Immunocontraception in Wild Horses: One Inoculation Provides Two Years of Infertility

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ABSTRACT Previous studies reported one year of contraception associated with a 1-injection porcine zona pellucida (PZP) vaccine. We have subsequently determined contraceptive effectiveness of a presumptive 1-injection, 2-year duration PZP vaccine in free-roaming wild horses (*Equus caballus*) in Nevada, USA. In January 2000, we captured, freeze-branded, treated, and subsequently released 96 adult females that received 1) a primary dose of vaccine emulsion consisting of aqueous PZP and Freund's Complete Adjuvant, and 2) booster doses of PZP and adjuvant in controlled-release polymer pellets. We determined PZP release characteristics of pellets in vitro, prior to field use. We determined reproductive success in treated and untreated females through October 2004 via measurement of estrone sulfate and progesterone metabolites in fresh feces collected from the ground and by twice-annual foal counts. Among treated females, annual reproductive success from 2001 though 2004 sequentially was 5.9%, 14.0%, 32.0%, and 47.5%. Untreated females showed average reproductive success of 53.8 ± 1.3% across this period. This study revealed that: 1) PZP acted as an effective contraceptive for 2 years posttreatment; 2) some residual contraceptive effect remained in year 3; and 3) fertility returned to control levels by year 4 posttreatment. It appears that controlled-release technology can replace both the second (1-month) and annual booster injection of PZP vaccine, thereby decreasing cost and increasing efficiency of use of this vaccine in wild horse management. (JOURNAL OF WILDLIFE MANAGEMENT 71(2):662–667; 2007)

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Since the passage of the Free-Roaming Wild Horse and Burro Protection Act in 1971, management of wild horses (*Equus caballus*) on public lands has proven biologically and politically challenging. Under its multiple-use mandate, the Bureau of Land Management (BLM) must maintain healthy, viable wild horse herds in a thriving ecological balance with many competing uses, including wildlife, livestock, and other commercial and recreational activities. With lethal horse population control being illegal and broadly unacceptable to the public, the BLM has relied principally on gathering, removing, and adopting wild horses to control their numbers on the range. However, the supply of wild horses has markedly exceeded adoption demand, forcing the BLM to hold horses in sanctuaries and other long-term holding facilities, primarily at taxpayer expense.

To help reduce population pressure on public lands and the adoption program, the BLM has been supporting horse contraception research since the 1970s (Kirkpatrick 2005). The use of porcine zona pellucida (PZP) immunocontraception for the purpose of safe and cost-effective regulation of free-roaming wild horse populations has been under investigation since 1988 (Turner and Kirkpatrick 1991). Porcine zona pellucida vaccine use in the horse was first reported 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 2–3 separate inoculations about a month apart yielded reversible infertility for one breeding season in a number of species including horses (Liu et al. 1989, Kirkpatrick et al. 1990), burros (*Equus 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, for managing free-ranging wildlife a 1-injection vaccine effective for 1–3 years is desirable.

One approach to a one-injection, multiple-year vaccine is to mimic the effects of booster injections by incorporating vaccine into controlled-release polymers. This methodology has involved 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 gradually erodes and releases the bioactive contents (Wang et al. 1991).

In previous studies with free-roaming wild horses, a single injection containing primer PZP adjuvant and booster PZP (no adjuvant) in controlled-release preparations of lactide-glycolide microspheres reduced fertility but was less effective than 2 separate inoculations (Turner et al. 1997). Since an important function of adjuvant is to enhance immuno-

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recognition of antigen and consequent antibody production, we expected that adjuvant presence in the controlled-release component of the vaccine would boost the effectiveness of this component. In a subsequent study, a vaccine preparation in which a carbomer adjuvant was added to the PZP-containing polymer microspheres proved as effective as 2 separate injections of PZP adjuvant (Turner et al. 2000). Further refinement of controlled-release components led to replacement of gradual-release polymer microspheres with bolus-release polymer mini-rods (pellets) in the 1-injection, 1-year vaccine (Turner et al. 2002). We hypothesized that these conditions would resemble multiple, separate injections of vaccine and would yield infertility similar to that obtained with multiple booster inoculations.

Our present objective was to test the effectiveness of a presumptive 2-year PZP vaccine that utilized a controlled-release PZP adjuvant component to provide multiple bolus-release booster events.

STUDY AREA

The study area was part of the Clan Alpine Herd Management Area in central Nevada, USA, and comprised about 7,800 km^2 centered at 39°32'N latitude and 117°55'W longitude. Elevations ranged from 1,300 m to 3,066 m. Vegetation on the portion of the range most used by study horses was composed largely of sagebrush (*Artemisia* sp.) communities, with pinyon pine (*Pinus edulis*) and juniper (*Juniperus* sp.) occurring at higher elevations. Grasses commonly utilized by horses in these communities were Indian rice grass (*Achnatherum hymenoides*), galleta (*Pleuraphis jamesii*), and desert needlegrass (*Achnatherum speciosum*). Average annual precipitation (2000–2004) was <22 cm at 2,000 m elevation. Water was available from several permanent springs in the study area. During the study period, we made >80% of the observations by aerial and ground surveys in 4 distinct areas comprising <3,000 km^2 and favored by the horses due to feed and water availability. The Carson City (Nevada) District of the Bureau of Land Management (BLM), United States Department of Interior administered the study area.

METHODS

Experimental Design

The study examined the antifertility effectiveness and longevity of a single-injection PZP vaccine containing a controlled-release component exhibiting several release windows. Every vaccine treatment contained an emulsion of aqueous PZP, Freund's Complete Adjuvant (FCA), and a controlled-release component containing PZP and the saponin adjuvant QS-21. We applied contraceptive treatment in the field (Jan 2000) after we randomly assigned >200 captured adult wild females to a vaccine treatment group ($n = 96$) or to an untreated control group ($n > 100$). We did not include a sham control group because we have previously shown (Turner et al. 1997) that wild females captured, handled, and given a placebo injection exhibited fertility rates that did not differ from those of untreated females not captured. We estimated fertility rates in the absence of contraceptive effects from foal counts made among treated and untreated females in May and October of 2000 (i.e., foals born to both were conceived before treatment in Jan 2000). We determined the effectiveness and duration of treatment by assessing reproduction via ground and aerial foal counts and fecal-hormone pregnancy tests at various times across the period from autumn 2000 through autumn 2004. We considered a female to have successful reproduction if she tested positive for pregnancy or was observed with her foal or both.

Horse population.—We performed this study under auspices of Animal Care and Use protocol (no. 100742) of the Medical University of Ohio and the standard animal management practices of the Bureau of Land Management. The study area was inhabited by about 600 horses with a harem-band social structure (Turner et al. 1981, Berger 1986). The peak breeding period was May and June, and the peak foaling period was April and May. We estimated the physical condition of females in the corral prior to inoculation and in the field during subsequent observations using the condition scale (1–10) developed by Henneke et al. (1983). Initial female condition ranged from fair (3) to good (6). Condition-3 females comprised <30% of those treated. None of the females appeared to deteriorate in condition across the study and many apparently improved, possibly due to increased feed availability after a January 2000 permanent removal of 233 (329 initially removed, 96 returned to range) horses from the range.

Because BLM selective-removal policies in place at the time dictated the removal of all females under 10 years of age, all females receiving treatment were >10 years of age. Six treated females were aged >20 years. In some wild horse herds, reproduction may be reduced in females >20 years old (T. Pogacnik, BLM Wild Horse and Burro Program files, unpublished data). However, data for the Nevada Wild Horse Range in central Nevada, where female physical condition was generally inferior to the Clan Alpine herd, showed fertility rates in females aged 10–20 were similar to those rates in females >20 (Turner et al. 2000).

PZP vaccine emulsion preparation.—We prepared PZP from porcine ovaries as previously described (Liu et al. 1989). The primer portion of the vaccine consisted of an emulsion of 0.5 cc FCA with 65 µg PZP in 0.5 cc phosphate buffer solution (PBS). We prepared the emulsion, 8 doses at a time within 24 hours of injection using 10-cc glass syringes joined with a plastic connector. After 100 plunger strokes, we loaded the emulsion into 10-cc plastic syringes for injection via a 14-gauge, 3.7-cm needle. We prewashed the needle (new needle each injection) and the injection site with 70% ethanol. The emulsion was kept chilled, but we hand-warmed it prior to injection.

Controlled-release pellet preparation.—We prepared the pellets by a heat-extrusion process employing a Dynisco extruder (Dynisco, Morgantown, PA). The pellets consisted of lyophilized PZP and QS-21 (a water-soluble saponin adjuvant) matrixed in homogenous lactide and glycolide

Table 1. Polymer pellet composition and in vitro bolus controlled-release characteristics for porcine zona pellucida (PZP) vaccine used in female wild horses in Nevada, USA, in 2000.

Pellet type based on exp release delay (months)	No. of pellets tested	Pellet mass (mg)	Polymer ratio[a] (lactide:glycolide)	PZP mass (µg)	QS-21[b] mass (µg)	Loading rate (PZP + QS-21) polymer (%)	Obs in vitro release delay[c] (months)	
							$\bar{x}$	Range
1	6	7	65:35	70	175	3.5	1.5	0.9–2.1
3	6	7	85:15	90	200	4.1	3.9	3.0–5.2
12	6	8.5	100:0	250	500	9.4	11.5	10.3–14.2

[a] Lactide used is a mixture of dextro- and levo-rotatory forms.
[b] QS-21 is a water-soluble saponin adjuvant.
[c] $\bar{x}$ and range are based on when >80% of total PZP release occurred during entire in vitro incubation period.

polymers. Pellet preparation and release characteristics (Table 1) are summarized below. We regulated the delay period for release of active ingredients from pellets by altering the ratio of lactide to glycolide. We chose to release windows at approximately 1 month and 3 months to mimic a second and third inoculation, which had yielded effective contraception for one breeding season in an earlier study (Kirkpatrick et al. 1990). Likewise, we chose an approximate 12-month window to mimic an annual booster inoculation shown previously to provide a second year of contraception (Kirkpatrick et al. 1992). We extruded pellets from a melted (<95° C) mixture of lactide and glycolide containing micronized PZP and QS-21. The cylindrical pellets, designed to fit inside the barrel of the 14-gauge hypodermic needle, were approximately 0.6 × 1.5 mm with an average weight of 7.5 mg. Pellets were of 3 types (Table 1), and we determined characteristics of PZP release at several polymer ratios in vitro by measuring PZP concentrations in the incubation medium across time using a sandwich-type enzyme-linked immunosorbent assay (ELISA) for PZP (Turner et al. 2002). The in vitro environment for a given pellet consisted of 3 cc of 37° C bacteriostatic PBS – 1% horse serum gently and continuously shaken (2 back-and-forth motions/sec). Every 5–7 days for up to 14 months, we removed the medium for assay and added 3 cc of new medium to the pellet vial. We performed the ELISA on 100 µl of each sample of medium. We did not measure the release of QS-21 from pellets. However, we expected release characteristics to be similar to PZP, based on similar water solubility (C. Kensil, Antigenics, Inc., personal communication).

Accessing, handling, and inoculating of horses.—The BLM gathered horses by helicopter, which permitted 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 2 January and 9 January 2000, the BLM gathered 329 horses by helicopter into portable corrals. In the corral system the BLM 1) separated the gathered males and females, 2) moved them singly through a stock chute, 3) estimated age through dentition and physical condition, 4) gave a 1-cc prophylactic Strepguard® (Miles Laboratories, Shawnee Mission, KS) injection intramuscularly, and 5) permanently marked 96 healthy females >10 years of age with consecutive numbers by freeze-branding. The brands were located on the upper left hip, were 8 cm in height, and were readable from a helicopter and through a spotting scope at >500 m distance on the ground.

We used a jabstick to inject 1 cc of freshly prepared emulsion of buffered PZP adjuvant (described above) into the left gluteus muscle and we simultaneously injected 1-month, 3-month, and 12-month pellets, which were sequestered in the needle barrel. We examined each needle postinjection to ensure pellet delivery. Three of the 96 deliveries by this method failed, and in those cases, we delivered the pellets by a second injection using PBS to propel the pellets. We photographed each marked female prior to release. The BLM maintained all females (treated or untreated) in portable corrals on grass–hay and water ad libitum for up to 4 days, and then released them into the range area from which they had gathered them. Before release, the BLM separated the females and their foals and retained the foals for eventual adoption. Within 24 hours of the release, representatives of the BLM surveyed the study area by helicopter to determine the horses' well-being and dispersal.

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 postconception (Kirkpatrick et al. 1991). We sampled females for pregnancy detection in autumn 2001 to determine treatment efficacy for year 2 and to verify for this herd the validity of foal counts for assessing reproductive rates, as previously reported for other herds (Turner et al. 1996, Turner and Morrison 2001). Between mid-September and mid-October of 2001, we observed females for defecation using binoculars and 20–40× spotting scopes. We took photographs of untreated females at observation when possible to enable later identification for foal presence or absence. We ensured that each sample collected was from the desired female by pairing observers such that one person maintained view of the sample through the scope while hand- or radio-signaling the other observer to the sample. If the location or specificity of the sample was in question, we did not collect the sample. 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). The combined measures

Table 2. Association of steroid metabolite levels in fecal samples collected in September and October 2001 with the presence of foals the following summer in female wild horses in Nevada, USA.

F condition	n^a	Fecal metabolite concentration[b] E_1C^c (pg/g feces wet wt) $\bar{x} \pm SE$	iPdG[c] (ng/g feces wet wt) $\bar{x} \pm SE$
Without foal	24	3.0 ± 0.1	649.3 ± 107.6
With foal	14	6.8 ± 0.9	10,083.0 ± 2,153.1

[a] F included freeze-marked ($n = 17$) and unmarked, but uniquely identifiable ($n = 21$), individuals.
[b] E_1C = estrone sulfate, iPdG = immunoreactive pregnanediol glucuronide.
[c] Values with vs. without foal were different ($t = 2.74$, $P < 0.01$).

Table 3. Hormonally determined pregnancy rates in the second year of treatment for free-roaming female wild horses in Nevada, USA, given in 2000 a single-injection porcine zona pellucida (PZP) contraceptive vaccine of presumptive 2-year duration.[a]

Condition	Total no. F	No. pregnant	No. not pregnant	Pregnancy rate (%)
PZP-treated[b]	17	3	14	16.6
Untreated control	21	11	10	55.0

[a] We immunized F in January 2000 and we collected fecals for pregnancy testing in September and October 2001. We considered a F to be pregnant if fecal estrone conjugates were >3.0 ng/g and immunoreactive pregnanediol glucuronide was >2,400 ng/g.
[b] Vaccine treatment consisted of intramuscular injections of liquid (PZP/Freund's Complete Adjuvant) plus 3 controlled-release pellets (PZP/QS-21 [a water-soluble saponin adjuvant]; 1-month, 3-month, or 12-month delay in release).

have proven >93% accurate in pregnancy diagnosis in several species (Easley and Kirkpatrick 1991), including the wild horse. We considered a female pregnant when values for a given sample were E1C > 3.1 ng wet feces and iPdG > 2,400 ng/g wet feces.

Foal counts.—The presence of a foal with a given marked female was determined by ground observations in May and October of 2000–2004. We concurrently counted foals among untreated, unmarked females. Double-counting of a given unmarked female was unlikely in the context of unique color and marking patterns across a given band of horses. In questionable cases, we compared our immunization-day photo-identification file with video of target females taken during field collections. We verified which foal was with which female in ground surveys by observing the horses until the foal clearly evidenced association with a given female by its repeated proximity to her during grazing and traveling or by nursing from her or both. We performed aerial counts of horses using a Bell 47 (Ft. Worth, TX) or Hughes Jet Ranger (Seattle, WA) helicopter, with both pilot and observer (1 or 2) having prior experience in aerial survey of horses. We established maternity by applying minimal pressure on a given band of horses initially, which permitted foals to pair with mothers as the band formed up and moved away from the helicopter. We approached more closely to permit reading of freeze-mark numbers, and then withdrew to permit reobservations of mother–foal pairings. We determined reliability of mother–foal pairing behavior as a basis for contraceptive assessment to be >95% when we reidentified and verified marked females with and without foals in ground surveys.

Statistical analysis.—Where appropriate, we have presented data as mean ± standard error. 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 or a binomial probability distribution (BPD; Zar 1984).

RESULTS

In vitro incubation of PZP-containing pellets yielded some PZP release (<10% of total) in the first 10 days, with no detectable release thereafter until a primary release event (72–85% of total release) for a given pellet. Considerable variation occurred in delay of release onset among pellets of a given type (Table 1). Duration of release averaged 8 days, 14 days, and 20 days for 1-month, 3-month, and 12-month pellets, respectively.

Among marked females, 18% ($n = 17$) were both pregnancy tested (Oct 2001) and assessed by foal counts (May and Oct 2002). In addition, among unmarked (untreated) females that were uniquely identifiable (from photographs taken during fecal collections), we assessed 21 individuals by both pregnancy test and foal presence or absence. Thus, we assessed 38 females both hormonally and by foal observation (Tables 2, 3). Average values for E_1C and iPdG were 2.3-fold and 15.5-fold, respectively, greater among females that produced foals than corresponding values for these metabolites in females that did not produce foals (Table 2). For both steroids measured, the average value for females that did produce foals was significantly greater ($t = 2.74$, $P < 0.01$) than for females that did not produce foals. In the spring of 2002, we were able to locate and reidentify 12 of the females that had tested pregnancy-positive and 8 of the females that had tested pregnancy-negative in October 2001. We observed all 12 of the pregnancy-positives and 1 of the 8 pregnancy-negatives to have a foal the following spring. Thus, diagnostic accuracy of hormone versus observation-based fertility assessment among these females was 19/20 (95%).

The percentages of reproductive success in 2001 were 5.2% for treated and 53.6% for untreated females (Table 4). These rates in 2002 were 14.9% (treated) and 58.5% (untreated) by observation, and 16.6% and 55.0%, respectively, by pregnancy test (Table 3). Statistics applied to observational rates revealed a significant difference between treated and untreated averages in both years (2001: $z = 5.66$, $P < 0.001$; 2002: $z = 4.81$, $P < 0.001$; Table 4). Reproductive success rates in treated females remained 19.5% below rates in untreated females in 2003, but this was not significant ($z = 1.15$, $P = 0.252$). In 2004, reproductive success rates in treated and untreated females were nearly identical (Table 4). Among untreated females,

Table 4. Fertility rates from 2000–2004 for free-roaming female wild horses in Nevada, USA, given a single-injection porcine zona pellucida (PZP) contraceptive vaccine of presumptive 2-year duration.

Yr[a]	Total ad observed[b]	Total foals observed	Untreated F		Treated F		Fertility rate (%)	
			With foal	No foal	With foal	No foal	Untreated	Treated
2000	107	27	11	9	16	17	52.4	48.5[c]
2001	127	40	37	32	3	55	53.6	5.2[d]
2002	236	41	31	22	10	57	58.5	14.9[d]
2003	128	28	22	21	6	13	51.1	31.6
2004	187	46	28	26	18	21	51.8	46.2

[a] Treatment was in Jan 2000; assessment period was approx. 2 weeks between May and Oct of each yr.
[b] No. includes M and F, representing the base population from which foal data were obtained each yr.
[c] No treatment effect expected on 2000 foal counts, because F were bred 6–8 months prior to treatment.
[d] Values for treated vs. untreated were different (2001: $z = 5.66$, $P < 0.001$; 2002: $z = 4.81$, $P < 0.001$).

the reproductive success rate averaged 53.5% across the entire study period. Females observed for foal presence across seasons and years were not necessarily the same individuals.

DISCUSSION

Inoculation of captured and released wild females with PZP vaccine resulted in a marked suppression of fertility that was significant across 2 breeding seasons, although year-2 infertility was not as great as in year 1. Although the reproductive rate in year 3 for treated females was not significantly below the rate in untreated females, the 19.5% absolute rate difference suggests a partial contraceptive carryover through year 3. The vaccine format employing controlled-release pellets was as effective in year 1 as that reported previously (Turner et al. 2000) for 2 separate inoculations. Fertility rates among both treated and untreated females foaling in 2000 (before vaccine effects) and among untreated females in other years were similar to those reported for other wild horse populations (Wolfe et al. 1989, Turner et al. 2000).

This study reconfirms the high accuracy of pregnancy diagnosis via fecal steroid metabolites in wild horses (Kirkpatrick et al. 1991, Turner et al. 2000), with pregnancy data versus foaling data in 95% agreement. These data are also evidence that fetal loss was unlikely to have inflated infertility rates since pregnancy diagnosis was well-correlated with foal counts. By measuring both E_1C and iPdG (Table 2), we minimized the chances of misdiagnosis.

Polymer pellet composition and characteristics (Table 1), and data obtained from in vitro incubation of PZP-containing pellets (previously reported by Turner et al. 2002) showed that all 3 pellet types released PZP in an approximately bolus fashion, with up to 80% of the PZP being released across a 7–10-day period for a given 1-month pellet and across a 12–30-day period for 3-month and 12-month pellets. While the in vitro pellet-release data may provide a reasonable approximation of in vivo activity, we cannot precisely extrapolate it to in vivo conditions (Sanders et al. 1986, Tracy et al. 1999); that is, actual release characteristics of the previous pellets in the females of this study are undetermined. However, the marked infertility in both years 1 and 2 indicate that pellet content release was sufficient to engage immune system memory of the PZP antigen (Kirkpatrick et al. 1992, Newman and Powell 1995).

Despite an increase in reproductive success across years 1–3 posttreatment in the population of treated females, the average reproductive success rate in treated females remained below untreated females. In year 4 posttreatment, the rates in treated and control females were nearly identical, indicating complete return to fertility. It is likely that slower return to fertility in some females reflects individual differences in immune system responsiveness or sensitivity across the population.

MANAGEMENT IMPLICATIONS

As suggested by Garrott (1991), the issues of cost-effectiveness and limitation of population growth must be included in assessing long-term contraceptive management potential. The availability of a 1-inoculation, multi-year PZP vaccine allows contraception to be incorporated at low cost into standard BLM management practices, which include the regular round up (gather) of wild horses for the purpose of removing some from the range. Administering a single-injection, multi-year contraceptive to females being returned to the range has the potential to limit management program costs by reducing frequency of horse roundups, number of horses entering the adoption program (or maintained in long-term holding facilities), and the number of horses using the range (Gross 2000). Associated benefits will include improved range quality and reduced stress to wild horse populations (due to better habitat and less human intervention).

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