

CHEMICAL FERTILITY CONTROL AND THE
MANAGEMENT OF THE ASSATEAGUE PONIES
(CA-1600-3-0005)

FINAL REPORT

FEB. 1986 - AUGUST, 1989

Attached is a final report for activities involved in Grant CA-1600-3-005. Part I describes the initial experiments involved in hormonal contraception. Fertility among mares bred by treated stallions was reduced by approximately 45% compared to control animals. Fertility among treated mares was 100%.

Part II describes the immunocontraception experiments between February 1988 and August, 1989. The contraceptive vaccine was > 93% effective in inhibiting fertility, was delivered remotely, is reversible, is safe when administered to pregnant mares, and cannot be passed along in the food chain.

Details of treatment and results are presented in the attached report.

INTRODUCTION

Between February of 1986 and August, 1987, a study was conducted on Assateague Island, to test the feasibility of inhibiting fertility of the horse population within the boundaries of the National Seashore. The objectives of this study included (1) testing the effectiveness of remotely delivered microencapsulated testosterone propionate (mTP) to stallions, and (2) testing the effectiveness of remotely delivered microencapsulated norethisterone (mNET) to mares. The research and theoretical considerations behind this study are set forth in the original proposal to Assateague National Seashore.

The study included the treatment of stallions with mTP in an attempt to lower sperm counts and decrease sperm motility, as reported previously by Kirkpatrick et al., (1982) and Turner and Kirkpatrick (1982) and treatment of mares with mNET in an attempt to inhibit ovulation.

THE MARE STUDY

Previous work (Ginther, 1979) indicated that elevated progesterone levels in mares leads to an inhibition of ovulation and subsequent infertility. The experimental design called for selecting six mares of proven fertility and administration of 2.0 g of mNET, IM, with a tranquilizer gun. The microencapsulated form of NET, prepared by Southern Research Institute (Birmingham, Alabama) releases over a six month period, from the time of administration (March 3-15, 1986), covering the entire breeding season. Norethisterone was chosen because of its potent biological activity, effectiveness in humans, and lack of side effects. The 2.0 g dose was calculated to maintain blood concentrations greater than 0.5 ng/ml plasma, the level necessary to inhibit ovulation in mares.

Administration of the drug to mares proved to be simple and was accomplished without difficulty. Two injections of approximately 5 mls of microcapsules (1 g of microcapsules = 0.326 gms of active drug) were given. The carrier was carboxymethyl

cellulose. The gun was a Pax Arms .527 cal. tranquilizer gun, which permitted regulation of the force with which the dart was fired and utilizing a self-injecting barbless dart. The mares were very approachable, reacted very little to the dart and were re-approachable from minutes to hours after the first darting. No injuries to horses occurred.

Three technical problems with the actual administration of the drug were encountered. First, administration occurred during several very cold days (10-15 F) and the viscosity of the carboxymethyl cellulose increased and sometimes interfered with fast injection. This necessitated keeping the carrier as warm as possible prior to delivery. A second problem involved the suspended microcapsules. Since the microcapsules are not dissolved, but merely suspended, they had a tendency to settle out and clump in the dart if not delivered within 10 minutes of initial mixing. Thus, when mares wandered off before darting could occur, the dart would have to be removed from the gun and the microcapsules resuspended. Third, use of barbless darts required considerable skill with the gun. Almost all tranquilizer guns are designed for delivering immobilizers. Consequently, a dart can be fired with substantial velocity and little trajectory and because of the barb it will remain in the animal after impact. The delivery of non-immobilizing drugs necessitates barbless darts, which will ultimately fall out. Thus, velocity must be decreased significantly and trajectories increased in order to "lob" darts into animals and have them remain. This problem can, and was overcome but points to the need for extreme familiarity with the gun. In actuality, doses of active drug delivered ranged from 0.652 to 1.3 g. Similar problems were encountered by other investigators, even with barbed darts (Seal et al., 1985).

The results of this arm of the study contraindicate the use of remotely delivered progestins in mares. Six of six mares delivered foals between April and August of 1987. A 100% foaling rate among six Assateague mares is an extremely unlikely event, with virtually no probability. These same six mares had a 50% foaling rate in 1986, and a

control population of 15 mares of known fertility had a similar 45.4 % foaling rate in 1987. This suggests that progestins actually enhanced fertility.

These results are explained by a similar study in western feral horses just completed by Vevea et al (1987) at the University of Minnesota. They placed subdermal silastic implants containing various doses of progesterone and estradiol, in doses far exceeding what were used on Assateague Island, in 120 feral mares. While elevated progestin levels occurred during the first five weeks in most horses, concentrations fell rapidly after that. Behavioral estrus and ovulation occurred in a large percentage of the test mares. The Minnesota data and the Assateague data suggest that mares merely increase their clearance of exogenous hormones. Thus, on Assateague, as fast as progestins were administered, mares increased their rate of clearance. In doing so, these mares lowered their own endogenous progestin levels to a point where ovulation was a certainty. It is important to remember that progestins concentrations above 0.5 ng/ml plasma are a leading cause of infertility in mares.

While this arm of the Assateague study failed - completely - to achieve the primary goal of infertility, several valuable lessons were learned. The ability of mares to increase progesterone clearance in response to exogenous progestins is, in itself, a valuable piece of scientific information. Of greater value to the future work of this nature was the discovery that mares could be treated remotely with considerable ease.

THE STALLION STUDY

In this arm of the study four stallions of proven fertility and in possession of harem bands were chosen. These four stallions collectively had 14 mares of proven fertility associated with them. Each stallion was to receive 3.0 g of mTP, delivered between February 4 and March 12, 1986, as described in the mare study. The mTP was designed to release over six months, causing oligospermia and decreased sperm motility. The dose of 3.0 g was selected because of (1) the remote delivery and limitations on volume (one

g of microcapsules = 0.5 g of testosterone propionate), and (2) the effectiveness of this dose in previous field trials with feral horses (Kirkpatrick et al., 1982; Turner and Kirkpatrick, 1982).

In addition to the technical problems already described in the mare study, certain of the stallions proved to be very difficult to administer the requisite four darts to. One stallion, K, who was acclimated to people and the State Park, presented a relatively easy target provided that several days passed between without harassment between darts. Another, OR, was moderately wary and caused some minor difficulty. Two others, M and OS were extremely wary and required many days over a 40 day period to complete injections. Darts which bounced out before completion of injection caused some loss of drug, and most doses fell short of the 3.0 g of active drug. Actual doses ranged from 2.0 to 3.4 g of active drug.

The behavior of treated stallions was tested for changes through the use of a reproductively associated behavior - elimination marking behavior (EMB) - as described by Turner et al., (1981).

In 1987 the 14 mares of proven fertility associated with the four treated stallions produced four foals, for a fertility rate of 28.9%. It is worth noting that one of these four foals was born about March 15 - extremely early - which means it was conceived only about 14 days after the completion of the drug treatment to the stallion (K). Thus, this early breeding, which is rare on Assateague, occurred before the pharmacological action of testosterone could take place. Nevertheless, this foal was counted among the four produced. The 28.9% foaling rate is significantly smaller than the 45.4 % foaling rate for a control population of 15 fertile mares for the 1987 season, and for the foaling rate for the experimental mares for the previous five years, which ranged from 42% - 50%. Thus, the decrease in foaling rates was somewhere between 37 % and 45 %, depending upon which control figure is used to calculate effectiveness. In any case, fertility was reduced from 1/3 to almost 1/2 among mares in the experimental group.

Quantifiable behavioral changes between treated stallions and seven control stallions were not significant ($P < 0.05$).

The decrease in fertility on Assateague Island, within a range of 37 % to 45 % is substantially less than the 83% decrease reported by Kirkpatrick et al (1982) for treated feral horses in Idaho. There are several probable reasons for this. First, in the Idaho tests the stallions were first immobilized and then given the mTP by hand, in doses of 3.0 to 10 g. There was no loss of drug during the administration. The Assateague stallions were all given doses at the lowest end of the effective dose range and this may have decreased the effectiveness of the treatment.

A second and more serious problem involved the social behavior of the horses. The entire experimental design was based upon two premises. First, the previous and extensive work of Keiper indicated great band stability and very little mare exchange. A second premise was that there would be minimal migration and range overlap between bands. Both these conditions existed and were well-documented prior to the initiation of this study. Both conditions changed significantly during the course of the study. Mare exchange became common and entire bands migrated north and south among experimental bands. Two events probably contributed to this instability. First, an increased density of horses, and stallions in particular suggests increased competition for mares and a loss of band fidelity. Second, a very large band (22 horses led by stallion T) fragmented during the spring of 1986, resulting in a large pool of unattended mares moving all about the study area. Thus, it is not at all certain that all four foals born to mares in the treated stallion groups were in fact sired by those stallions.

Despite a clear reduction in fertility, and despite over 10 years of research with microencapsulated testosterone and advocacy for its use, we see several shortcomings associated with the remote delivery of this drug. There is no question about the pharmacological effectiveness of the mTP. A controlled laboratory study and two field

tests have confirmed this fact. The shortcomings are of a logistical nature, caused by remote delivery, and affect field usefulness. The volume of drug required for effective pharmacological action necessitates a minimum of four 7.0 ml darts just to reach the low end of the effective dose range. Four darts is not in itself a problem - there appears to be little likelihood of serious injury to horses - but because of the wild nature of the stallions, delivery takes a great deal of time and effort and is clearly not cost effective. Larger darts might be considered but the danger of injury to horses increases. Even more important is the changing nature of the social behavior of the Assateague horses. The increase in stallion density--5 bachelor bands with a total of 15 stallions, 16 harem stallions, and at least 11 other stallions over the age of one in a total population of a little over 100 horses in the study area--and the decrease in band fidelity by mares means that effective use of mTP must target a very large population of stallions - perhaps as many as 30. The logistics of this challenge and the man hours of labor required would be enormous.

These results point to the need for several changes in the approach to fertility control in Assateague's feral horses. First, the treatment should focus upon mares, and not stallions. Mares are considerably easier to approach and treat and interband movements will not interfere with effectiveness. Second, a drug or vaccine should be utilized which is water-based, making injection easier. Third, this drug should be able to be delivered in smaller volumes than 6-7 ml, making complete injection more likely. Fourth, the drug should ideally be deliverable in a single administration, and fifth, its action must be reversible.

The tables which follow illustrate salient data gathered during the course of this study.

REMOTELY DELIVERED IMMUNOCONTRACEPTION IN FERAL HORSES

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The remotely-delivered porcine zonae pellucidae (PZP) vaccine described here is the first successful application of immunocontraception to uncaptured free-roaming wildlife. The PZP vaccine was administered to free-roaming feral mares on a coastal barrier island by means of darts, and was greater than 93% effective in inhibiting fertility among mares receiving either two or three inoculations. The vaccine's effects are reversible, do not effect pregnancies in progress, do not effect social behavior and the vaccine cannot be passed through the food chain. These results suggest immunocontraception is a safe and effective method of controlling feral horse populations.

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Reducing fertility among free-roaming feral horses (*E. caballus*) has been the goal of numerous studies over the past 16 years. Initial experiments 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) (1). Despite the pharmacological effectiveness of treatment, which decreased sperm count and motility, considerations of cost-effectiveness and the stresses caused by immobilization or capture made it clear that management of horse populations by fertility control would be difficult to achieve unless the contraceptive agent could be delivered remotely. In a second study (2) the pharmacological effectiveness of mTP in stallions was confirmed, but difficulty was encountered in remotely delivering a sufficient mass of the steroid.

More recently attention has turned to contraception in the feral mare. Experiments with ethinylestradiol-progesterone Silastic® implants (3) show pharmacological promise but the techniques used require 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 (4), and solubilized porcine zona pellucidae (PZP) injections inhibited fertility in 13 of 14 domestic or captive mares (5).

The objectives of this study were to (a) test the contraceptive efficacy of a porcine zona pellucidae (PZP) vaccine in free-roaming

feral mares, under field conditions, (b) determine the effectiveness of remote delivery, (c) determine the vaccine's contraceptive effectiveness in pregnant and non-pregnant mares, and (d) evaluate the vaccine's safety for use in pregnant mares.

Twenty-nine mares of proven fertility were selected for the study from among the approximately 150 feral horses inhabiting Assateague Island National Seashore, (MD). The ages and fertility records, some dating back as far as 1974, were known for almost all animals. The mares chosen for treatment were purposely selected for high fertility rates, with the treatment group averaging 10% higher rates than the overall herd rate over the preceeding three years. The PZP vaccine was prepared from porcine ovaries as previously described (5) and stored frozen at -50°C until used.

Between February 29 and March 10, 1988, all 29 mares received an initial inoculation of vaccine. The vaccine was prepared as an emulsion, made up of 0.5 cc of vaccine (equivalent to approximately 5000 zonae or $64.3\text{ }\mu\text{g}$ of protein) in phosphate buffer and 0.5 cc of Freund's Complete Adjuvant. The two components were mixed in the field, using two 10 cc glass syringes joined with a plastic connector and after 100 strokes the emulsion was loaded into a 6 cc self-injecting plastic dart with a 1.5 inch barbless needle. The mares were darted from the ground, in the hip region using a Pax-Arms® .527 caliber capture gun. The needles were rinsed with 70% EtOH prior to being loaded into the rifle. Eight of the mares were acclimated to humans and vaccine delivery was accomplished with a 3 cc syringe and a jab-stick. Between the 12th and 21st of March, 26 of the 29 mares received a second inoculation, as described

above. The second inoculation was 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. Between the 16th and 25th of April, 18 of the 26 mares which received the second inoculation also received a third inoculation, which was identical to the second. Identifying markings were recorded for each horse and the animals were observed throughout April for adverse effects and particularly for the presence of abscesses at the sites of injection.

During October of 1988, five months after the last inoculation and two months after 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 29 treated and six 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 sulfate (E_1S) and indexed to creatinine concentrations (6) and for non-specific progesterone (Po) metabolites (7) and pregnancy determinations were made on the basis of the urinary E_1S and non-specific Po metabolite concentrations. In August of 1989, the mares were again located, identified and observed for the presence of foals. Foal production for 1989 was compared to foal production for the same group of mares for 1988 and 1987, with six control mares for the same two previous years, and with all untreated mares for 1989. Finally, in August of 1989, a random sample of seven treated mares was pregnancy tested by means of urinary steroid metabolites in order to test reversibility of the vaccine's antifertility effect.

A total of three abscesses were observed among the 29 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 six to nine days after treatment. Complete healing had occurred within 14 days following treatment.

Of the 29 treated mares, 15 were pregnant at the time of inoculation (51.7 %) and all 15 produced foals in the spring of 1988, one to five months after the last inoculation of PZP vaccine. The six control mares produced three foals (50 %). By October, 1988, a foal belonging to one 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 of 1989, as yearlings.

Urinary E_1S and non-specific Po metabolite concentrations in mid-October, 1988, indicated a total of two pregnancies among the 29 treated mares. None of the 18 mares receiving three inoculations were pregnant, one of eight receiving two inoculations was pregnant, and one of three mares receiving one inoculation was pregnant. Three of six control mares were pregnant. The 30 non-pregnant treated and control mares had mean urinary E_1S concentrations of 0.11 ± 0.34 (SEM) $\mu\text{g}/\text{mg}$ creatinine versus 3.47 ± 0.735 $\mu\text{g}/\text{mg}$ Cr. among the five pregnant mares. Mean urinary non-specific Po metabolite concentrations were 3.6 ± 0.499 ng/mg Cr for the 30 non-pregnant mares versus 215.8 ± 83.4 ng/mg Cr for the five pregnant mares. In both cases, the differences between

concentrations in pregnant and non-pregnant mares were significant at the $P < 0.001$ level of confidence. Accuracy of urinary pregnancy testing is given in Table 1. By August, 1989, two live foals were present among the 29 treated mares, and three foals among the six control mares, precisely as predicted by the urinary hormone metabolite measurements. Overall fertility rate of the 29 treated mares was 6.8 % after treatment, versus 55.5 % for each of the two previous pre-treatment years 1987 and 88, versus 50% for the six control mares in 1989, and versus 53.8% for all 13 untreated sexually mature mares in the study area in 1989. The three mares receiving only one inoculation were not part of the experimental design, and when the experimental population is confined to the 26 mares receiving two or three inoculations, the overall fertility rate for treated mares is 3.8 %. Foaling data for treated and untreated mares are given in Table 2. Three of seven randomly selected treated mares were determined to be pregnant in August of 1989, based on urinary estrone conjugates.

This study provides the first description of successful fertility inhibition among uncaptured free-roaming wildlife by means of remotely delivered immunocontraception. The success of the PZP vaccine in suppressing fertility among the Assateague feral mares is based on its ability to inhibit fertilization or possibly implantation (8). The porcine zona pellucida consists of three glycoproteins. One of those, ZP3, is the receptor molecule for sperm surface molecules (9). Equine antibodies raised against PZP are thought to block the sperm receptor sites on the equine ovum, thereby preventing fertilization (5). To date, PZP has been used to

successfully inhibit fertility in a number of mammals, including four species of non-human primates and *in vitro* fertilization in humans (10).

No previous studies have been conducted in which pregnant animals of any species were vaccinated with PZP. Two major questions to be answered by this study were whether the immunosuppression which accompanies pregnancy would interfere with the effectiveness of the antifertility effects of PZP vaccine, and whether the pregnancies would be successful and the foals healthy. These are important considerations since the use of this vaccine for management purposes will by necessity include pregnant mares among the treated animals. Pregnancy did not alter antifertility effects of the vaccine nor was the normal progress of pregnancies and health of the foals affected by vaccine administration.

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 the horses. Another advantage is the reversibility of the vaccine's contraceptive effects. Earlier studies with captive horses (5) demonstrated that treated horses that failed to conceive after PZP treatment could breed successfully the following year, as did at least three of seven mares in this study. The issue of reversibility is politically, as well as biologically important since it is unlikely that public opinion will favor irreversible sterilization among feral horses. Despite the return of normal fertility among PZP-treated

horses reported by others (5) and in this study, the long-term effects of chronic PZP immunocontraception are not defined at present and point the way toward future studies. In some species, such as the rabbit (11) and dog (12), 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, but these effects have not been demonstrated in non-human primates. Histological studies of ovaries among captive PZP-treated horses revealed no changes three years after treatment, and plasma Po values during treatment were consistent with normal cyclicity (5).

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 incidence of exchange of mares between bands was within normal limits for the Assateague herd. The focus of PZP contraception is fertilization and no hormones are involved which might impinge upon the brain and change behavior directly.

A final advantage of the PZP vaccine is the protein nature of the contraceptive antigen. This precludes the possibility of passage of the antifertility agent through the food chain. In any realistic management by contraception, some treated animals will die from natural causes and a variety of scavengers, including eagles, buzzards, small carnivores and in some areas mountain lions and bears, will feed upon the carcass. Protein, unlike steroids, and particularly synthetic steroids, cannot be passed along intact to the predators' tissues and cannot accumulate therein. In addition,

protein vaccination avoids the possible urinary and fecal contamination of the environment by poorly metabolized steroids, and especially those synthetic estrogenic steroids which have high potency and high resistance to biodegradation.

These results suggest that PZP immunocontraception is an attractive alternative for the management of feral horse populations, however, the requirement for at least two inoculations for successful fertility inhibition is a current weakness. The technology to convert the PZP antigen into a single-dose vaccine already exists in the form of microencapsulation. This process, which provides a sustained release of drug has been used successfully with contraceptive steroids (1) and antigenic protein (13). 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 (14). The effectiveness and safety of this form of immunocontraception can also be improved through the use of monoclonal proteins, since the pure receptor protein, ZP3, can be produced instead of the entire spectrum of zonae proteins which were used in this study.

Experiments are currently underway to assess the effectiveness of a single annual booster inoculation, once antigen recognition has occurred. If a booster is effective, 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.

Coupled with remote monitoring of ovarian dynamics and pregnancy

testing now possible by means of urinary and fecal steroid metabolites, the remote delivery of PZP offers complete non-capture technology for feral horse contraception and evaluation of reproductive effects of treatment. This in turn makes public acceptance of contraceptive control of wildlife more likely than with approaches that require capture and handling.

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