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# Antigen recognition in feral mares previously immunized with porcine zonae pellucidae

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**Summary.** Twenty-six free-roaming feral mares were immunized against porcine zonae pellucidae (PZP) between February and May, 1988. Eight sexually mature mares received 2 inoculations 2 weeks apart, and 18 mares received 3 inoculations at intervals of 2 and 4 weeks. Analysis of urinary oestrone conjugates ($E_1C$) and non-specific progesterone metabolites (iPdG) in samples collected in October, 1988, revealed that none of the 18 mares that received 3 and only 1 of the 6 mares that received two inoculations were pregnant, whereas 3 of 6 sham-injected control mares and 5of 11 untreated mares were pregnant. In February and March, 1989, 14 of the immunized mares that were not pregnant were given a single booster inoculation of PZP. Urinary $E_1C$ and iPdG analysis, from samples collected in August and October, 1989, revealed only a single pregnancy among the 14 boosted mares, whereas 33% of mares treated in 1988, but not given a booster inoculation in 1989, and 7 of 16 untreated controls, were pregnant. Foaling was reflected accurately by the urinary steroid metabolite analyses. These results suggest that once recognition of PZP antigen occurs in mares, subsequent annual inoculations will raise antibody titres sufficiently to inhibit fertility.

## Introduction

Fertility control of feral horses has been the focus of numerous studies over the past two decades. Initial experiments concentrated on contraceptive androgens that were delivered to stallions as injectable, microencapsulated compounds capable of sustained release (Kirkpatrick *et al.*, 1982; Turner & Kirkpatrick, 1982). More recently, microencapsulated progestagens have been delivered to mares (Kirkpatrick & Turner, 1991), and silastic implants containing ethinyloestradiol have been placed in mares (Plotka *et al.*, 1989). All of these methods resulted in varying degrees of pharmacological success. However, the microencapsulated steroids required extremely large doses that interfered with remote delivery, and the silastic implants required restraint and surgery. In addition, concern exists about the long-term effects of steroids on the health of the animal.

An alternative to steroid-induced fertility control is immunocontraception. A conjugated form of a gonadotrophin-releasing hormone (GnRH) has been used successfully to stimulate antibodies in captive feral mares (Goodloe *et al.*, 1988) and solubilized porcine zonae pellucidae (PZP) injections inhibited fertility in 13 of 14 captive mares (Liu *et al.*, 1989). The objectives of this study were to (a) test the contraceptive efficacy of a PZP vaccine in free-roaming feral mares under field conditions; (b) determine the effectiveness of remote delivery; (c) determine the contraceptive efficiency and safety of the PZP vaccine when administered to both pregnant and non-pregnant mares and (d) evaluate the contraceptive efficiency of a single annual booster inoculation.

## Materials and Methods

Twenty-six feral mares of proven fertility, roaming free on Assateague Island National Seashore, Maryland, were selected for the study from among approximately 100 feral mares inhabiting the island. The mares chosen for treatment were selected because of their high foaling rates, which annually averaged 10% higher than the mean herd rate for the preceding 3 years. The PZP vaccine was prepared from porcine ovaries as described previously by Liu *et al.* (1989*a*). Briefly, porcine ovaries were sliced finely and the oocytes separated by filtration in cold phosphate buffer. The zona pellucida protein was heat solubilized and stored at -5°C until used.

Between 29 February and 10 March, the 26 mares received an initial inoculation of vaccine that was prepared as an emulsion of 0·5 ml phosphate buffer containing approximately 5000 zonae pellucidae (64·3 µg protein) and 0·5 ml Freund's Complete Adjuvant. The two components were emulsified in the field, using two 10 ml glass syringes joined with a plastic connector. After 100 strokes, the emulsion was loaded into a 3·0 ml self-injecting plastic dart with a 3·81 cm barbless needle. The needles were rinsed with 70% ethanol prior to being loaded into the rifle. The mares were darted from the ground in the hip region, using a Pax-Arms 0·327 calibre capture gun (Telonics Inc., Mesa, Arizona). Eight of the mares were treated in the presence of humans and the initial injection was accomplished with a 3 ml syringe and a jab-stick. Thereafter, further injections were given by dart. Between 12 and 21 March, the 26 mares received a second inoculation, as described above except for the addition of 0·5 ml phosphate buffer solution and substitution of 0·5 ml of Freund's Incomplete Adjuvant for the complete adjuvant. Between 16 and 25 April, 18 of the 26 mares received a third inoculation that was identical to the second. Six additional mares received only phosphate buffer and adjuvant in 1 or 2 inoculations. Identifiable markings were recorded for each horse and the animals were observed throughout April for adverse effects and the presence of abscesses at the sites of injection (Fig. 1).

During October 1988, 5 months after the last inoculation and a minimum of 2 months after completion of the breeding season, the mares were located and identified and the presence or absence of foals was recorded. Urine samples were collected from each of the 26 mares and 6 control mares by extracting the urine from the soil or aspirating it directly from the ground immediately after micturition. The unextracted urine samples were assayed for oestrone conjugates ($E_1C$) by radioimmunoassay (Kirkpatrick *et al.*, 1988), for non-specific metabolites of progesterone (iPdG) by enzyme immunoassay (EIA) as described by Kirkpatrick *et al.* (1990*a*), and for creatinine (Cr) by the microcolorimetric method of Taussky (1954). Oestrone conjugate and iPdG concentrations were given as µg/mg Cr and ng/mgCr, respectively, to account for differences in urine concentrations. Pregnancy determinations were based on $E_1C$ concentrations in excess of 1·0 µg/mg Cr and iPdG concentrations in excess of 100 ng/mg Cr.

Twenty-five of the 26 mares were not pregnant, based on urinary $E_1C$ and iPdG evaluations, and 14 of them were selected for a single booster inoculation, given between 17 February and 10 March, 1989. This injection was identical to the initial inoculation and was given with 0·5 ml Freund's Complete Adjuvant.

In August and October, 1989, urine samples were collected from the 14 booster-inoculated mares, the remaining 12 previously treated mares that did not receive booster inoculations and 16 other sexually mature mares that were not

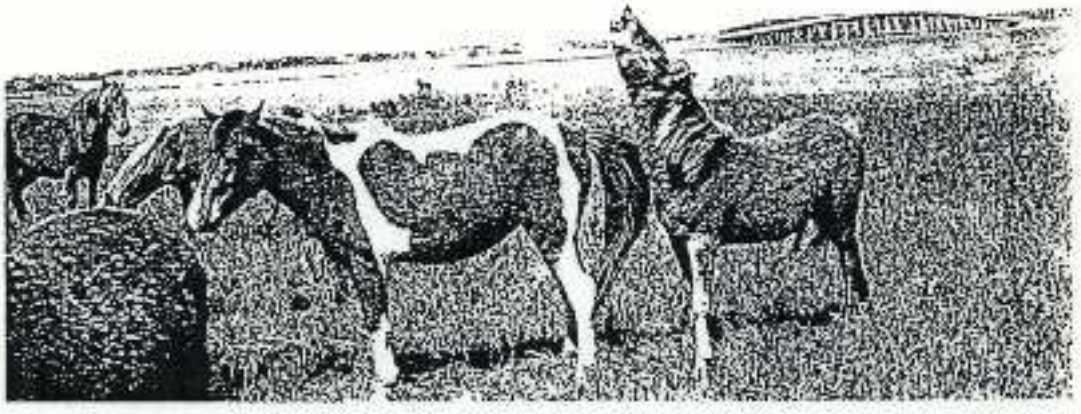

Fig. 1. A band of PZP-treated mares with a young stallion showing interest.

Table 1. Pregnancy and foaling rates for booster-inoculated, control and untreated mares.

| Treatment group | No. of mares | No. mares diagnosed pregnant (%)* | No. mares that foaled |
|---|---|---|---|
| Booster PZP | 14 | 1 (7) | 1 (7) |
| Control (sham injected) | 6 | 3(50) | 3 (50) |
| Untreated | 16 | 7(44) | 7 (44) |

* Based on urinary $E_1C$ and iPdG measurements.

treated previously. The samples were assayed by EIA for the oestrone conjugates, oestrone 3-glucuronate and oestrone 3-sulphate as described by Munro *et al.* (1991). For the $E_1C$ assay, each urine sample was diluted 1:100 in distilled $H_2O$ and a 20-µl aliquot was taken for assay. The antibody (R522, B.L. Lasley, University of California, Davis) showed equal cross-reactivity for both the glucuronide and sulphate conjugates of oestrone. The inter- and intra-assay coefficients of variation were 13% (n = 10) and 10% (n = 12) respectively. For the iPdG assay, urine samples were diluted 1:1 with distilled $H_2O$, and a 20-µl aliquot was taken for assay. The inter- and intra-assay coefficients of variation were 11·45% (n = 10) and 10·04% (n = 15), respectively. Results were given as µg $E_1C$, or ngiPdG/mg Cr. Differences in foaling rates were tested for significance by means of binomial probability distribution.

## Results

The initial inoculations given in 1988 resulted in only a single pregnancy among the 26 treated mares. None of the 18 mares receiving three inoculations was pregnant on the basis of urinary $E_1C$ and iPdG concentrations and none delivered foals in 1989. Only one of the 8 mares that received two inoculations was diagnosed pregnant on the basis of urinary $E_1C$ and iPdG and this was the only mare to produce a foal in 1989. The overall fertility rate after treatment was 3·8% (compared with 53·8% for the same mares for each of the two previous pre-treatment years [1987 and 1988]), 50% for the 6 control mares in 1989, and 45·4% for 11 untreated sexually mature mares in the study area in 1989. These differences in foaling rates between the 26 PZP-treated mares and the other groups were all highly significant ($P < 0·0002 - 0·0019$). Foaling and pregnancy data based on the urinary $E_1C$ and iPdG measurements showed 100% correlation. Details of the results of these initial inoculations have been reported previously (Kirkpatrick *et al.*, 1990b).

Thirteen of the 14 mares (92·8%) that received the single PZP booster inoculation in February, 1989, were diagnosed not pregnant in August or October, 1989, on the basis of urinary $E_1C$ and iPdG concentrations, and they did not produce foals in 1990. The one PZP booster-treated mare that did foal in 1990 was diagnosed pregnant in October, 1989, with urinary $E_1C$ concentrations of 4·95 µg/mg Cr and iPdG concentrations of 511·7 ng/mg Cr. Urinary $E_1C$ concentrations for the 13 booster-treated non-pregnant mares ranged from undetectable to 0·414 µg/mg Cr, and urinary iPdG concentrations ranged from 4·94–26·5 ng/mg Cr. The pregnancy and foaling rates for the 6 control and 16 untreated mares were 50% and 43·7% respectively (Table 1). The differences in foaling rates for the 14 PZP booster-inoculated mares *vs* the 6 control mares and the 16 sexually mature, untreated mares in 1990, and the mean foaling rate for all sexually mature mares on Assateague Island between 1975 and 1982 (57·1 ± 3·9%, Keiper & Houpt, 1984) were significant ($P < 0·001 - 0·018$).

## Discussion

Liu *et al.* (1989) demonstrated the contraceptive efficacy of PZP immunization in captive feral and domestic mares and Kirkpatrick *et al.* (1990b) demonstrated similar efficacy in dart-injected free-roaming feral mares. This study is the first reported attempt to suppress fertility in mares with a single

annual booster inoculation following a lapse of 12 months after initial immunization. National Park regulations prohibited the capture or handling of the Assateague Island horses, thereby preventing the collection of blood and the measurement of antibody titres. However, the contraceptive efficacy of the booster inoculation experiment was consistent with the results of the previous two trials (Liu *et al.*, 1989; Kirkpatrick *et al.*, 1990b) and suggest anamnestic increases in anti-PZP antibody titres.

Measurement of antibody production in mares given a series of 3 intramuscular injections of PZP emulsified in Freund's Adjuvant indicated that peak titres occur approximately 3 months after initial inoculation, wane thereafter and fall to basal levels about 9–12 months later (Liu *et al.*, 1989). This pattern of rise and fall of antibody titres in mares in response to PZP immunization is similar to those seen in primates (Sacco *et al.*, 1983) and other animal models (Henderson *et al.*, 1988). Comparisons of the results of PZP immunization with those in other species must, however, be made with care because of differences in the quantity of antigenic protein administered, the nature of the adjuvant and the purity of the PZP protein. Three of the 12 mares immunized initially in 1988, but not given a booster inoculation, became pregnant between April and June, 1989, indicating a decline of antibody titres and a return to normal fertility 13 to 17 months after initial immunization. A fourth mare delivered a foal in January, 1990, thereby indicating that she had conceived in February, 1989, some 11 months after her last inoculation. These pregnancies suggest extremely low antibody titres in the mares concerned because it has been demonstrated previously that only low concentrations of anti-PZP antibody are required to inhibit fertility (Dunbar & Schwoebel, 1988). The 33% foaling rate for the mares that did not receive booster inoculations is consistent with the overall foaling rates for Assateague Island mares (Keiper & Houpt, 1984).

The production of only a single foal among the 14 PZP booster-treated mares was statistically significant and suggests that antibody titres increase rapidly to concentrations that provide contraceptive protection. The duration of antibody titres with contraceptive action following booster inoculations remains unknown and will require controlled studies with captive populations. The causes of the single failure among the 26 mares immunized originally, and the 1 failure among the 14 mares receiving a booster inoculation, are not understood. The most likely explanation is tissue capsulation of the antigen at the injection site resulting in poor absorption into the circulation. Although the dart needles were washed in 70% ethanol, delivery by dart results in puncture wounds that include skin bacteria or hair or both. The 26 mares used in this study were darted a total of 84 times, in addition to 15 dart-injection attempts that penetrated the skin but bounced out before injection was complete. A total of five small abscesses (10–25 mm in diameter) were observed, which appeared 48 h after injection and drained for 6–9 days before healing completely by 14 days. No abscesses appeared in the two treated mares that became pregnant.

It is generally agreed that the mechanism of action of PZP contraception is the production of antibodies that block the ZP3 sperm receptor on the zona pellucida (Florman & Wassarman, 1985), probably by causing steric hindrance of sperm-receptor attachment (Skinner *et al.*, 1984). Because urinary $E_1C$ concentrations are not diagnosed until after Day 35 of gestation (Evans *et al.*, 1984), the results of pregnancy testing to date in the present study suggest, but do not prove, that PZP immunization of mares results in conception failure. Alternatively, antibodies may be interfering with folliculogenesis to prevent the maturation of ova, thereby resulting in infertility.

The effects on the endocrine functions of the mare's ovaries of annual PZP immunization over the course of several years are not known. Liu *et al.* (1989) found no evidence of abnormal ovarian histology, or any significant changes in progesterone patterns in seasonally oestrous mares that received only the 3 initial inoculations. However, the dog (Mahi-Brown *et al.*, 1985), rabbit (Skinner *et al.*, 1984) and baboon (Dunbar *et al.*, 1989) all demonstrated significant changes in ovarian steroid secretion and oocyte differentiation after PZP immunization, thereby implying that ovarian components other than mature ova had been affected. Although behaviour was not evaluated systematically in this study, 4 of the 14 booster-treated mares were observed showing behavioural oestrus and copulation. Studies currently are in progress to evaluate ovarian steroid secretion patterns

in seasonally oestrous mares following 3 consecutive years of PZP immunization.

In conclusion, the results of this study indicate that contraception in feral mares can be accomplished by dart gun administration of PZP Freund's Adjuvant emulsion. The technique does not harm pregnant animals and the effects can be extended with single annual booster inoculations.

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## References

Dunbar, B.S. & Schwoebel, E. (1988) Fertility studies for the benefit of animals and human beings: development of improved sterilization and contraceptive methods. *J. Am. vet. med. Ass.* 193, 1165-1170.

Dunbar, B.S., Lo, C., Powell, J. & Stevens, J.C. (1989) Use of a synthetic peptide adjuvant for the immunization of baboons with the denatured and deglycosylated pig zona pellucida proteins. *Fertil. Steril.* 52, 311-318.

Evans, K.L., Hughes, J.P., Couto, M., Kasman, L.H. & Lasley, B.L. (1984) Pregnancy diagnosis in the domestic horse through direct urinary estrone conjugate analysis. *Theriogenology* 22, 615-620.

Florman, H.M. & Wassarman, P.M. (1985) O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. *Cell* 41, 313-324.

Goodloe, R., Warren, R.J. & Sharp, D.C. (1988) Sterilization of feral horses by immunization against LHRH. *Proc. Ann. Conf. Wildl. Dis. Ass.* University of Georgia, p. 25.

Henderson, C.J., Hulme, M.J. & Aitken, R.J. (1988) Contraceptive potential of antibodies to the zona pellucida. *J. Reprod. Fert.* 83, 325-343.

Keiper, R. & Houpt, K. (1984) Reproduction in feral horses: an eight-year study. *Am. J. vet. Res.* 45, 991-995.

Kirkpatrick, J.F., Turner, J.W., Jr. & Perkins, A. (1982) Reversible fertility control in feral horses. *J. equine vet. Med.* 2, 114-118.

Kirkpatrick, J.F., Kasman, L., Lasley, B.L. & Turner, J.W., Jr. (1988) Pregnancy determination in uncaptured feral horses. *J. Wildl. Manag.* 52, 305-308.

Kirkpatrick, J.F., Lasley, B.L. & Shideler, S.E. (1990a) Urinary steroid evaluations to monitor ovarian function in exotic ungulates: VII. Urinary progesterone metabolites in Equidae assessed by immuno-assay. *Zoo. Biol.* 9, 341-348.

Kirkpatrick, J.F., Liu, I.M.K. & Turner, J.W., Jr. (1990b) Remotely-delivered immunocontraception in feral horses. *Wildl. Soc. Bull.* 18, 326-330.

Kirkpatrick, J.F. & Turner, J.W., Jr. (1991) Fertility control in deer. In: *Deer Management in an Urbanizing Region: Problems and Alternatives for Traditional Management*. Ed. S. Lieberman. Humane Society of the U.S., Washington, D.C.

Liu, I.K.M., Bernoco, M. & Feldman, M. (1989) Contraception in mares heteroimmunized with porcine zona pellucida. *J. Reprod. Fert.* 89, 19-29.

Mahi-Brown, C.A., Yanagimachi, R., Hoffman, J.C. & Huang, T.T.F. (1985) Fertility control in the bitch by active immunization with porcine zonae pellucidae: use of different adjuvants and patterns of estradiol and progesterone levels in estrous cycles. *Biol. Reprod.* 32, 761-772.

Munro, C.J., Stabenfeldt, G.H., Cragun, J.R., Addiego, L.A., Overstreet, J.W. & Lasley, B.L. (1991) Relationship of serum estradiol and progesterone concentrations to the excretion profiles in their major urinary metabolites as measured by enzyme immunoassay. *Clin. Chem.* (In press).

Plotka, E.D., Vevea, D.N., Eagle, T.C., Tester, J.R. & Siniff, D.B. (1989) Effects of hormone implants on estrus and ovulation in feral mares. *J. Wildl. Dis.* 24, 507-514.

Sacco, A.G., Subramanian, M.G., Yurewicz, E.C., DeMayo, F.J. & Dukelow, W.R. (1983) Heteroimmunization of squirrel monkeys (*Saimiri sciureus*) with a purified porcine zona antigen (PPZA): immune response and biologic activity of antiserum. *Fertil. Steril.* 39, 350-358.

Skinner, S.M., Mills, T., Kirchick, H.J. & Dunbar, B.S. (1984) Immunization with zona pellucida proteins results in abnormal ovarian follicular differentiation and inhibition of gonadotropin-induced steroid secretion. *Endocrinology* 115, 2418-2432.

Taussky, H.H. (1954) A microcolorimetric determination of creatinine in urine by the Jaffe reaction. *J. biol. Chem.* 208, 853-861.

Turner, J.W. & Kirkpatrick, J.P. (1982) Steroids, behaviour and fertility control in feral stallions in the field. *J. Reprod. Fert. Suppl.* 32, 79-87.