiple comparison test for proportions and binomial probability distribution (Zar 1984).

RESULTS

Data obtained from in vitro incubation of PZP microspheres (Fig. 1) showed that <5% of the PZP was released until about 4 months, when a single, bolus-type release event occurred over a period of 3–6 days for a given set of microspheres. Each of the 3 major peaks (Fig. 1) is the release from 1 of the 3 sets of microspheres. Released amounts were from 37 to 130× greater in major release periods than in quiescent periods. The total window in which release occurred for the 3 sets of microspheres tested was 39 days. The interval between the release event for set 1 and set 2 was 12 days, and the interval separating set 2 and set 3 was 24 days.

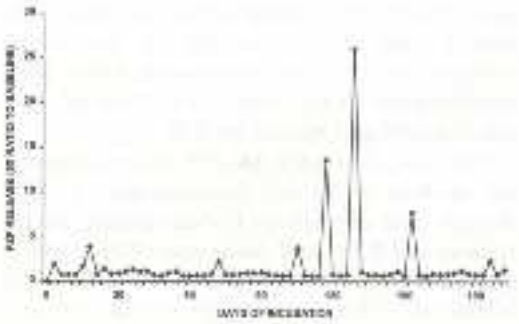

Fig. 1. Average release pattern in vitro for 3 identical, separately incubated lactide:glycolide (65:35 ratio) microsphere populations containing 80 µg porcine zona pellucida per population. Each microsphere population exhibited 1 major bolus release (visible in the average shown) during the incubation.

During 13–17 days of captivity and at the time of their release, none of the 2-injection mares ($n = 155$) showed abscesses. None of the mares observed in the field thereafter showed injection-site marks, suggesting that abscesses had not occurred. Following the treatment of mares in January 1996, severe drought prevailed in Nevada through 1997. However, we observed 80% of the 267 marked mares between May 1996 and October 1997. This reidentification rate was clearly aided by the drought. In 1 instance covering 48 hr, >50% of all marked mares visited a single watering site.

Among 222 marked mares, 30.2% were pregnancy tested (Oct 1996), 85.6% were assessed by foal counts (May and Oct 1997), and 24.3% were assessed by both methods. In the 1996 breeding season, reproductive success in 2-injection mares was 12.8% (without CAR) and 10.6% (with CAR). In the microsphere group (C) 11.3% of mares were reproductively successful (Table 1). In comparing reproductive success in 1996 (foals conceived before treatment) and 1997 (foals conceived while treatment active), the overall

Table 1. Effect of 3 versions of porcine zona pellucida (PZP) contraceptive vaccine on fertility of feral mares in Nevada in 1996 and 1997.

Treatment[a]		Mares located[b]	Mares reproductively successful[c,d]	
Injection 1	Injection 2	*n*	*n*	%
PZP/FCA	PZP/FIA	78	10	12.8
PZP/FCA + Carbopol®	PZP/FIA	47	5	10.6
PZP/FCA + Microspheres	NONE	97	11	11.3

[a] Adjuvants were Freund's Complete Adjuvant (FCA), Freund's Incomplete Adjuvant (FIA), and Carbopol microspheres contained PZP and CAR.

[b] Mares which were located for sample collection and observation for foal presence, May through October.

[c] A positive fecal pregnancy test or presence of a foal was the criterion for reproductive success.

[d] No significant differences among groups for $P < 0.05$, Tukey-type multiple comparison test for proportions.

Table 2. Association of steroid metabolite levels in fecal samples collected in September and October 1996 with the presence or absence of foals the following summer in feral mares in Nevada.

		Metabolite concentration[a]			
		E_1C		iPdG	
		(pg/g feces wet wt.)		(ng/g feces wet wt)	
Mare condition	*n*	$\bar{x}$	SE	$\bar{x}$	SE
Without foal	46	1,037.5	134.4	203.9	14.9
With foal	8	3,329.4	534.8	676.9	47.0

[a] E_1C = estrone sulfate, iPdG = immunoreactive pregnanediol glucuronide.

percentage of reproductively successful mares was 11.6% (26/222) in 1997 and 66.8% (165/250) in 1996, a 5.7× difference. This assessment involved mares that were located for sample collection and/or observation between May 1996 and October 1997. Mares sampled in 1996 and 1997 were not necessarily the same individuals. Among untreated mares the reproductive success rate in 1996 was 59.4% (38/64) and in 1997 was 62.5% (45/72) based on foal counts.

In 1996, we collected fecal samples from 67 marked mares. In the 1996 samples obtained from mares that were reidentified and had produced foals in 1997 ($n = 8$), the average values for E1C and iPdG were 3.2 times and 3.3 times greater than the respective average values associated with reidentified mares that did not produce foals ($n = 46$) in 1997 (Table 2). For both steroids measured, the average value for mares that produced foals was greater ($P < 0.01$) than for mares that did not produce foals. All mares that were diagnosed as pregnant by fecal steroids and were reidentified the following spring had a foal. Five mares that were diagnosed as not pregnant had a foal the following spring. Overall accuracy was 49/54 (91%).

DISCUSSION

Fertility rates among both treated and untreated mares foaling in 1996 (before vaccine effects) and among untreated mares in 1997 were slightly higher than those reported for other feral horse populations (Wolfe et al. 1989), but were similar to those observed in horse roundups of previous years in the Nevada Wild Horse Range (Bureau of Land Management database, Las Vegas District, unpublished data). The similarity in foaling rates between untreated mares in 1996 and 1997 demonstrated that environmental factors or disease were not contributory to low foaling rates in treated mares in 1997.

We previously reported the use of PZP microspheres in feral horses in Nevada (Turner et al. 1997). In that study, fertility in the 1-injection microsphere-treated group was 4.4× higher than in the group given 2 separate inoculations. In contrast, the marked and equivalent infertility of the microsphere-treated and 2-injection groups of the present study indicated that the microspheres were as effective as a second injection. While comparisons between different studies must be cautious, we consider noteworthy the differences between the microspheres in these separate studies. In the previous study, the microspheres contained no adjuvant and exhibited a continuous-release pattern (Wang et al. 1991, Turner et al. 1997). Microspheres used in the present study contained adjuvant and exhibited a bolus-release pattern, with the change in release patterns from continuous to bolus achieved by an approximate 10× increase in the polymer:PZP ratio in the microspheres. We have not determined to what extent either or both of the above characteristics contributed to the improved effectiveness of the microspheres used in the present study.

Based on in vitro microsphere data, PZP release from microspheres in mares was likely much later than the second injection given to the 2-injection group. However, Tracy et al. (1999) reported shorter delays in proteins released in vivo than in

vitro. Also, Sanders et al. (1986) used 20% ethanol in vitro to increase plasticity of polymers (more similar to in vivo conditions) and observed that release occurred earlier in that circumstance. This suggests that delay of release in the present study (in vivo) was less than the 4 months observed in vitro.

The time interval between exposures is not important if it does not exceed the immune system's ability to engage t-cell mediated immunomemory of the antigen (Newman and Powell 1995). The similar infertility effect in both groups indicated that such an interval was not exceeded in this study, and Kirkpatrick et al. (1992) showed that this interval may be 2–3 years for PZP vaccine. One circumstance in which it is possible that a long delay between first and second exposure could result in weakened contraceptive effect is when titers generated by initial antigen exposure have decreased, and exposure 2 does not occur until the breeding season has begun. It is likely that the bolus release occurred prior to the onset of breeding in this study, since treatment was in January and breeding for the majority of mares began in May.

In rabbits, Friiz (1997) observed similar initial anti-PZP antibody responses with FCA or CAR as adjuvants. Any vigorous additive effect of adjuvants in this study should have been observable, because fertility rates remained above 10% after treatment. However, the fertility rates in Groups A and B were not different, indicating that there was no additive adjuvant effect.

This study confirmed the high accuracy of pregnancy diagnosis via fecal steroid metabolites in feral horses (Kirkpatrick et al. 1991*b*). It also provided evidence that fetal loss was unlikely to have inflated infertility rates, since pregnancy diagnosis was well correlated with foal counts. The marked difference (>3×) between non-pregnant and pregnant state in the measured metabolite levels favors reliable pregnancy determination, and by measuring both E1C and iPdG we minimized the chances of misdiagnosis. All cases of disagreement were false negatives—i.e., pregnancy diagnosis was negative but a foal was present. We were unable to determine in these cases whether (1) the pregnancy test was incorrect, (2) we mismatched the foal with the mare, or (3) conception occurred too late in the year to be detectable at the time of fecal collection.

The present study did not include a sham control group or testing of return to fertility. Return to fertility was not monitored, since we previously have reported (Kirkpatrick et al. 1991*a*, Turner et al. 1997) in studies using 2 exposures to PZP that return to fertility occurred. The latter study employed the same controlled-release polymers as in the present study to provide the second PZP exposure. A sham control group was not included in the present study, since we have previously shown (Turner et al. 1997) that feral mares captured and handled and given a placebo injection exhibited fertility rates (based on fecal analysis) not different from those of untreated mares that were never captured.

MANAGEMENT IMPLICATIONS

A 2-injection contraception protocol has presented logistical and economic problems for routine use in feral horses, because of the need to keep gathered horses for ≥ 2 weeks. The results of the present study indicate that a single-injection vaccine with a controlled-release component can provide effectiveness equivalent to a 2-injection protocol, permitting treatment and immediate release of each horse. Incorporation of PZP vaccination into the existing BLM roundup procedures reduces foal production with minimal cost of accessing horses for contraception. Reduced foal production will enhance humaneness of management by reducing the frequency of roundups and the number of horses in long-term captivity.

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