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EQUINE IMMUNOCONTRACEPTION USING PORCINE ZONA PELLUCIDA: A NEW METHOD FOR REMOTE DELIVERY AND CHARACTERIZATION OF THE IMMUNE RESPONSE

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SUMMARY

The purpose of this study was to investigate a new method of remote vaccine delivery and characterize the antibodies produced by the horse following vaccination with porcine zona pellucida (pZP). Three mares were initially immunized with 400 μg and boosted with 200 μg of pZP combined with a synthetic trehalose dicorynomycolate glycolipid adjuvant and packaged in a biodegradable bullet. Four control mares were vaccinated in the same manner, but with placebos. Contraception efficacy was determined by pregnancy rates. Serum antibody levels were measured by enzyme linked immunosorbent assay (ELISA) and expressed as an absorbance at a dilution of 1:1,000 of the mares' serum. Immunoblots using pZP and equine zona pellucida (eZP) were used to determine specificity and cross-reactivity of the antibodies. The delivery system investigated proved to be easy to administer, effective, and did not result in abscess formation or produce any clinical problems. Treatment with pZP resulted in a decreased conception rate during the first year (0/3) and second year (0/3) when compared to control mares for each year (3/4 and 4/4, respectively). ELISA revealed that the treated mares had absorbance levels that varied from 0.20 to 2.28 and lasted for at least 2 breeding seasons. The sera from these mares reacted with all three pZP glycoproteins and varied in their specific response to eZP glycoproteins. This variation was demonstrated by recognition of the 90-106 and 45-55 kDa eZP bands by serum of one mare while the 75-90 and 40-50 kDa glycoproteins bands were recognized with another mare's serum. In summary, immunocontraception for two breeding seasons was achieved using a new remote delivery system for vaccination of mares with pZP and resulting serum antibodies cross-reacted with specific eZP glycoproteins.

In 1971, feral horse populations came under government protection with the passage of the Wild Free-Roaming Horse and Burro Act which directs the Secretaries of Agriculture and Interior to protect, manage, and control these populations on public land. In the early 1970's the population was estimated to be around 17,500 horses and burros and increased to an estimated 42,756 horses and 7,665 burros by 1985.[1]

In 1985, over 18,000 excess animals were removed and plans were made for subsequent yearly removal of additional animals. The Wild and Free-Roaming Horse and Burro Act of 1971 was an act meant to protect dwindling populations of feral horses. It did not provide a means for managing the populations once their numbers had been reestablished and does not allow for any type of management that involves death of the animals. Since 1985 excess animals have been removed and put up for public sale through the "Adopt-A-Horse" program. However, many

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of the animals are not desirable for domestic use and remain in holding pens until natural death. Reduced budget trends reflected an increased concern in the United States Congress about management levels and the rapidly escalating cost of maintaining unadopted excess animals. In 1985, over $5 million was spent to maintain up to 10,000 animals at a time.[1]

Traditional methods of animal population control including hunting, trapping, relocating, and poisoning are often less than desirable.[2] Controlled hunting cannot be used in urban areas or National Parks. Trapping, particularly with leg-holding devices, is now illegal in many states, provinces, and countries. Live-trapping and relocation of animals is expensive and works only where sufficient suitable habitat for relocation exists. Poisoning is unacceptable because it is notoriously nonspecific.

Recently, a great deal of emphasis has been placed on immunological methods of fertility control to regulate population growth for both humans and wildlife. Long-term contraception via stimulation of the immune system has been investigated in feral mares.[3,4,5,6] These investigations used heat-solubilized porcine zona pellucida (pZP) as the contraceptive immunogen. The most detailed investigation was reported by Liu et al.[3] using a series of four injections containing either 2000 or 5000 pZP each, along with either aluminum hydroxide gel or Freund's as the adjuvant. Contraception occurred in 86% (12/14) of the fertile mares studied; however, one of the mares developed an abscess at the injection site. Although mare anti-pZP sera cross-reacted with intact equine oocytes, the highest reactive serum dilution for equine oocytes was 1:20 while it was 1:800 for porcine oocytes. Serum titers, reported as relative absorbance levels, at 8 months post-injection remained high (>0.8) for seven of the feral mares while two had near zero levels (<0.05). Inhibition of fertilization in vitro could not be studied in the horse system due to inconsistent controls. Therefore, the porcine system (boar spermatozoa with porcine oocytes) was used for the in vitro studies. Preincubation with mare antisera containing increasing levels of antibody resulted in an increased block of spermatozoa binding up to 99%.

Subsequently, Kirkpatrick et al.[4] investigated the applicability of this type of immunocontraceptive vaccine to feral horse populations. This vaccine also contained 5,000 heat-solubilized pZP (64.3 μg protein) emulsified with an equal volume of Freund's complete adjuvant. A final volume of 1 cc was loaded into a 3.0 cc self-injecting, plastic dart tipped with a 3.81 cm barbless needle. Three of the 26 horses treated developed abscesses at the injection site and difficulty was encountered in relocating mares initially injected. The result was that 18 mares received three injections while 8 received only two. Vaccination had no deleterious effect on current pregnancies since 57.6% (14/26) of the mares delivered live foals approximately 1 to 3 months after the final injection. The foaling rate for the year following vaccination decreased significantly for treated mares (3.8%) when compared to the previous two pretreatment years (53.8%), as well as placebo treated (50.0%) and untreated sexually-mature mares in the same area during the posttreatment year (45.4%). A booster injection given to 14 mares one year after the initial vaccinations again significantly decreased foaling rates to 7% (1/14) for boosted mares as compared to 50% (3/6) for placebo treated and 44% (7/16) for untreated mares.[5]

During the third year of study Kirkpatrick et al.[6] administered a second annual booster to 10 mares. In this study behavioral estrus and ovarian function were monitored by observation and urinary hormone analysis, respectively, for 7/10 treated and 4/20 control mares. Results indicated that none of the ten mares boosted for a third consecutive year became pregnant as opposed to 55% (11/20) of control mares. They also could find no evidence of ovulation during the ovulatory season and urinary estrogen concentrations in 4 of 7 treated mares indicated acyclicity. The authors suspected possible altered ovarian function as the cause. This would be consistent with data from similar vaccines used in other species.[7,8,9]

Thus far, studies using immunocontraceptive vaccines in the horse have involved either animal capture or remote delivery via dart.[3,4,5,6] Animal capture was not an applicable method for delivery of a vaccine to large numbers of wild animals due to the high cost, labor, and risk of injury to both horses and handlers. Although the dart offered the advantage of remote delivery, it has been shown to be difficult to deliver. In addition, dart recovery was less than optimal,[10] which led to the potential for environmental contamination.

The current investigation was undertaken to test a new remote delivery system for mare immunocontraception that would not lead to environmental contamination, and that would decrease the incidence of abscess formation at the injection site. In addition, this investigation sought to more precisely characterize the antibody response produced following vaccination.

MATERIAL AND METHODS

Immunogen: The immunogen used was heat-solubilized whole porcine zona pellucida (pZP) provided by Dr. Lowell Miller.[a]

Immunization: The seven sexually-mature mares used in the investigation were determined to be reproductively sound based on transrectal palpation, ultrasound, uterine culture, and uterine biopsy. Three mares each received two initial vaccinations containing 200 μg of pZP each. Thus, the total amount of protein given for the initial vaccination was 400 μg pZP per mare. These mares were boosted 4.5 weeks later with one injection also containing 200 μg pZP.

[a]Denver Wildlife Research Center, Denver, CO.

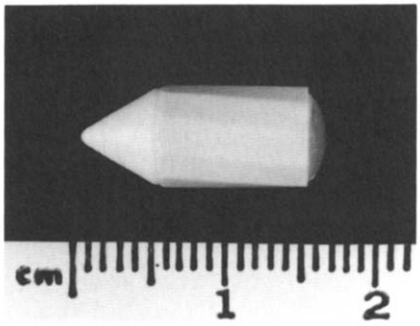

Figure 1. Biobullet in which the immunogen and adjuvant were packaged for remote delivery of the vaccine.

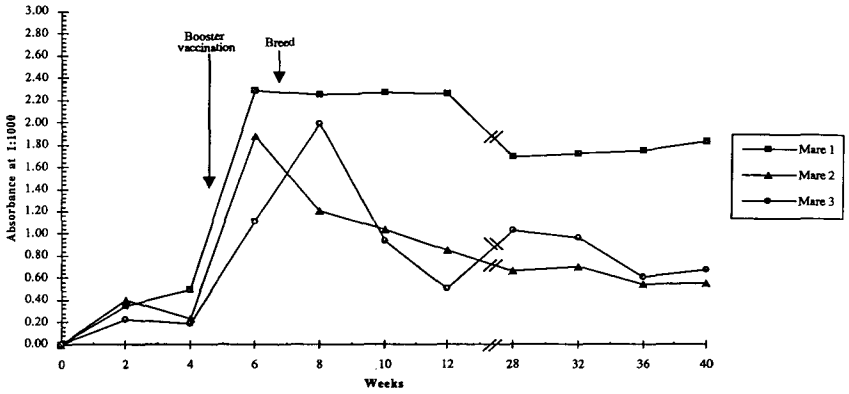

Figure 2. Absorbance levels at 1:1,000 dilution of serum of mares vaccinated with pZP as determined by ELISA. The time points for the booster vaccination of all mares and onset of the breeding trials are designated at weeks 4.5 and 6.5, respectively.

One of the mares was given a second booster injection containing 100 μg pZP 15 weeks after the initial injection due to the establishment of a pregnancy following the first two injections.

The remaining four mares were control animals that were given one initial placebo injection followed by a similar booster placebo injection 2.5 weeks later. These vaccinations contained no protein, but were otherwise identical to the injections given to the pZP group. Due to the constraints of a limited breeding season and unforeseen delays in production of the immunizing agent, control mares could not be boosted at the same interval as the experimental mares.

The immunization adjuvant[b] contained synthetic trehalose dicorynomycolate (TDM) glycolipid (25 mg/ml) and squalene oil. Each dose contained 20 μl of adjuvant (500 μg TDM) and was packed into a biobullet[c] for injection (Figure 1). The biobullet was composed of active and inactive components. The inactive components included calcium carbonate and hydroxypropylcellulose. The biobullets weighed approximately 575 mg and were 0.653 cm in diameter and 1.5 cm long. They were administered with a 0.25 caliber air gun from a distance of 20 feet. Mares were vaccinated in the semimembranosus and semitendinosus muscles which was confirmed by a trickle of blood at the injection site. Mares were then checked repeatedly for abscess formation and lameness.

Contraceptive Evaluation: Each mare (control and experimental) was bred by the same sexually-mature stallion of proven fertility. Two different stallions were used for two breeding seasons (1993 and 1994). All mares were bred on the first estrus occurring at least 2 weeks following the booster injection. Control mares were bred during one estrous cycle while treated mares were bred during two consecutive estrous cycles, to confirm contraceptive effects. In the second year, the treatment mares were bred through four consecutive estrous cycles while the control mares were bred through 1 estrous cycle. In all cases estrus was detected using a teaser stallion and rectal palpation. Follicular development and ovulation were followed, on an every-other-day basis, by transrectal palpation and ultrasonography. Mares were bred based on follicular size and texture. Pregnancy rates were determined by transrectal ultrasonography at 15 and 23 days post-ovulation. The presence of a viable embryo was confirmed at 23 days by a heartbeat which can be detected from 20 to 21 days of pregnancy in the horse.[11] Any experimental group pregnancies were terminated by i.m. injection of prostaglandin F2a.

Serum Collection: Blood was drawn from mares prior to immunization, then at weekly intervals until completion of the first year's breeding trials and monthly thereafter. For serum collection, blood was allowed to clot at room temperature for 12 hours. Following centrifugation for 10 minutes at 2000 x g, serum was decanted and stored at -20°C until further evaluation.

Antibody Titration: Serum antibody levels of mares were measured by enzyme linked immunosorbent assay (ELISA) as described by Harlow and Lane.[12] Plates[d] were coated with 5 μg/ml pZP in coating buffer (100 mM boric acid, 25 mM $Na_2B_4O_7$-10H²O, and 75 mM NaCl; pH 8.5; 50 μl per well) and incubated at 4°C overnight.

Plates were blocked for 30 minutes with 10 mM trizma base containing 1% bovine serum albumin. Fifty μl of the primary antibody (serum) dilution were added to wells in duplicate and incubated for 2.5 hours. Initially, the primary antibody was serially diluted (1:50; 1:100; 1:500; 1:1,000; 1:5,000; 1:10,000; 1:50,000; 1:100,000; 1:500,000) for quantitation of specific polyclonal antibody levels. Absolute antibody levels were reported as absorbance at a 1:1,000 dilution of the serum for this study.

[b]provided by Dr. Terry Ulrich, Ribi ImmunoChem Research, Inc., Hamilton, MT.
[c]Ballistivet, Inc., White Bean Lake, MN.

[d]Immulon 4; Dynatech Laboratories; Chantilly, VA.
[e]BSA; fraction V; Sigma Chemical Co.; St. Louis, MO.

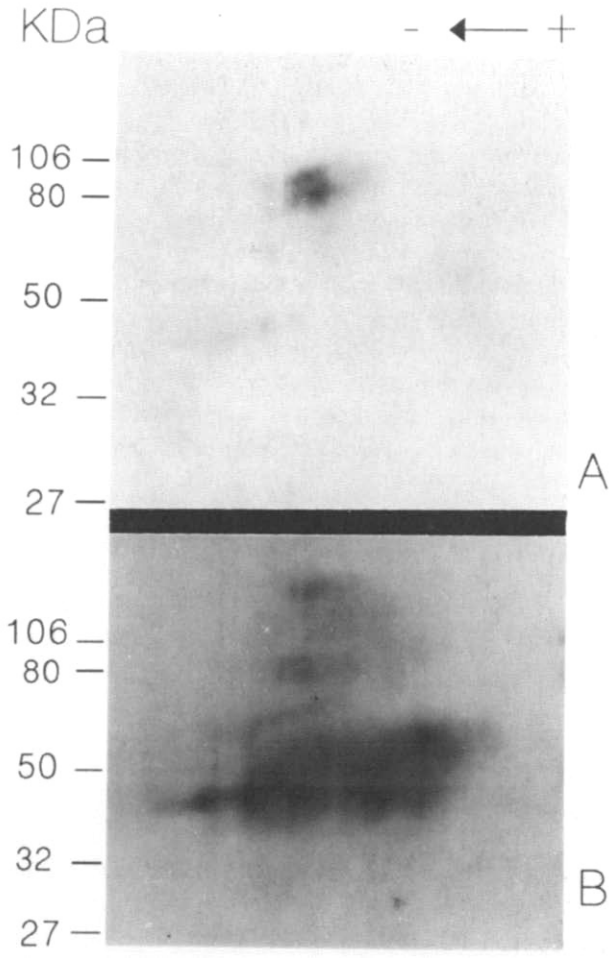

Figure **3.** Immunoblots of mare serum against the immunogen (pZP): A) Immunoblot using serum from Mare 1 as the primary antibody, B) Positive control using rabbit anti-heat solubilized whole pZP as the primary antibody.

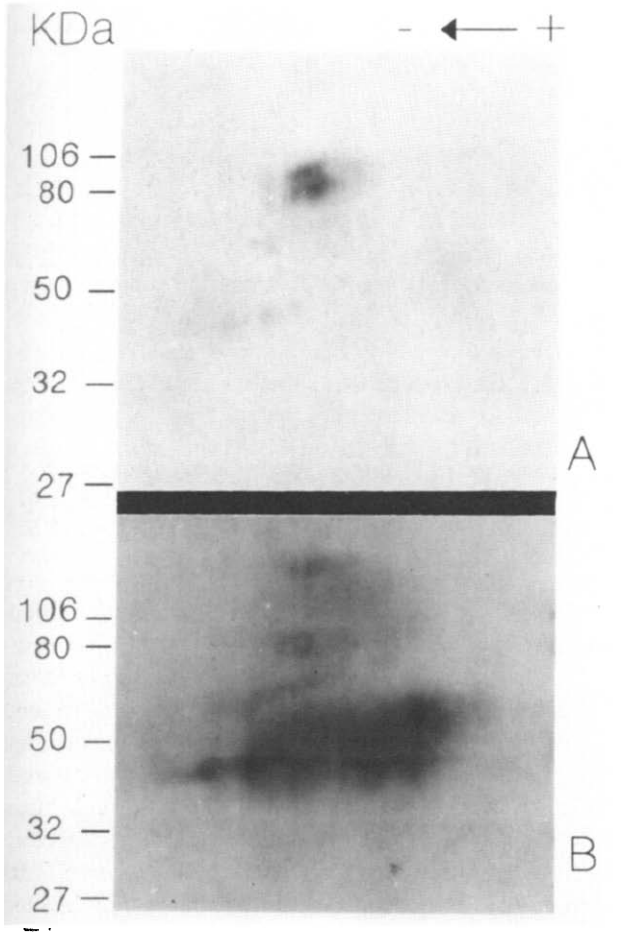

Figure **4.** Specific cross-reactivity of mare serum against eZP: A) Immunoblot using serum from Mare 1 as the primary antibody, B) Positive control immunoblot using rabbit anti-heat solubilized whole pZP as the primary antibody.

The secondary antibody used was alkaline phosphatase labeled, rabbit anti-horse IgG.[f] This antibody was diluted 1:10,000 and incubated in the wells for 2.5 hours. P-nitrophenyl phosphate[g] was the substrate used for color development in glycine buffer (0.1 M glycine, 1 mM $MgCl_2$, and mM $ZnCl_2$; pH 9.8) as recommended by Sigma Chemical Company. Following a 30-minute incubation period, absorbance was read at 405 nm.

As a positive control for the assay, three wells on each plate were coated with a 1:100 dilution of mare serum and treated in the same manner as all other wells.

Immunoblots: Immunoblot techniques incorporated two-dimensional polyacrylamide gel electrophoresis (PAGE) of pZP and eZP.[13] Equine oocytes were isolated from horse ovaries as described by Miller et al.[14] One hundred oocytes were transferred to 25 µl SDS solubilization buffer (2% SDS, 10% glycerol, 0.2% mercaptoethanol and 50 mM cyclohexylamino ethane sulfonic acid; pH 9.5) and incubated at room temperature for 2 hours with intermittent agitation. They were then held in boiling water for 10 minutes, the slurry was centrifuged at maximum speed in a micro centrifuge for 15 minutes, and the supernatant applied to isoelectric focusing (IEF) gels (10 µl/gel) after adding 5 µl of 0.1% bromophenol blue. Heat-solubilized whole pZP (1 mg/ml) was diluted 1:1 with the SDS solubilization buffer, 5 µl of 0.1% bromophenol blue added and applied to IEF gels (5 µl/gel).

The first dimension IEF was performed in a BioRad[h] mini IEF unit. The gels were formed according to O'Farrell.[15] The electrode solutions were 0.2% phosphoric acid and 0.1 M sodium hydroxide. IEF was performed at 750 volts until the dye reached the acid end of the gel and the gels then were equilibrated with SDS-PAGE sample buffer for 5 min before running in the second dimension.

For the second dimension SDS-PAGE, the equilibrated IEF gel was applied directly to a 12% polyacrylamide gel containing 0.2% bisacrylamide. For determining the molecular mass of protein spots, prestained low MW protein markers[h] were run alongside the IEF gel. The gel

[f]Sigma Chemicals; St. Louis, MO; #A-6063.
[g]Sigma Chemicals; St. Louis, MO; #N-9389.
[h]BioRad, Mellville, N.Y.

composition and gel running conditions were according to Harlow and Lane[12] in a BioRad mini gel apparatus.

For blotting, proteins were electrophoretically transferred to nitrocellulose sheets[g] using a BioRad trans-blot SD semi-dry transfer cell. Immunostaining was performed in glass dishes with gentle shaking at room temperature. The protein blots were left overnight in the blocking solution, which was 3% BSA dissolved in TBST (tris buffered saline containing 0.05% Tween-20). The blots were then transferred to suitably diluted primary antiserum or preimmune antiserum and incubated for 2 hours. The dilution of primary antibody used was calculated based on ELISA results and the protein being blotted. Serum from Week 6 post-immunization was used for all mares. For pZP, dilutions were 1:8,000 for Mare 1, 1:2,000 for Mare 2, and 1:1,000 for Mare 3. For eZP, dilutions were 1:2,000 for Mare 1, 1:400 for Mare 2, and 1:200 for Mare 3. Following three washes in TBST, blots were transferred to alkaline phosphatase conjugated secondary antibody (affinity purified, rabbit anti-horse IgG;[i] diluted in TBST to 1:10,000) and incubated for 2 hours. Finally, they were washed in TBST and water, and stained for alkaline phosphatase using bromo-chloro-indolyl phosphate (BCIP) and nitrobluetetrazolium (NBT) according to Harlow and Lane.[12] The blots and gels were photographed on 4x5 Polaroid 55 film.

The positive control used for immunoblots incorporated a rabbit anti-heat solubilized whole pZP[j] as the primary antibody, which was known to recognize all eZP and pZP proteins.[7,13] The secondary antibody was alkaline phosphatase labeled, affinity purified, goat anti-rabbit IgG.[h]

RESULTS

Of the seventeen bullets delivered remotely to the seven mares, none resulted in abscess formation. The bullets penetrated the skin entirely and vaccination was followed immediately by a trickle of blood at the injection site that allowed for easy remote confirmation of vaccination. The mares showed no signs of lameness either immediately following vaccination or at any time post-vaccination.

Serum titration demonstrated that high levels (≥1.80) were achieved in the three mares following the initial two vaccinations (Figure 2). Mare 1 and Mare 2 had maximum levels of 2.38 and 1.85, respectively, while Mare 3 had an initial maximum level of 1.96. Injection of Mare 3 with a third vaccination resulted in an absorbance of 1.00 at 26 weeks. In all cases maximum titers were reached by 2 to 3 weeks following the booster injection. To date serum antibody levels have persisted for 40 weeks, although the immunocontraceptive effect is still persisting 14 weeks after the last ELISA data point in Figure 2.

Breeding trials began 2 weeks following the booster injection. Pregnancy data did not necessarily reflect antibody levels since Mares 1 and 2 did not become pregnant, while Mare 3 did. However, following a third injection, Mare 3 also did not become pregnant.

In the first year, 1 of 7 breeding attempts (14.3%) in experimental mares resulted in a pregnancy while 3 of 4 attempts (75%) in control mares resulted in pregnancy. Thus, the contraceptive effect for all breedings of experimental mares was decreased as compared to control mares. In the second year, the mares were again bred after resumption of normal cyclicity. For the control mares, 1 breeding for each mare resulted in a pregnancy (4/4, 100%). In the vaccinated mares, 0 of 12 breeding attempts resulted in a pregnancy (0%).

Specific characterization of the polyclonal antibodies produced by the vaccination was performed by immunoblots against pZP (the immunogen) and eZP (for cross-reactivity). Immunoblots of mare serum against pZP indicated that the antibodies of all three treated mares recognized bands at approximately 90 kDa and 55 kDa (Figure 3A). This pattern of recognition was identical to the positive control (rabbit anti-heat solubilized pZP; Figure 3B). When tested against eZP, serum from Mare 1 reacted primarily with a band at 75-90 kDa, but also with a band at 40-50 kDa (Figure 4A). In contrast, the sera of Mares 2 and 3 reacted primarily with a band at 90-106 kDa, but there was also a band at 45-55 kDa (data not shown). Treatment of control eZP blots with rabbit anti-heat solubilized pZP confirmed that all three eZP proteins (90->106 kDa, 75-90 kDa nd 45-80 kDa) were present on the nitrocellulose (Figure 4B).

DISCUSSION

There are several characteristics which are important when considering a contraceptive vaccine for animals.[16] First of all, it must be easy to deliver, effective, and safe for the administrator and the animals receiving it. In addition, the agent should be inexpensive due to the large number of animals to be treated. Finally, the agent should be remotely administrable, i.e. without capture or chemical immobilization of the animal in order to maximize treatment efficiency while minimizing danger and harassment of the animal. Biobullet immunization against reproductive proteins would satisfy these requirements since (1) it would require only periodic treatments, (2) it would be inexpensive, (3) it could be delivered remotely, (4) it would not cause infertility in nontarget species, and (5) it would be safe to the target and secondary animals (i.e., no detectable cross-reactivity to normal somatic tissues and no detectable pathology).

Immunocontraception has been achieved in several species using pZP as an antigen. A few representative

[h]BioRad, Mellville, N.Y.
[i]Sigma Chem. Co.; St. Louis, MO.
[j]donated by Dr. B.S. Dunbar, Baylor College of Medicine, Houston, TX.

reports include white-tailed deer,[17] dogs,[8] and squirrel monkeys.[18] Although previous studies with pZP have demonstrated that it can be used to achieve successful immunocontraception in the horse,[3,4,5,6] several aspects of the vaccination needed further investigation and improvement in order to meet the needs of an applicable wildlife vaccine.

This report demonstrated that a new method of remote delivery resulted in high and prolonged (2 breeding seasons) levels, as well as immunocontraception, in the limited number of horses used in this study. A relatively low rate of conception (1 of 19 attempts) was achieved. These data support further study using larger numbers of animals (both domestic and feral) to determine whether this contraceptive effect is significant, as has been previously reported for pZP proteins administered by other routes of delivery.[3,4,5,6]

In this study, vaccines were emulsified with the TDM adjuvant and packaged in a biobullet for remote delivery. This was in contrast to previous reports using Freund's adjuvant and a dart when administered remotely.[3,4,5,6] Advantages of the new method included the finding that the biobullet/TDM adjuvant vaccination used in this study did not result in abscess formation as did Freund's adjuvant.[3,4] Secondly, confirmation of vaccination with the biobullet was easily performed from a remote location since injection was immediately followed by a trickle of blood at the injection site. Although in this study vaccination was performed from only 20 feet, the biobullet can be administered from 100 feet. Thirdly, the prolonged levels produced by the vaccine allowed immunocontraception for 2 complete breeding seasons and removed the necessity to boost the animals in the second season. Finally, because both the immunogen and bullet were biodegradable, no hazard to the environment would be imposed by bullets that miss their targets and remain unrecovered. Further, rabbits fed the adjuvant and antigen at the same dose as the biobullet did not develop detectable antibodies in their serum (ELISA and Western blot analysis) and there were no significant effects of this serum on in vitro fertilization of rabbit oocytes.[k] These advantages offered significant improvements over the previous administration techniques used in the horse.

To date, only one description of the immunological response produced in mares following vaccination with pZP exists.[3] Our investigation further characterized the response using serum titration and immunoblots. Liu et al.[3] gave at least four vaccinations (2,000 to 5,000 pZP) to nine feral mares and six domestic horses (four fertile mares, one infertile mare, one gelding). All nine feral mares had equivalent titers as determined by ELISA absorbance levels, but by 10.5 months seven mares (78%) still had relatively high titers while two (22%) had low titers. By 10 months post-vaccination, the titers of three of the domestic animals had decreased to one half the maximal level. The other three animals had titers at least 75% of their maximal levels. In addition, the authors stated that they found a high degree of individual variation in response to vaccination. This was in agreement with the results of our investigation using serum titrations to determine antibody levels.

Liu et al.[3] also found pregnancy data to be reflective of antibody levels. Of the feral mares, one of eight was pregnant 10 months post-vaccination, and this mare had an undetectable antibody level. Of the four domestic fertile mares, two did not conceive while the other two conceived only following a decrease in their antibody level. The results of our study agreed with those of Liu et al.[3] in that one of the three experimental mares became pregnant. However, antibody levels and Western blot analysis for Mare 3 were similar to that for Mare 2, which did not become pregnant. For this reason additional parameters should be incorporated in determining whether a single serum sample indicates contraception. The use of "titers" to describe a vaccine's cause and effect for an immunocontraceptive agent has been replaced by absorbance levels at 1:1,000 serum dilution for this study. When compared to disease process "titers," the above data demonstrate that "titers" alone from a single serum sample would not be predictive of immunocontraception. This is true for variations of animal-to-animal as well as one vaccine to another. The only real determinant of immunocontraceptive effect is the lack of production of offspring. Not only do the above-vaccine recipients have differences in the levels of their antibodies, but they also have variable responses to the specific proteins. Furthermore, if you consider possible complicating factors such as 1) individual antibody levels at their site of action, e.g., anti-pZP levels in the oviduct, 2) individual variations in type of immune (IgG or IgA) response, 3) immune competence of the recipient, or 4) effect of nutrition, stress, and debilitation on antibody levels, then the need for a standardized assay is more apparent.

Cross-reactivity of antibodies raised against pZP with eZP was first reported by Liu and Shivers.[19] That study found that antibodies raised in rabbits against pZP would bind the ZP of intact equine oocytes. Subsequently, Miller et al.[13] found that a similar antibody (rabbit anti-heat solubilized whole pZP), which was known to recognize all pZP families (93 kDa, 55a kDa and 55ß kDa; Skinner et al.[7]), also recognized all three eZP families (93-120 kDa, 73-90 kDa and 45-80 kDa). In addition, an antibody raised against the 55-120 kDa pZP protein recognized only the 45-80 kDa eZP protein. Together these data strongly indicated that pZP and eZP have common epitopes. This supported the findings of previous studies of contraception in horses following vaccination with pZP[3,4,5,6] and the current investigation. It remained to be determined, however, exactly which ZP protein family was being recognized by the serum of vaccinated horses. The current

[k]Fayrer-Hosken, 1994 personal communication.

investigation provided the first report of this information.

Our results indicated that serum from mares vaccinated with heat-solubilized whole pZP recognized both pZP protein bands. However, the same serum primarily recognized either the 93-120 kDa or 73-90 kDa molecular weight eZP family in addition to a slight recognition of 40-80 kDa family. The lowest molecular weight family in the pig,[20,21] hamster,[22] and mouse[23] is believed to be the oocyte receptor for the sperm. In the horse, antibodies specific for the lowest molecular weight pZP protein also recognized the lowest molecular weight eZP,[13] which may indicate a similar role.

In other species the eZP vaccine has been shown to cause contraception by interrupting folliculogenesis.[7,8] In this study, however, contraception was known not to be due to inhibition of ovulation since there was normal follicular development and ovulation of the mares as determined by rectal palpation and ultrasonography. These data indicate that further characterization of the equine pZP vaccine using gamete interaction studies and ovarian immunohistochemistry are needed in order to fully understand its mode of action and possible long-term side effects.

REFERENCES

1. Boyles JS: Managing America's wild horses and burros. *Eq Vet Sci* 6:261-265, 1986.
2. Kirkpatrick JF and Turner JW Jr: Comparative reproductive biology of North American feral horses. *Eq Vet Sci* 6:224-230, 1986.
3. Liu IKM, Bernoco M and Feldman M: Contraception in mares heteroimmunized with pig zonae pellucidae. *J Reprod Fert* 85:19-29, 1989.
4. Kirkpatrick JF, Liu IKM and Turner JW Jr: Remotely-delivered immunocontraception in feral horses. *Wildl Soc Bull* 18:326-330, 1990.
5. Kirkpatrick JF, Liu IKM, Turner JW and Bernoco M: Antigen recognition in feral mares previously immunized with porcine zonae pellucidae. *J Reprod Fert* (Suppl) 44:321-325, 1991 .
6. Kirkpatrick JF, Liu IKM, Turner JW Jr, Naugel R and Keiper R: Long-term effects of porcine zonae pellucidae immunocontraception on ovarian function in feral horses (Equus caballus). *J Reprod Fert* 94:437-444, 1992.
7. Skinner SM, Mills T, Kirchick HJ and Dunbar BS: Immunization with zona pellucida proteins results in abnormal ovarian follicular differentiation and inhibition of gonadotropin-induced steroid secretion. *Endocrinology* 115:2418-2432, 1984.
8. Mahi-Brown CA, Yanagimachi R, Hoffmann JC and Huang TTG: Fertility control in the bitch by active immunization with porcine zona pellucida: Use of different adjuvants and patterns of estradiol and progesterone levels in estrous cycles. *Biol Reprod* 32:761-772, 1985.
9. Dunbar BS, Lo C, Powell J and Stevens VC: Use of a synthetic peptide adjuvant for the immunization of baboons with denatured and deglycosylated pig zona pellucida glycoproteins. *Fert Steril* 52:311-318, 1989.
10. Turner WJ Jr and Kirkpatrick JF: New developments in feral horse contraception and their potential application to wildlife. Wildl Soc Bull 19:350-359, 1991.
11. Ginther OJ: *Reproductive biology of the mare.* Cross Plains, Wisconsin: Equiservices. 1992.
12. Harlow E and Lane D: *Immunoassays. In: Antibodies, a laboratory manual.* New York: Cold Spring Harbor Laboratory. p. 553-612, 1988.
13. Miller CC, Fayrer-Hosken RA, Timmons TM, Lee VH, Caudle AB and Dunbar BS: Characterization of equine zona pellucida glycoproteins by polyacrylamide gel electrophoresis and immunological techniques. *J Reprod Fert* 96:815-825, 1991.
14. Miller CC, Dunbar BS, and Fayrer-Hosken RA; Large scale isolation of equine zonae pellucidae. *Theriogenology* 41(4):785-794,1994.
15. O'Farrell PH: High resolution two dimensional electrophoresis of proteins. *J Biol Chem* 250:4007-4021, 1975.
16. Turner JW Jr and Kirkpatrick JF: Fertility control as a management tool for feral horse populations. *Eq Vet Sci* 6:278-284, 1986.
17. Turner JW Jr, Liu IKM, and Kirkpatrick JF: Remotely delivered immunocontraception in captive white-tailed deer. *J Wildl Manage* 56:154-157, 1992.
18. Sacco AG, Subramanian MG, Yurewicz EC, DeMayo FJ and Dukelow WR: Hetero-immunization of squirrel monkeys (Saimiri sciureus) with a purified porcine zona antigen (PPZA): immune response and biological activity of antiserum. *Fert Steril* 39:350-358, 1983.
19. Liu IKM and Shivers CA: Antibodies to the zona pellucida in mares. *J Reprod Fert* (Suppl) 32:309-313, 1982.
20. Sacco AG, Subramanian MG and Yurewicz EC: Association of sperm activity with a purified pig zona antigen (PPZA). J *Reprod Immunol* 6:89-103, 1984.
21. Berger T, Davis A, Wardrip NJ and Hedrick JL: Sperm binding to the pig zona pellucida and inhibition of binding by solubilized components of the zona pellucida. *J Reprod Fert* 86:559-565, 1989.
22. Moller CC, Bleil JD, Kinloch RA and Wassarman PM: Structural and functional relationship between mouse and hamster zona pellucida glycoproteins. *Dev Biol* 137:276-286, 1990.
23. Bleil JD and Wassarman PM: Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm. *J Cell Biol* 102:1363-1371, 1986.