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REMOTELY-DELIVERED IMMUNOCONTRACEPTION IN FERAL HORSES

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Reducing fertility among free-roaming feral horses (*Equus caballus*) has been the goal of numerous studies over the past 16 years (Kirkpatrick et al. 1982, Goodloe et al. 1988, Plotka et al. 1989). Initial experiments by Kirkpatrick et al. (1982) and Turner and Kirkpatrick (1982) resulted in an 83% decrease in foaling among feral mares bred by stallions which were first immobilized and then treated with injectable microencapsulated testosterone propionate (mTP). Although the treatment decreased sperm count and motility, the high costs and the stress caused by immobilization or capture made it clear that the contraceptive agent needed to be delivered remotely. In a second study Kirkpatrick and Turner (unpublished data) demonstrated the pharmacological effectiveness of mTP in stallions, but difficulty was encountered in remotely delivering a sufficient mass of the steroid.

Recently, attention has turned to contraception in the feral mare. Experiments with ethinylestradiol-progesterone Silastic® implants (Vevea et al. 1987, Plotka et al. 1988, Plotka et al. 1989) showed pharmacological promise, but the technique required capture, restraint, and field surgery to place the implants intraperitoneally. An alternative to steroid-induced fertility control is immunocontraception. A conjugated form of luteinizing hormone releasing hormone (LHRH) has been used successfully to raise antibodies in captive feral mares (Goodloe et al. 1988), and solubilized porcine zonae pellucidae (PZP) injections inhibited fertility in 13 of 14 domestic and captive feral mares (Liu et al. 1989).

The success of the PZP vaccine in suppressing fertility is based on its ability to inhibit fertilization or possibly implantation (Sacco et al. 1984). The porcine zona pellucida consists of 3 glycoproteins. One of those, ZP3, is the receptor molecule for sperm surface molecules (Florman and Wassarman 1985). Equine antibodies raised against PZP are thought to block the sperm receptor sites on the equine ovum, thereby preventing fertilization (Liu et al. 1989). To date, PZP has been used to successfully inhibit fertility in a number of mammals, including 5 species of nonhuman primates and in vitro fertilization in humans (Sacco 1987).

The objectives of this study were to (a) determine the effectiveness of remote delivery, (b) test the contraceptive effectiveness of a PZP vaccine in free-roaming feral mares, (c) determine the contraceptive effectiveness of the vaccine in pregnant and nonpregnant mares, and (d) evaluate the safety of the vaccine for use in pregnant mares.

MATERIALS AND METHODS

Forty-six sexually mature mares were selected for the study from among the approximately 100 feral mares inhabiting Assateague Island National Seashore, Maryland. The ages and fertility records, some dating back as far as 1974, were known for almost all animals on the island (Keiper and Houpt 1984). Ages ranged from 3 to 18 (mean = 9.12, SD = 4.45 years). The mares chosen for treatment were not randomly selected. Instead, they were selected because of their high fertility rates, which averaged about 10% higher (51.7%) than the overall herd rate (approximately 40%) annually for the preceding 3 years. The PZP vaccine was prepared from porcine ovaries (Liu et al. 1989) and stored frozen at −5 C until used in the field.

The inoculation was prepared as an emulsion, of 0.5 cc of vaccine (equivalent to approximately 5,000 zonae or 64.3 μg of protein) in phosphate buffer and 0.5 cc of Freund's Complete Adjuvant. The second and third inoculations were the same as the first except for the addition of 0.5 cc of phosphate buffer solution and substitution of 0.5 cc of Freund's Incomplete Adjuvant for the complete adjuvant. The 2 vaccine components were mixed in the field, using 2 10-cc glass syringes joined with a plastic connector. After 100 strokes the emulsion was loaded into a 3.0-cc self-injecting plastic dart which was tipped with a 3.81-cm barbless needle. The needles were rinsed with 70% EtOH prior to being loaded into the rifle.

National Park Service regulations prohibited the capture or handling of any horses during the course of the study. Twenty-one mares were darted from the ground, at distances from 25 to 30 meters, in the hip region, using a Pax-Arms® 0.527-caliber capture gun. Between 29 February and 10 March 1988, 26 mares received an initial inoculation of vaccine. Eight of the mares were acclimated to humans, and the initial vaccine delivery was accomplished with a 3-cc syringe and a jab-stick and thereafter by dart. Between 12 and 21 March, 26 of the 29 mares received a second inoculation by dart, as described above. Three of the mares became extremely wary, could not be approached for the second inoculation, and were dropped from the experiment. Between 16 and 25 April, 18 of the 26 mares which received the second inoculation received a third inoculation, which was identical to the second. The 6 control mares, which were selected from the original 46 mares, received only phosphate buffer and adjuvant in 2 inoculations, between 3 March and 29 March. Identifying markings were recorded for each horse, and the animals were observed throughout April for abscesses at the sites of injection.

A minimum of 2 inoculations is required in horses in order to raise sufficiently high antibody titers for a minimum of 6 months (Liu et al. 1989). The schedule of inoculations used in this study was based on the spacing of inoculations in the 1 previous study with horses (Liu et al. 1989) and the breeding and foaling activity patterns of the Assateague horses, which peak in May and June (Keiper and Houpt 1984). The first inoculation causes antigen recognition and temporary increases in antibody titers. The second inoculation causes increased titers which last several months, and each subsequent inoculation increases the duration of high titers.

During October 1988, 5 months after the last inoculation and 2 months after the breeding season, the mares were located and identified, and the number of foals was recorded. Urine samples were collected from each of the 26 treated and 6 control mares, without capture, by extracting the urine from the soil or aspirating it directly from the ground immediately after urination. The urine samples were assayed for estrone conjugates (E_1C) and indexed to creatinine (Cr) concentrations (Kirkpatrick et al. 1988) and for nonspecific progesterone (Po) metabolites (iPdG) (Kirkpatrick et al. 1990). Pregnancy determinations were made on the basis of the urinary E_1C and iPdG concentrations.

In August 1989, the mares were again located, identified, and observed for the presence of foals. The 1989 foal production for the treated mares was compared to foal production (1) for the same group of mares for 1987 and 1988, (2) for the 6 control mares for 1989 and (3) with 11 untreated mares for 1989. The validity of these comparisons is based on long-term records of reproductive success among the Assateague horses (Keiper and Houpt 1984) which demonstrate that foaling patterns are consistent from year to year and that the probability of a mare having a foal is independent of her foaling success the previous year. Finally, in August 1989, a random sample of 7 uncaptured treated mares was tested for pregnancy by means of urinary steroid metabolites (Kirkpatrick et al. 1988, Kirkpatrick et al. 1990) in order to test reversibility of the vaccine's antifertility effect. Differences in foaling rates among treated, control, and untreated groups were tested for significance by means of binomial probability distribution (Freedman et al. 1978:231, 236).

RESULTS

Three abscesses were observed among the 26 horses treated. The abscesses appeared at the site of injection approximately 48 hours following the third treatment, were about 10–25 mm in diameter, and drained from 6 to 9 days after treatment. Complete healing had occurred within 14 days following treatment.

Of the 26 treated mares, 14 were pregnant at the time of inoculation (57.6%) and all 14 produced foals in the spring of 1988, approximately 1–3 months after the last inoculation of PZP vaccine. The 6 control mares produced 2 foals in 1988. By October 1988, a foal belonging to 1 of the treated mares had disappeared and was presumed dead. Another foal belonging to a treated mare died during the fall of 1988 as a result of a leg injury. All other foals born to treated or control mares were in good health in August 1989 as yearlings. During the 18 months following inoculation, only 3 mares moved to different bands.

Urinary E_1C and nonspecific Po metabolite concentrations in mid-October 1988 indicated there was 1 pregnancy among the 26 treated mares. None of the 18 mares receiving 3 inoculations were pregnant, and 1 of 8 receiving

Table 1. Foaling rates for treated and untreated mares for pretreatment and post-treatment years, Assateague National Seashore, 1987 through 1989.

Group	Inoculations/horse	No. horses	% of mares producing foals (no. foals)		
			Pretreatment		Post-treatment
			1987	1988	1989
Treated	3	18	50.0 (9)	51.1 (11)	0.0 (0)
Treated	2	8	62.4 (5)	37.4 (3)	12.4 (1)
Control	0	6	33.3 (2)	33.3 (2)	50.0 (3)
Untreated	0	11			45.4 (5)

2 inoculations was pregnant. Three of 6 control mares were pregnant. Mean urinary E_1C and iPdG concentrations of nonpregnant treated and control mares (0.12 ± 0.35 SE $\mu g/mg$ creatinine [Cr] and 3.42 ± 0.486 ng/mg Cr, respectively; $n = 28$) were lower than those of pregnant mares (3.41 ± 0.723 $\mu g/mg$ Cr and 227.82 ± 89.7 ng/mg Cr, respectively; $n = 4$) ($t = -12.59$, 30 df E_1C; $t = -9.47$, 30 df, iPdG, $P < 0.001$).

By August 1989, 1 and 3 live foals were present among the 26 treated and 6 control mares, respectively, as precisely predicted by the urinary hormone metabolite measurements (Table 1). Post-treatment foaling rate for the treated mares (3.8%, $n = 26$) was less ($P < 0.002$) than that for the 2 pretreatment years (53.8%), for control mares in 1989 (50.0%), and for untreated sexually mature mares in the study area in 1989 (45.4%). Three of 7 randomly selected treated mares were determined to be pregnant in August 1989, based on urinary estrone conjugates and iPdG.

DISCUSSION

The choice of Freund's Complete Adjuvant for the first inoculation and Freund's Incomplete Adjuvant for the second and third was based on the work of Liu et al. (1989). While only 5 abscesses were noted in this study, the evaluation of other adjuvants which are less likely to cause abscesses is an important direction for future research.

No previous studies have been conducted in which pregnant animals of any species were vaccinated with PZP. In this study the immunosuppression which accompanies pregnancy did not interfere with the effectiveness of the antifertility effects of PZP vaccine, the pregnancies were successful, and the foals healthy. These are important considerations because the use of this vaccine for management will likely include pregnant mares among the treated animals.

A major advantage of the PZP vaccine is the small volume required and the aqueous base, both of which facilitate administration by dart. Remote delivery eliminates the need to capture horses, the attendant costs, and the likelihood of injury to horses, although our experience did not include long-distance darting of extremely wary feral mares. An advantage of PZP is the reversibility of the vaccine's contraceptive effects. Liu et al. (1989) demonstrated that captive treated horses that failed to conceive after PZP treatment could breed successfully the following year, as did at least 3 of 7 free-ranging mares in this study. The issue of reversibility is politically as well as biologically important because it is unlikely that public opinion will favor irreversible sterilization among feral horses.

A final advantage of the PZP vaccine is the protein nature of the contraceptive antigen. This characteristic precludes the possibility of passage of the antifertility agent through the food chain. In most circumstances some treated animals will die from natural causes and a variety of predators and scavengers will feed upon the carcass. Protein, unlike steroids, and particularly synthetic steroids, cannot be accumulated intact in the predators' and scavengers' tissues. In addition, protein vaccination avoids urinary and fecal contamination by poorly metabolized steroids, and especially those synthetic estrogenic steroids which have high potency and high resistance to biodegradation.

Despite the return of normal fertility among PZP-treated horses reported by others and in this study, the long-term effects of continuous PZP immunocontraception have not been described. In the domestic rabbit (*Oryctolagus cuniculus*) (Wood et al. 1981), the domestic dog (*Canis familiaris*) (Mahi-Brown et al. 1985), and the baboon (species not given; Dunbar et al. 1989) there are data that suggest the antibody response of the treated animal attacks not only the mature ovum, but oocytes and other ovarian tissues, with resulting changes in estradiol and progesterone secretion. These effects have not been demonstrated in any of the 4 other species of nonhuman primates studied or in horses. Histological studies of ovaries among captive PZP-treated horses revealed no changes 3 years after treatment, and plasma progesterone values during treatment were consistent with normal cyclicity (Liu et al. 1989).

Behavioral integrity of treated animals is important, particularly in the case of social animals such as the horse. Bands with treated horses remained intact during the 18-month duration of this study, and the exchange of 3 mares between bands was within accepted limits for the Assateague herd during the previous 3 pretreatment years.

These results suggest that PZP immunocontraception is a possible alternative for controlling fertility in feral horse populations. However, the requirement for at least 2 inoculations for successful fertility inhibition is a weakness, and the current limitations of remote delivery are impediments for the use of PZP in management. The 3 mares which received only 1 inoculation were extremely wary and not approachable for a successful second inoculation. If this form of immunocontraception is to become an effective management tool for controlling feral horse populations, it must first be developed as a single-dose vaccine. Technology to convert the PZP antigen into a single-dose vaccine currently exists in the form of microencapsulation. This process, which provides a sustained release of drug, has been used successfully with contraceptive steroids (Kirkpatrick et al. 1982) and antigenic protein (Eldridge et al. 1989). Recently, the specific porcine zona antigenic proteins have been produced with monoclonal tissue cultures, eliminating the need for time-consuming preparation from fresh ovarian tissue and providing a potentially inexpensive source of the vaccine (Takagi et al. 1988). The effectiveness and safety of this form of immunocontraception can also be improved through the use of monoclonal proteins, because the pure receptor protein, ZP3, can be produced instead of the entire spectrum of zonae proteins which were used in this study. Experiments are under way to assess the effectiveness of a single annual booster inoculation, once antigen recognition has occurred. If a booster is effective, as it appears to be on the basis of urinary steroid metabolites, it is probably possible to incorporate the booster in an initial inoculation which delivers an initial bolus of antigen, a second pulse of microencapsulated antigen a month later, and the microencapsulated booster a year later.

CONCLUSIONS

This study provides the first description of successful fertility inhibition among uncaptured free-roaming mammals by means of remotely delivered immunocontraception. Remote inoculation of feral mares with PZP was an effective means of fertility inhibition and did not affect intact pregnancies. The process was reversible, it did not affect social integrity of horse bands, and the vaccine cannot be passed through the food chain. The impact of PZP contraception is on fertilization, and no hormones are involved which might impinge upon the brain and change behavior directly. Coupled with remote pregnancy testing by means of urinary and fecal steroid metabolites, the remote delivery of PZP offers a potential noncapture technology for feral horse contraception. This in turn makes public acceptance of contraceptive control of mammalian wildlife more likely than with approaches that require capture and handling.

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