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Effects of Two Porcine Zona Pellucida Immunocontraceptive Vaccines on Ovarian Activity in Horses

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ABSTRACT Feral horse population growth rates as high as 25% are of concern to those responsible for managing range lands as well as conservation groups. Current methods to control these populations include adoption and long-term holding, which are both costly and controversial. Porcine zona pellucida (pZP) immunocontraception may have the greatest potential to control fertility because it has proven to be effective in other studies and vaccines are easy and safe to administer. One pZP vaccine formulation, SpayVac® (ImmunoVaccine Technologies, Inc., Halifax, NS, Canada), has demonstrated single-dose, multi-year contraceptive efficacy in other wildlife species, which would make it both practical and economical for field application. Over a 7-month period during the breeding season, we assessed the effect on ovarian activity of 2 formulations of SpayVac, 1 non-aqueous with modified Freund's adjuvant (MFA) and the other, an aqueous emulsion with MFA, compared to controls ($n = 7$ per group). Comparative reproductive parameters included serum concentrations of progesterone (P_4) determined by enzyme-linked immunosorbent assay (ELISA), ovarian activity assessed by transrectal ultrasound and palpation, as well as gross and histological examination of ovaries upon necropsy ($n = 9$ or 3 mares from each group) or after ovariectomy ($n = 12$ or 4 mares from each group). We determined serum antibody titers using ELISA. Mean serum concentrations of P_4 were less in the non-aqueous MFA treatment group compared to control mares ($P < 0.025$). Ovaries collected from control mares weighed more ($P = 0.002$) and had greater variation ($P = 0.003$) than those from either vaccinated group. Both treatment groups also had smaller ovaries and fewer follicles compared to controls ($P < 0.001$). Three to 4 months after vaccination, 93% of SpayVac-injected mares ceased cycling; whereas all control mares continued to cycle throughout the study. Relatively constant antibody titers were reached by week 6 post-vaccination, although we found appreciable variation within treatment groups, especially 4–8 weeks post-vaccination. Based on our study, the SpayVac formulations impair ovarian function but do not affect other major organ systems, and could provide a safe and effective immunocontraceptive option for mares. Additional research to elucidate the vaccine's mechanism of action, actual contraceptive efficacy, and long-term effects are still needed. © 2013 The Wildlife Society.

KEY WORDS *Equus caballus*, feral horses, immunocontraception, mares, Oregon, porcine zona pellucida, SpayVac®.

Feral horse (*Equus caballus*) populations that inhabit federal lands in the western United States are protected by the Wild Free-Roaming Horses and Burros Act of 1971. This act requires that the bureau of land management (BLM) manage the populations "… to achieve and maintain a thriving natural ecological balance …" Because feral horses generally lack effective predators, populations can increase by 20–25% annually (Jenkins and Ashley 2003) and rapidly exceed established appropriate management levels (Vincent 2010). Therefore, achieving the intent of this act is problematic. Currently, the only publicly acceptable means of controlling feral horse populations is live removal. Younger horses may be adopted by members of the public, but about half of the 84,741 horses removed between 2000 and 2009 ended up in long-term holding (Vincent 2010). The Government Accountability Office reported that off-range holding costs consume most of the BLM's Wild Horses and Burros budget

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(Government Accountability Office 2008). Expenditures related to long-term holding increased from $7 million in 2000 to $21 million in 2007, and that figure is expected to continue to increase rapidly.

Contraception has long been recognized as a potentially useful approach for controlling the fertility of feral horse populations, thus reducing or possibly even eliminating the need to remove horses from their natal ranges (Kirkpatrick and Rutberg 2001). Several techniques have been tried in horses, including hormone implants (Plotka et al. 1988), intrauterine devices (Killian et al. 2008), and immunocontraception using either gonadotropin-releasing hormone (GnRH; Killian et al. 2008) or porcine zona pellucida (pZP; Kirkpatrick 1995, Kirkpatrick et al. 1996) proteins as antigens. At present, immunocontraception based on pZP glycoproteins appears to be the most practical and effective (Barber and Fayrer-Hosken 2000*a*, Fraker and Brown 2011), with few side effects (Ransom et al. 2010). ZP-specific vaccines likely have fewer side effects compared to GnRH vaccines (like GonaCon^{TM}; National Wildlife Research Center, Fort Collins, CO), because GnRH receptors are located in a variety of tissues in addition to reproductive organs, including the nervous system (Lopez et al. 2007), bladder (Bahk et al. 2008), and heart (Skinner et al. 2009). The potential effect of pZP-based immunocontraception on social structure and behavior in herd animals has been explored by others who reported either no differences (Kirkpatrick et al. 1995, Powell 2000) or minimal differences (Ransom et al. 2010) between vaccinated and control mares with respect to activity budgets, hierarchy within the herd, or interactions with stallions, unlike GnRH vaccines, which suppressed behavioral and physiological estrus (Elhay et al. 2007, Botha et al. 2008). With pZP vaccines, antibody occupation of ZP receptors is thought to disrupt sperm binding (Dunbar and Schwoebel 1988, Skinner et al. 1990, Benoff 1997), which normally initiates the acrosome reaction and subsequent fertilization (Bedford 2008). Previous studies with pZP vaccines have demonstrated their general safety (Kirkpatrick et al. 1992, Kirkpatrick and Turner 2007), specifically in pregnant mares (Kirkpatrick and Turner 2002, 2003), and injection-site reactions have been reported as the primary side effect of concern (Roelle and Ransom 2009). Currently 6 different free-ranging wildlife species are managed by pZP immunocontraception (Kirkpatrick et al. 2011).

A simulation study by Gross (2000) of the Pryor Mountain herd found that contraceptive vaccines reduced variance in population size and did a better job of stabilizing feral horse populations at target sizes than did removal (average coefficient of variation was 8% compared to 17%, respectively). Model projections for immunocontracepted horses on Assateague Island predicted an average rate of population decline of 13% per year, which suggested that the management target of 80–100 horses would be reached in 5–8 years (Ballou et al. 2008). Slow rates of population decline have been attributed to reduced mortality and significantly increased longevity among treated mares (Kirkpatrick and Turner 2008).

Zona pellucida glycoproteins are weak antigens and consequently, most pZP vaccines require both potent adjuvants and booster doses to maintain serum antibody titers at contraceptive levels (Ransom et al. 2011). Boosters can be administered by traditional means (dart or hand injection) or through the use of slow-release pellets, which can be implanted when the primary dose is delivered. An immunocontraceptive vaccine that can sustain antibody titers over multiple years with 1 treatment is advantageous because horses would require fewer treatments, thereby minimizing cost as well as stress and risk of injury to horses and workers. Efforts to produce a sustained release of antigen incorporated pZP in either microcapsules or pellets (Liu et al. 2005); however, more effective oil-based adjuvants, such as modified Freund's adjuvant (MFA), cannot be incorporated into these matrices (Kirkpatrick et al. 2011). Mares treated with a 1-ml injection of 65-μg pZP in a Freund's complete adjuvant (FCA) emulsion and the simultaneous injection of booster pellets designed to release pZP antigens at 1 month, 3 months, and 12 months had annual reproductive rates for 2001–2004 of 5.9%, 14.0%, 32.0%, and 47.5% compared to an average of 53.8 ± 1.3% for untreated mares (Turner et al. 2007). However, the effectiveness of the slow-release pellets has been inconsistent (Turner et al. 2008, Ransom et al. 2011). SpayVac® (ImmunoVaccine Technologies, Inc. [IVT], Halifax, NS, Canada) is a pZP vaccine that uses a unique liposome technology (DepoVax®) and has delivered single-dose, long-lasting immunocontraception in a variety of species including fallow deer (*Dama dama*; Fraker et al. 2002) and gray seals (*Halichoerus grypus*; Brown et al. 1997*a*). Annual reproductive success 1–4 years after treatment for mares given a single 1-ml injection of SpayVac using 400 μg of pZP and AdjuVac^{TM} (National Wildlife Research Center) as the adjuvant was 0%, 17%, 17%, and 17% compared to 75%, 75%, 88%, and 100% for untreated mares (Killian et al. 2008).

Although pZP vaccines have been tested in different species for almost 30 years, few studies have been conducted to determine potential effects on the reproductive system in mares. Adverse effects on ovarian function have been described in a number of species receiving pZP vaccinations, including hamsters (*Mesocricetus auratus*; Hasegawa et al. 1992), dogs (*Canis lupus familiaris*; Mahi-Brown et al. 1985), rabbits (*Oryctolagus cuniculus*; Sehgal et al. 1989), white-tailed deer (*Odocoileus virginianus*; Curtis et al. 2007), sheep (*Ovis aries*; Stoops et al. 2006), and primates (*Macaca radiate*; Upadhyay et al. 1989). Mares boostered annually with a pZP vaccine demonstrated a lack of or altered estrous cycles based on urinary non-specific progesterone metabolite and estrone conjugate concentrations (Kirkpatrick et al. 1992). Porcine ZP antibodies can be passed through the placenta and colostrum (Sacco et al. 1981). However, the ZP in the fetal horse is not yet developed (Deanesly 1975), and Kirkpatrick et al. (1996) demonstrated that offspring of mares that had been vaccinated while pregnant were fertile. Killian et al. (2008) reported a greater incidence of uterine edema, possibly associated with reduced serum concentrations of progesterone, in SpayVac-treated mares as

compared to controls. Before initiating a larger-scale study using SpayVac, the BLM requested that research be conducted with domestic mares using different SpayVac formulations to confirm safety and examine potential effects on ovarian activity.

We used 2 different SpayVac formulations in this study: 1) an aqueous pZP liposome MFA mixture and 2) a non-aqueous formulation comprised of lyophilized pZP liposome mixture, which is reconstituted with MFA prior to injection. The latter formulation was of particular interest because it has an extended shelf life and need not be stored frozen—important considerations for a vaccine that is to be used under field conditions. The objectives of this 7-month study were to 1) compare the ability of aqueous and non-aqueous SpayVac formulations to raise serum concentrations of pZP antibodies, 2) examine all major organ systems to ensure that the vaccines did not negatively affect health, and 3) assess ovarian function based on follicular development and progesterone secretion.

STUDY AREA

We maintained animals at a 22-ha ranch located approximately 80 km northeast of Corvallis near Stayton, Oregon. The elevation in this region was approximately 138 m, and temperatures peaked during summer months increasing from average highs of 15.8° C and lows of 4.4° C in April to highs of 27.1° C and lows of 10.8° C in July–August. Precipitation also declined during the summer from an average of 12.7 cm in April to less than 2.5 cm in July–August. We randomly assigned 21 mares to 3 groups of 7 maintained on fenced 2-ha pastures comprised of sandy loam with river rock. Each pasture contained a 4.9-m × 7.3-m 3-sided shelter. Another shelter enclosing 2 wooden palpation stocks was located in an adjacent, separate corral where we routinely examined mares during the study.

METHODS

Animals

We used 21 domestic mares 3–14 years of age in this 7-month study (Table 1), which began in early March and ended in late September–early October 2010 over the course of 1 breeding season. We fed horses daily with alfalfa (*Medicago sativa*) in the morning and grass hay in the evening, and a salt-mineral block as well as water was available ad libidum. Routine veterinary care as well as hoof-trimming and grooming was provided as needed by the Silver Creek Animal Clinic, Silverton Oregon, located near the study site. We conducted this research under a protocol approved by the Institutional Animal Care and Use Committee [IACUC] at Oregon State University (3924).

We conducted preliminary physical examinations on all of the mares and confirmed reproductive soundness by trans-rectal palpation and ultrasonography (Robinson and Sprayberry 2009). We collected 10-ml blood samples from the jugular vein using 18-gauge needles and a Vacutainer tube guide (VWR International, West Chester, PA); we used samples to confirm the absence of pre-vaccination serum pZP antibody titers, determine serum concentrations of progesterone, and perform complete blood counts (CBCs) and serum biochemical profiles for evaluation of health status. We collected samples for serology in Vacutainer tubes (VWR International), which were allowed to clot overnight at 4° C, centrifuged for 10 minutes at 1,300*g*, and stored at −20° C until time of analysis. We collected whole blood samples for CBCs in Vacutainer tubes containing

Table 1. Mare identification number, treatment group, breed, estimated age (year), and weight (kg) of mares in Oregon in 2010.

Mare ID	Treatment	Breed	Approximate age	Approximate or actual weight[a]
14	MFA-A[b]	Arab	14	359
20[c]		Thoroughbred	5	457
22		Paint	5	377
23[c]		Arab	6	377
24		Thoroughbred	7	455
25[c]		Quarter horse	8	416
26		Quarter horse	4	436
8	MFA-NA[d]	Thoroughbred	14	477
9[c]		Paint	8	591
10		Quarter horse	10	491
11		Quarter horse	6	423
12		Paint	7	432
13[c]		Arab	8	341
18[c]		Quarter horse	6	418
15[c]	Control MFA	Quarter horse	3	443
21		Quarter horse	5	425
16[c]		Arab	13	493
19		Paint	5	409
27[c]	Control saline	Thoroughbred	8	450
28		Dun	4	400
29		Palomino	5	432

[a] We obtained actual weights for necropsied mares.
[b] SpayVac® in an aqueous formulation with modified Freund's adjuvant (MFA).
[c] Necropsied at the end of the study (other mares were ovariectomized).
[d] SpayVac in a non-aqueous formulation with MFA.

ethylenediaminetetraacetic acid (EDTA) and analyzed them the same day.

We administered combination prophylactic health vaccines against eastern, western, and Venezuelan encephalomyelitis; influenza; tetanus; and rhinopneumonitis EHV-1 and EHV-4 (Pfizer Animal Health, Fort Dodge, IA) intramuscularly (IM) in the left side of the neck. We also administered oral paste dewormer (Parid EQ Paste; 1.87% ivermectin; Butler Schein Animal Health, Chicago, IL), and lice powder as needed (Farnam Companies, Inc., Phoenix, AZ) at least 2 weeks prior to injection with either a SpayVac vaccine or control solution.

We randomly assigned mares to 1 of 3 experimental groups ($n = 7$) and fitted each mare with a plastic identification collar. Mares in each group received 1 of the following single IM injections in the right side of the neck: 1) 0.5 ml of SpayVac in a non-aqueous formulation using MFA, 2) 1.0 ml of SpayVac in an aqueous emulsion using MFA, or 3) controls receiving 0.5 ml of normal (0.9%) saline ($n = 3$) or normal saline with MFA ($n = 4$). We examined injection sites frequently for evidence of increased heat and/or swelling.

We collected 10-ml jugular venous blood samples from each mare at 1-week intervals for 7 months to determine serum concentrations of progesterone (P4) as well as pZP antibody titers. We collected and processed these samples as described above for serology and kept them frozen at -20° C until time of analysis. At the end of the study, we re-assessed health status by determining biochemical profiles and CBCs, and we estimated weights for all mares using standard measurements obtained with a tape measure (Ellis and Hollands 1998).

We performed monthly transrectal palpation and ultrasonography to image reproductive organs, record ovarian changes during the estrous cycle, and note patterns of uterine edema (Samper 1997). We used a Mindray DP-3300 Ultrasound System with a 4–6-MHz rectal linear transducer (Apexx Veterinary Equipment, Englewood, CO) to visualize the uterus and ovaries, and recorded follicle measurements (in mm) from both ovaries at the time of each examination. We classified a mare as being in anestrus (not reproductively cycling) when follicles were less than 20 mm in diameter without a corpus luteum (CL) present. For the safety of mares and handlers, we restrained the horses in stocks for these procedures. Initially, we routinely sedated mares with a 0.02- to 0.04-mg/kg intravenous (IV) injection of Dormosedan® (detomidine hydrochloride; Butler Schein Animal Health), but over the course of the study, the mares adapted to these procedures and we only administered Dormosedan on an as needed basis.

At the end of the study, a veterinarian ovariectomized 4 mares from each experimental group ($n = 12$) using a routine standing colpotomy procedure to obtain ovaries for subsequent gross and histopathological examinations. We withheld feed for 48 hours prior to surgery, and administered booster tetanus vaccinations (Equine Tetguard; Butler Schein Animal Health) IM prior to surgery. Handlers restrained mares in familiar palpation stocks that allowed for access to the horse's jugular vein for injections and to the perineal area to perform surgery. Surgical preparation included wrapping the tail with gauze and tying it up to keep it out of the surgical field, rectal palpation to manually evacuate the feces, scrubbing the perineal area with chlorhexadine (2% Chlorhexadine; Vet Solutions, Inc., Bedford, TX), and flushing the vagina 3 times with a dilute (0.04%) chlorhexadine solution. We administered IV injections of dormosedan (0.02–0.04 mg/kg), torbugesic® (0.01–0.04 mg/kg; 10 mg/ml butorphanol tartrate; Butler Schein Animal Health), and Anased® (0.9–1.2 mg/kg; 100 mg/ml xylazine hydrochloride; Butler Schein Animal Health) for sedation and analgesia during surgery. We monitored heart rate, respiratory rate, and response to the procedure regularly. The veterinarian performed a sterile surgery with an incision in the anterior–dorsal–lateral wall of the vagina (Embertson 2006). He injected 2% lidocaine (Butler Schein Animal Health) into the ovarian pedicle for local analgesia using an IV injection set with a Luer-Lok® adapter and needle (Beckton, Dickinson and Company, Franklin Lakes, NJ), and removed the ovaries using a chain ecrasure. After removal, we immediately dissected away the mesovarium and weighed each ovary. We removed a section of the ovulation fossa and fixed it in 10% neutral buffered formalin for subsequent histological analysis.

The entire procedure with each mare took 20–30 minutes, and the vaginal opening was left to heal by second intention. Following surgery, we administered 1.2–1.5 mg/kg Flunixiject® IV (50 mg/ml flunixin meglumine; Butler Schein Animal Health) and 10 mg Buprinorphine Sustained Release® subcutaneously (10 mg/ml; buprenorphine hydrochloride; ZooPharm, Inc., Fort Collins, CO) for post-surgical analgesia. Each mare also received an IM injection of Excede®, a long-duration antibiotic (6.6 mg/kg; ceftiofur crystalline-free acid; Butler Schein Animal Health), which was effective for at least 4 days. We visually observed all mares for 14 days for post-operative complications including pain, bleeding, or infection. We monitored heart rate, respiratory rate, and body temperatures daily for 1 week. At 10 days post-surgery, we performed a vaginal examination to confirm healing of the incision in the vaginal wall.

We euthanized the remaining mares ($n = 9$) for necropsy by sedating them with Anased (0.9–1.2 mg/kg; 100 mg/ml xylazine hydrochloride) and then using an overdose of Euthasol® (pentobarbital sodium; Butler Schein Animal Health) calculated at 1 ml/4.5 kg body weight and administered into the jugular vein.

Vaccine Preparation

We obtained frozen pig ovaries from Sioux-Preme Packing Company (Sioux Center, IA) and shipped them to IVT under storage at -20° C. The laboratory at IVT isolated pZP and prepared the vaccine as previously described by Brown et al. (1997*b*). They incorporated purified pZP in phosphate buffered saline (PBS; pH 7.4) at a final concentration of 200 μg per dose. They then added lipids containing lecithin and cholesterol at a ratio of 10:1 (0.2 g lecithin and 0.02 g cholesterol/dose; Lipoid, Newark, NJ) to the pZP solution to form multilamellar liposomes.

Technicians mixed half of the prepared pZP liposomes mixture with MFA (Calbiochem, La Jolla, CA) to form a water-in-oil (aqueous) emulsion (1:1, v/v; 1 ml/dose). They lyophilized the second half of the prepared pZP-liposomes mixture, and reconstituted the lyophilized (non-aqueous) liposomes with MFA for a final deliverable dose volume of 0.5 ml. They produced vehicle control vaccines as above without the addition of the pZP antigen (0.5 ml/dose).

To control quality, the laboratory used a bicinchoninic acid-containing protein assay and gel electrophoresis. Standard bioburden testing according to United States Pharmacopeia Convention (USP) methods further ensured purity and safety of vaccines. We obtained permits required for the transportation of pZP products across the USA–Canada border and received an Experimental Use Permit for SpayVac (OEUP 09-06) from the Oregon Department of Agriculture. IVT shipped all SpayVac vaccines (lots VM6-100330-1 [MFA aqueous] and WF16-100219-10 [MFA non-aqueous]) and control solutions (lots DPX6-100325-1 [MFA control]) in pre-loaded ready-to-inject syringes.

Laboratory Analyses

The Oregon State University (OSU) Veterinary Diagnostic Laboratory completed the CBC and serum chemistry analyses using automated analyzers.

Determination of pZP antibody titers by ELISA.—We dissolved lyophilized pZP (IVT) in Dulbecco's phosphate buffered saline (DPBS) to a concentration of 1.0 mg/ml; the mixture was gently shaken, heated for 30 minutes at 37° C and diluted to 1 μg/ml with coating buffer (1.59% $NaHCO_3$, 2.93% Na_2CO_3, and 0.2% NaN_3). We aliquoted 100 μl of pZP in coating buffer into each well of a flat-bottom Costar® 96-well EIA/RIA plate (Corning Incorporated, Corning, NY), which was then covered and incubated overnight at 4° C. We washed the Plate 5 times with Tris-buffered saline-Tween 20 (0.001 M Tris, 0.004 M Tween-20, and 0.15 M NaCl; TBS-T), and blocked wells with 100 μl of 5% skim milk in TBS-T for 1 hour at 37° C. Following 2 washes with TBS-T, we aliquoted 100 μl of serial serum dilutions ranging from 1:10 to 1:1,280 for control mares and 1:4,000 to 1:2,048,000 for MFA aqueous and non-aqueous injected mares, respectively, into the wells, and incubated the plate overnight at 4° C. We washed the plate 5 times with TBS-T, added 100 μl of alkaline phosphatase (AP)-conjugated Protein G (EMD Chemicals, Inc., Gibbstown, NJ) in AP buffer (0.01 M Tris, 0.1 M NaCl, and 0.01 M $MgCl_2$) to each well, and then incubated the plate for 1 hour at 37° C. We washed the plate 5 times with TBS-T and twice with AP buffer, and added 100 μl of 1 mg/ml p-nitrophenyl phosphate, disodium salt (EMD Millipore, Billerica, MA) in AP buffer to each well. We incubated the plate in the dark for 1 hour at 37° C and read it at an absorbance of 405 nm using a Benchmark microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA). We defined endpoint titers as the reciprocal of the greatest dilution above a cutoff value calculated from the measured absorbances. We calculated cutoff values using the 95% confidence interval as described by Frey et al. (1998). Using 1 or 2 pre-immune columns ($n = 8$ or 16) from the plate, we calculated the cutoff value using the following equation: $\text{cuttoff value} = \bar{X} + \text{SD}\left(t \times \sqrt{1 + (1/n)}\right)$.

Immunohistochemical (IHC) validation of anti-ZP antibody production.—To validate the IHC staining protocol, we obtained ovaries during routine necropsies of 3 mares with non-reproductive conditions at the OSU Veterinary Diagnostic Laboratory. We collected a tissue sample, no more than 1 cm thick, at the ovulation fossa and fixed it for 24 hours in 10% neutral buffered formalin. We trimmed fixed samples into cassettes for routine processing and paraffin embedding. We faced blocks for tissue exposure, sectioned them at 4 μm, and loaded them onto charged slides for hematoxylin and eosin (HE) staining. We scanned stained slides for mature follicles (i.e., ovum past primordial and primary stage) using light microscopy. We sometimes needed deeper cuts into the block to identify such follicles. Once we identified a mature follicle, we loaded the next few 4-μm sections from the block onto ProbeOn slides (Fisher Scientific, Pittsburgh, PA) for IHC staining.

We anchored tissue to slides by baking at 60° C for 1 hour, using a series of solvent solutions that changed from paraffin to xylene to alcohol and then to diH_2O, blocking for endogenous peroxidases with 3% H_2O_2 for 10 minutes, washing 6–8 times in TBS-T, and blocking with serum free protein block (Dakoblock, Dako, Inc., Carpentaria, CA) for 10–30 minutes. We applied filtered equine sera (primary antibody) diluted 1:100 and let slides sit overnight at 4° C. We then washed slides 6–8 times in TBS-T, and applied peroxidase-conjugated rabbit polyclonal antibody to horse immunoglobulin G (HRP®; Abcam, Cambridge, MA) as a secondary antibody (1:100 dilution) for 30 minutes at room temperature. After washing 6–8 times in TBS-T, we applied Novared (Vector Laboratories, Inc., Burlingame, CA) for 5 minutes. We again washed slides in TBS-T, and applied hematoxylin for 5 minutes as a counterstain. We performed sequential washes in TBS-T, distilled H_2O, and more TBS-T, completed another rundown series (diH_2O to alcohol to xylene), and then coverslipped slides with Canada balsam mounting media to complete IHC slide preparation.

We used serum collected from vaccinated or pre-vaccinated mares as the primary antibody in this protocol. We pooled equal volumes of sera from 3 mares in each of the 2 vaccination treatment groups to create representative samples per treatment; a pool of pre-vaccination sera served as the negative control sample. In this protocol, we used treatment samples that we collected in early August, whereas we obtained pre-vaccination samples from mares at the start of the study in March. In all instances, we filtered the pooled serum, divided it into 1 ml aliquots, and kept it frozen until we performed IHC assays.

We also attempted to detect bound endogenous anti-ZP antibody in ovaries collected from vaccinated mares using the same protocol, with the exception of primary antibody application. Ovaries from non-vaccinated mares in the study served as negative controls.

Progesterone assay.—We determined serum concentrations of P_4 using a progesterone ELISA kit (Alpha Diagnostic International, San Antonio, TX). Cross-reactivity of the antiserum was 100% to progesterone with the next highest cross-reactivities of 1.5% to 11-deoxycorticosterone, 0.7% to 17-α-hydroxyprogesterone, 0.15% to pregnenolone, and less than 0.1% for all others. Intra- and inter-assay coefficients of variation were 9.0% and 14.2%, respectively, and assay sensitivity was 0.2 ng/ml.

Statistical Analyses

We conducted principal components analyses of CBC and serum chemistry data collected at the beginning and end of the study to see if mares differed in their health condition among treatment groups in any systematic way. We analyzed data using the non-parametric Wilcoxon rank sum test (GraphPad Prism®, GraphPad Software, La Jolla, CA) to determine whether mares were in anestrus or not reproductively cycling (i.e., follicles less than 20 mm in diameter without a CL present). We performed all other analyses using the Number Cruncher Statistical System software program (2000; NCSS LLC, Kaysville, UT). We analyzed differences in pZP antibody titers and serum concentrations of P_4 among treatment groups by using repeated measures analysis of variance (ANOVA) where treatment, time and the treatment × time interaction were the main effects. Because of unequal variance, we analyzed pZP antibody titers using a log transformation. We used 1-way ANOVA to analyze for differences in ovarian weight among treatment groups. If we observed significant effects in the ANOVA, we determined differences between means using Fisher's least significant difference procedures. We defined significance as $P < 0.05$.

Necropsy and Histopathology

We weighed mares on a scale prior to euthanasia. The same pathologist performed all necropsies and tissue collections within 2 hours of euthanasia. The pathologist focused special attention on the vaccination site with enbloc resection of the affected area, which was then photographed, measured, and sampled for histology. In 2 instances, the nature of the exudate within this site prompted bacterial culture of the lesion. The pathologist performed a complete necropsy on each mare with inspection of the various body systems and noted any gross lesions. Collection of tissue samples into 10% neutral buffered formalin included any significant gross lesions as well as samples of the vaccination site; prescapular, anterior mediastinal, and tracheobronchial lymph nodes; heart; lung; liver; kidney; adrenal gland; mammary gland; pituitary; ovary; oviduct; uterus; and cervix.

The pathologist weighed the ovaries, photographed any large follicles, noted presence of corpora lutea, and collected ovarian histopathologic samples near the ovulation fossa, avoiding any large follicular structure that may have been present. Ovarian tissue was fixed for 24 hours prior to processing, and other tissues were fixed a minimum of 48 hours. The histology laboratory routinely processed post-fixation tissue samples into paraffin blocks; technicians stained sections at a thickness of 6–7 μm with HE for microscopic examination.

RESULTS

Animals

Horses ranged in weight from 341 to 591 kg (Table 1), and mean body weight did not differ among treatment groups ($P > 0.5$; 1-way ANOVA). Weights estimated by tape measure were approximately 13 kg more than weights determined using the scale. Ages ranged between 3 and 14 years and did not differ among treatment groups ($P > 0.5$; 1-way ANOVA). Although we identified various combinations of mild anemia, neutrophilia, lymphopenia, and hypocalcemia in some of the mares at the beginning of the study, all health parameters were normal at the end of the study for all mares. Principal components analysis did not reveal any visually distinct groupings among the mares assigned to the different treatment groups at either the start or end of the study.

Mares had localized reactions to the MFA injections, which varied in intensity and duration. Four of the MFA non-aqueous- and 2 of the MFA aqueous-injected mares exhibited no grossly visible response. One of the MFA non-aqueous treated mares developed a 9-cm × 10-cm area of inflammation 2 weeks post-vaccination, which completely resolved in the following 2 weeks. Two of the MFA non-aqueous-, 4 of the MFA aqueous-, and 1 of the control MFA-injected mares developed areas of inflammation that ranged in size from 5 cm × 10 cm to 11 cm × 14 cm, varied from firm to tissue-soft consistency, and interestingly, fluctuated through time so that grossly palpable areas of swelling on the neck completely resolved and then re-appeared 1–2 weeks later. One of the MFA aqueous treated mares developed an abscess approximately 10 cm × 15 cm in size that ruptured 16 weeks post-vaccination. We cleaned the abscess, flushed it regularly with cleansing solution, and it healed completely within 7 weeks. We found no correlations between injection site reactions and sizes of mares, vaccine titers, or effects on the reproductive system.

Ovaries collected from control mares ($n = 7$) weighed more ($P = 0.002$) and had greater variation ($P = 0.003$) than either MFA vaccinate group (mean ± SD; 48.8 ± 23.99 g vs. 21.3 ± 3.89 g and 26.2 ± 6.59 g for control vs. non-aqueous and aqueous injected mares, respectively) and were grossly smaller in size. We did not detect significant differences ($P > 0.10$) between treatment groups (Fig. 1).

Placebo controls continued to cycle normally throughout the trial. However, by the end of the study, mares treated with either SpayVac formulation had developed significantly ($P < 0.001$) smaller ovaries and fewer follicles (Fig. 2a) that could be distinguished ultrasonographically from the control mares (Fig. 2b). Follicle sizes were noticeably smaller for the MFA-non-aqueous and -aqueous treatment groups 3 months post-vaccination (a few 5–7 mm in diameter) compared to controls (several approximately 10–30 mm in diameter with multiple small follicles). Consistent with a reduction in the number of mares cycling, far fewer corpora lutea were visible ultrasonographically in treatment groups during this time (Table 2).

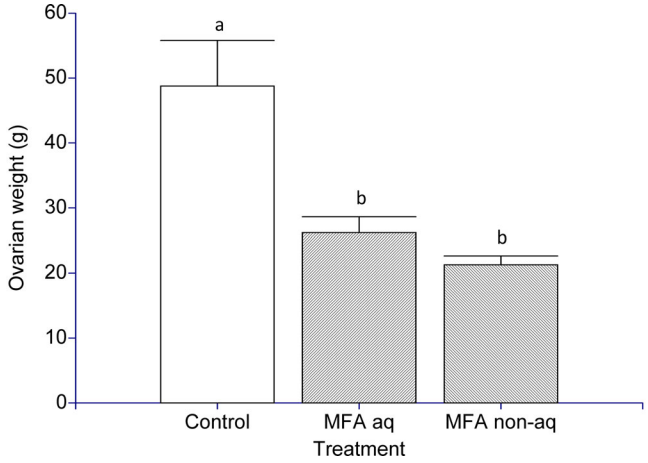

Figure 1. Weights of ovaries (means ± SE) collected from ovariectomized and necropsied mares injected with vehicle (control) or SpayVac® in aqueous or non-aqueous suspensions with modified Freund's adjuvant (MFA aq or MFA non-aq, respectively). Means without similar superscripts differ ($P \leq 0.001$).

Laboratory Analyses

Mean antibody titers for both treatment groups differed significantly ($P < 0.001$) from those of the control group, which did not develop pZP antibody titers. Most treated mares reached a relatively constant antibody titer value by week 6 (Fig. 3), although we found appreciable variation within treatment groups especially between 4 and 8 weeks post-vaccination (Table 2). Mares vaccinated with the MFA aqueous formulation tended to develop greater antibody titers slightly earlier than MFA non-aqueous treated mares (Table 2). Mean pZP antibody titers 8 weeks post-vaccination were greater in MFA non-aqueous treated mares as compared to MFA aqueous treated mares (mean ± SE; 403,429 ± 274,429 vs. 86,857 ± 20,346, respectively) and remained greater for the duration of the project (Fig. 3); however, mean differences between these 2 treatment groups were not statistically significant ($P = 0.15$).

Immunohistochemistry confirmed an immune response in pooled serum samples from both treatment groups by specific demonstration of the ZP in contrast to a lack of positive staining when treated with pre-vaccination sera (Fig. 4). However, we could not obtain convincingly positive reactions in any of the ovaries retrieved from vaccinated mares when we attempted to demonstrate endogenously bound anti-ZP antibody. We tried various antigen retrieval methods to amplify availability of receptors, including heat antigen retrieval and proteinase K digestion, but we found no improvement in signal. This aspect of the study was hampered by limited availability of developing follicles in vaccinated mares in comparison to control mares. We had to examine large numbers of HE sections to detect follicles, and in many instances, these structures had a poorly defined ZP.

Mean serum concentrations of P_4 were significantly lesser ($P < 0.025$) in the MFA non-aqueous treatment group compared to control mares (mean ± SE; 1.81 ± 0.30 ng/ml vs. 6.33 ± 0.59 ng/ml, respectively; Fig. 5), but differences were not significant ($P > 0.10$) for the MFA aqueous treatment group mean (4.05 ± 0.53 ng/ml). Significant decreases ($P < 0.001$) in serum concentrations of P_4 in MFA non-aqueous and aqueous treated compared to control mares coincided with a loss of estrous cyclicity observed as early as 14 weeks post-vaccination (Fig. 6; Table 2).

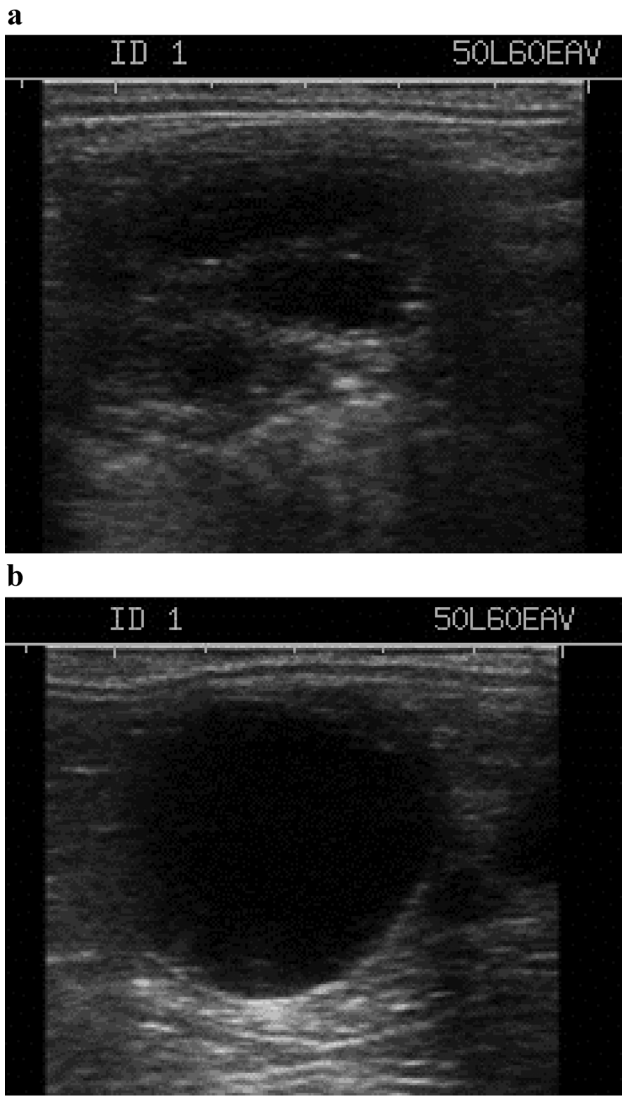

Figure 2. Transrectal ultrasound images of ovaries taken on 15 September 2010 illustrate decreased ovarian activity in (a) a SpayVac® aqueous treated mare as compared to (b) a control mare with a fully developed secondary follicle (hash marks represent 1-cm increments).

Necropsy and Histopathology

Gross findings.—The main gross abnormality observed in the study involved the vaccination site, where a chronic inflammatory process created a mass effect in all but 3 mares, visible upon dissection. Necropsy findings revealed injection site reactions in 3 vaccinated mares that did not demonstrate gross visible lesions earlier. Fibrous tissue altered the fascia and muscular tissue at these injection sites, delineating areas of granulomatous to purulent inflammation characterized by a friable to semi-liquid yellow exudate. Most of these lesions were composed of multifocal to confluent nodules ranging

Table 2. Treatment groups (control and treatments receiving SpayVac® in an aqueous or non-aqueous formulation with modified Freund's adjuvant [MFA]) and their mean porcine zona pellucida (pZP) antibody titers (±SE), ovarian activities, and serum concentrations of progesterone (P_4; mean ± SE) in mares based on weeks post-vaccination.

	Control[a] (n = 7)		MFA aqueous (n = 7)			MFA non-aqueous (n = 7)		
Weeks	Ovaries	P_4 (ng/ml)	pZP titer[b]	Ovaries	P_4 (ng/ml)	pZP titer	Ovaries	P_4 (ng/ml)
0	Active[c] CL[d]	1.8 ± 1.3	<4,000	Active CL	1.4 ± 0.8	<4,000		1.3 ± 1.0
2	Active CL	4.3 ± 1.7	6,857 ± 738		2.3 ± 1.8	5,143 ± 738	Active CL	2.1 ± 1.5
3	Active CL	3.3 ± 1.5	25,143 ± 7,494	Active CL	4.3 ± 2.2	25,714 ± 10,023		0.8 ± 0.3
4	Active CL	4.8 ± 2.7	112,000 ± 67,069	Active CL	4.1 ± 1.3	30,857 ± 9,470	Active CL	1.7 ± 1.2
6	Active CL	8.9 ± 4.4	121,143 ± 65,493	Active CL	10.8 ± 3.9	146,286 ± 62,010	Active CL	2.5 ± 1.4
8	Active CL	7.8 ± 2.6	86,857 ± 20,316		6.1 ± 3.3	403,429 ± 275,429	Active CL	4.9 ± 2.1
10	Active CL	5.9 ± 2.1	80,000 ± 18,142	<Foll[e] CL	6.3 ± 3.7	182,857 ± 25,860	<Foll CL	6.5 ± 2.4
14	Active CL	14.7 ± 6.2	59,429 ± 14,274	Active CL	2.1 ± 1.0	173,714 ± 30,323	<Activity	1.4 ± 1.4
18	Active CL	5.4 ± 2.5	66,286 ± 32,786	<Activity[f]	0.4 ± 0.2	164,571 ± 33,800	NSS	0.4 ± 0.2
22	Active CL	2.2 ± 2.0	86,857 ± 31,589	NSS[g]	1.3 ± 0.8	100,571 ± 12,930	NSS	0.3 ± 0.1

[a] pZP titers were not observed in control mares.
[b] The lowest serum dilution used to quantify pZP titers in mares vaccinated with MFA aqueous or non-aqueous treatments was 1:4,000.
[c] Active = majority of mares have follicles in various stages of development.
[d] CL = corpora lutea.
[e] <Foll = majority of mares have no small follicles (<10 mm).
[f] <Activity = majority of mares have multiple small follicles but no corpora lutea; many have no significant structures.
[g] NSS = no significant structures (or few follicles <5 mm) found in the majority of mares' ovaries.

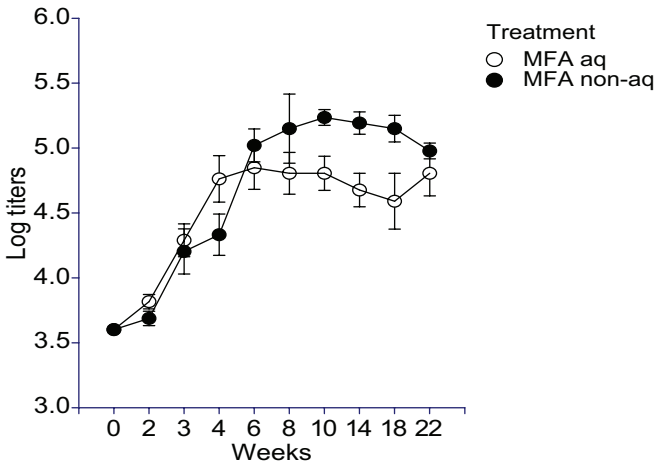

Figure 3. Mean porcine zona pellucida antibody titers (±SE) expressed as log titers in mares vaccinated with SpayVac® in aqueous or non-aqueous suspensions with modified Freund's adjuvant (MFA aq or MFA non-aq, respectively).

from a few millimeters to over 3 cm in diameter. Neither of the lesions that were cultured yielded any growth of bacterial pathogens.

Prescapular nodes on the same side of the body as the SpayVac injection site were not grossly enlarged in any of the mares. However, mares 15 and 23 exhibited granulomatous lesions on the cut surface of enlarged mediastinal and/or tracheobronchial nodes.

In 2 mares, gross lesions were identified that corresponded to clinical observations of lameness: a sub-acute laminitis in mare 9 and an erosive arthritis with meniscal tear in mare 27. Other gross lesions noted in various animals were interpreted to be within the background or incidental pathology that would be expected in any random sampling of mares. Such findings were not present in every animal but included: fibrous tags on the anterior surface of the liver or on serosal surfaces of some abdominal viscera, hepatic granulomas, haemomelasma ilei lesions, intra-abdominal lipomas, paraovarian cysts, and intragastric bot fly larvae.

Histopathology findings.—Typical vaccination site lesions were described as classic granulomatous foci with or without lightly mineralized necrotic debris and collagenous replace-

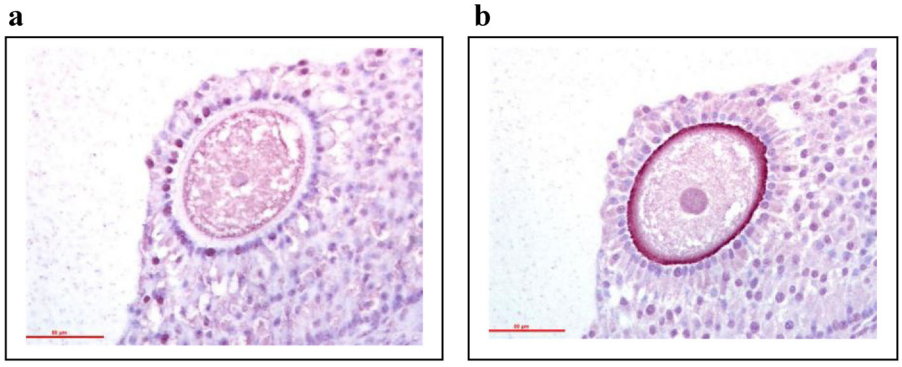

Figure 4. Immunohistochemistry staining demonstrating the zona pellucida in mare follicles treated with (a) pre-immune sera as compared to (b) immune sera.

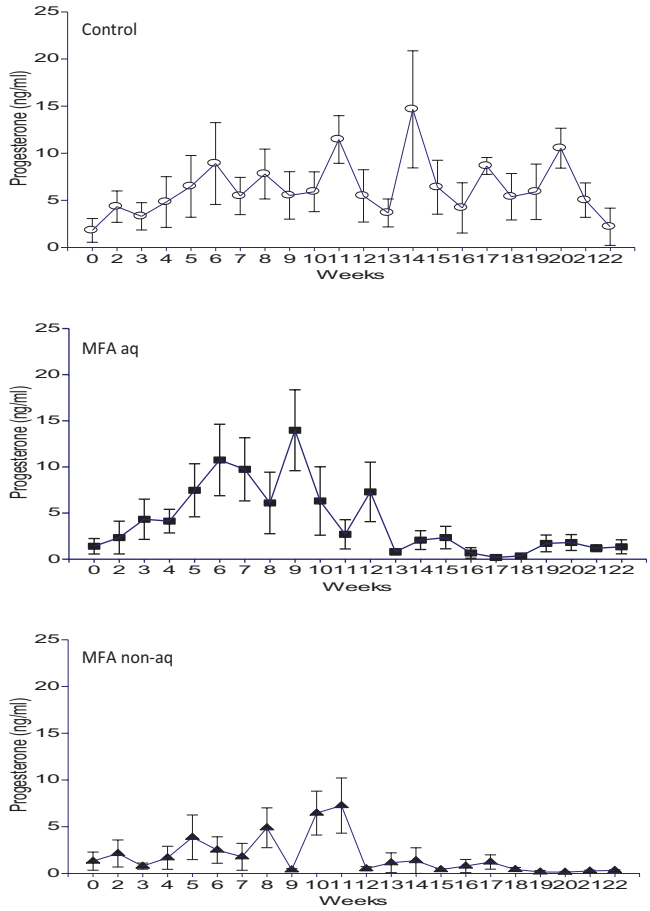

Figure 5. Serum concentrations of progesterone (means ± SE) in mares injected with vehicle (control) or SpayVac® in 2 different formulations (modified Freund's adjuvant [MFA] aqueous [aq] or MFA non-aqueous [non-aq]).

ment of muscle tissue. However, often areas within the same mass tended toward a more suppurative character, and in some mares, this suppurative response predominated.

The lymph nodes comprising the prescapular complex were moderately hyperplastic in approximately half of the mares sampled, but this lymphoid follicular hyperplasia included control animals and could not be directly linked to vaccine treatment. In contrast, multiple mares had lymphadenitis of the anterior mediastinal node (5 of 7 sampled), either of an eosinophilic or granulomatous nature. Two of 3 mares with granulomatous lymphadenitis were from the MFA aqueous treatment group; the other was an MFA control animal. All mediastinal nodes were altered by mild hyperplasia to some degree, including a saline control mare. Tracheobronchial nodes were also generally hyperplastic, but interpretation of these nodes was complicated by histiocytic to granulomatous infiltrates focused on dust particles and other particulate matter (pneumoconiosis response). In only 1 instance (MFA aqueous treated mare 23) was the granulomatous infiltrate not linked to particulate debris and was therefore interpreted as a likely consequence of vaccine exposure.

Minor lobular hyperplasia of the mammary gland was seen in 3 mares from both treatment groups. This was a mild, non-diffuse change and was interpreted as a reflection of their reproductive history rather than a current endocrine abnormality.

The myocardium of 3 mares (9, 13, and 25) featured multifocal perivascular to interstitial infiltrates of lymphocytes, plasma cells, and a few histiocytes. In 2 animals, this change was associated with rare necrotic myocytes, whereas mare 9 had only tight cuffing of a few interstitial vessels. Similar changes may be seen with viral or parasitic infections, but a treatment connection is unlikely.

Mild multifocal lymphoplasmacytic interstitial nephritis was seen in the majority of mares involving both treatment groups and likely reflected another background lesion unrelated to the vaccine.

No oophoritis was found in any of the ovaries examined. Ova and primary follicles often had vacuolated cytoplasm, but this was likely a processing artifact. Perhaps the most surprising feature of the ovaries examined was the general lack of developing follicles in treated mares. Given the size of equine ovaries and the tendency for follicles to cluster together within the stroma, this could be dismissed as an oddity of collection or sectioning. However, when combined with the knowledge that a similar phenomenon was seen during the examination of large numbers of ovarian sections in the IHC aspect of this study, this finding appears to be a treatment related effect. All sections of the oviduct and cervix were normal. We found some mild endometritis in individuals, including the control saline mare.

Most other lesions corresponded to background changes such as hepatic granulomas, which appear to have originated from parasite migration. Mare 25 also had a few lymphofollicular nodules in the pulmonary interstitium that were likely parasite-related.

DISCUSSION

Efforts to create longer-lasting contraceptive vaccines with increased efficacy have involved modifying formulations (Henderson et al. 1988, Botha et al. 2008), adjuvants (Allison and Gregoriadis 1974, Gupta et al. 1993, Lyda et al. 2005), and delivery matrices (Turner et al. 2007, 2008; Killian et al. 2008). Previous studies have demonstrated a positive correlation between levels of pZP antibodies produced and contraceptive effect via binding of sperm receptor sites (Liu et al. 1989, Turner et al. 1997); however, other contraceptive mechanisms of action may involve altered ovarian function. This study demonstrated the ability of 2 single-dose SpayVac formulations to elicit a strong antibody response and affect ovarian function in mares, although we did not examine actual contraceptive efficacy.

Liposomes are multi-lamellar, concentric spheres made up of phospholipid bilayers separated by aqueous compartments and may themselves serve as immunological adjuvants (Allison and Gregoriadis 1974, Shek and Sabiston 1982, Gupta et al. 1993). VacciMax® (the aqueous version of DepoVax used in SpayVac), when administered to rabbits, created a significantly greater humoral response compared to that produced by alum-adjuvant vaccines, and this enhanced

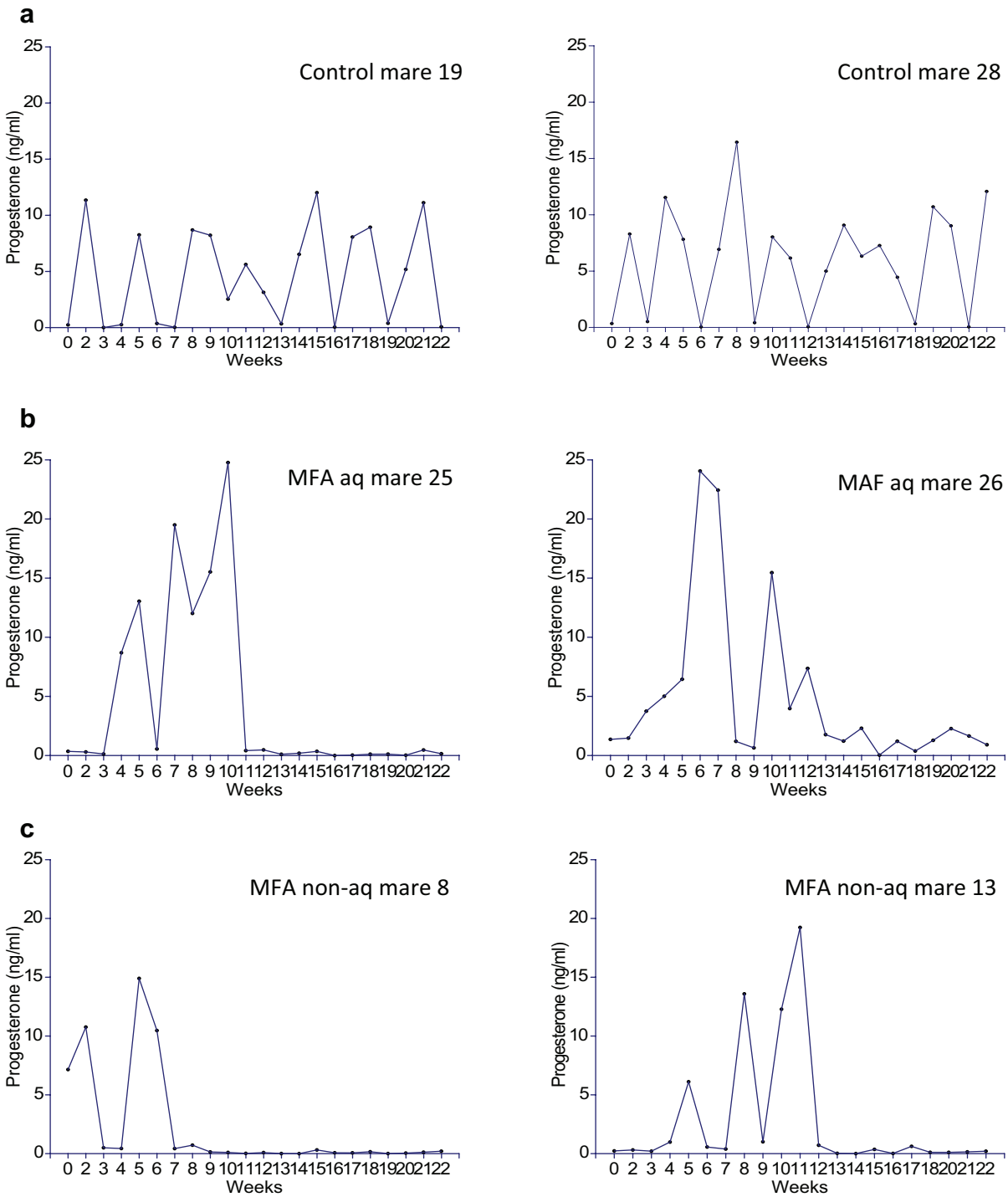

Figure 6. Fluctuations in serum concentrations of progesterone in individual mares from (a) control, (b) modified Freund's adjuvant (MFA) aqueous (aq), and (c) MFA non-aqueous (non-aq) treatment groups.

response was correlated with an increased number of ZP-specific plasma cells in the bone marrow (MacDonald et al. 2010; http://www.deliveringbreakthroughs.com/library/humoral%20poster%20London%20dec05.pdf). SpayVac may similarly stimulate an increased production of plasma cells in mares. Most long-lived plasma cells are found in the bone marrow, and differentiation from a plasmablast into a long-lived plasma cell (as opposed to a short-lived plasma cell) can be a T-cell dependent or independent process (Bortnick et al. 2012). A variety of transcription factors (e.g., Blimp-1) and cytokines (e.g., interleukin 5 and 6; tumor necrosis factor) have been shown to contribute to the longevity of plasma cells (Radbruch et al. 2006), and absence of an inhibitory factor (FcγRIIb) has resulted in increased numbers and greater persistence of long-lived plasma cells in mice (Xiang et al. 2007). We did not quantify

plasma cells in this study, but future research to explore precisely how SpayVac may stimulate their production and/or longevity in mares should be conducted.

Other proposed mechanisms for maintenance of antibody titers include sustained release of antigen (Murphy 2008), self-boosting via seasonal exposure to endogenous ZP (Killian et al. 2008), and/or recruitment of follicular dendritic cells. SpayVac's liposome matrix is believed to provide sustained release of antigen, obviating the need for frequent boosters. Indeed, many of the mares in this study exhibited varying degrees of an inflammatory reaction at the injection site that waxed and waned through time. Additionally, follicular dendritic cells draining lymph nodes around the site of injection might be the key to long-lasting immunity, as proposed by Miller et al. (2009) based on a 7-year trial using different pZP vaccine formulations in white-tailed deer. Antigens that induce a strong T-cell response initiate the mobilization and maturation of dendritic cells, which migrate into areas being assaulted (Ng et al. 2008). Follicular dendritic cells are found primarily in lymph nodes and the spleen, their receptors trap antigen opsonized by complement or antibodies, and they interact closely with B cells, which must bind the antigen presented by dendritic cells to become future memory cells (Banchereau and Steinman 1998). Many of the mediastinal lymph nodes of the mares in this study exhibited mild hyperplasia, but this included control mares, and other causes could not be ruled out. Regardless, multiple mechanisms are likely responsible for producing the strong immune response seen in the SpayVac treated mares.

Immune responses are highly idiosyncratic and dependent upon an animal's physiological state. Consequently, differences in immune response may contribute to different effects based on variations in pZP vaccine formulations. Previous studies have demonstrated that antibody titers in groups of horses and several deer species vary significantly in response to vaccination with the same immunogen, dose, and adjuvant (Kirkpatrick et al. 2011). A variety of internal physiologic factors can influence an animal's ability to mount an immune response. The range of antibody titers in this study also varied extensively within treatment groups with standard deviations often exceeding means up through the first 8 weeks post-vaccination. Modeling (Ballou et al. 2008) and field trial studies (Kirkpatrick and Turner 2008) have shown that 100% contraceptive efficacy is not required to effectively manage fertility; however, antibody titers need to consistently remain at a species-specific percentage above reference serum values to achieve reasonable rates of contraception (Liu et al. 1989).

Because pZP is a poor immunogen, a powerful adjuvant is required to stimulate an immune response. One of the first studies with pZP in mares used an aluminum hydroxide (alum) gel as an adjuvant, but 4 boosters given 2–4 weeks apart and another at 6–10 months were required to maintain contraceptive efficacy (Liu et al. 1989). AdjuVac uses *Mycobacterium avium* in a water-oil emulsion (Miller et al. 2004). However, the current adjuvant of choice for many contraceptive vaccines, including SpayVac, is MFA, which is made with fractionated cell walls from *Mycobacterium butyricum*, a common soil bacterium that does not result in false tuberculosis-positive test results. A recent study in horses compared pZP vaccines formulated with either MFA or FCA and found consistently greater antibody titers in the MFA vaccinate group, although differences were not significant (Lyda et al. 2005).

Local injection site reactions are not uncommon, and a variety of factors have been proposed as contributors to their formation, including concurrent vaccinations that may result in cross-reacting epitopes (Miller et al. 2008), water-in-oil emulsions (Gupta et al. 1993), and oils comprised of shorter chain carbon lengths (Fukanoki et al. 2000). Miller et al. (2008) suggested that epitopes on the surface of killed *Streptococcus equi* in strangles vaccines were cross-reacting with epitopes on *M. avium*, contributing to the injection site reactions seen in mares simultaneously vaccinated with GonaCon (Killian et al. 2006). However, in our study, mares were given only viral preventive health vaccines prior to administration of SpayVac, so injection site reactions were unlikely potentiated by this mechanism. We observed inflammation at the injection site in a control-MFA treated mare, so reactions are attributed at least in part to the use of MFA. Although injection-site reactions to MFA may be common, abscesses are rare, ranging from 0.5% (Turner and Kirkpatrick 2002) to 5.5% (Roelle and Ransom 2009) in different horse populations treated with either FCA- or MFA-potentiated pZP vaccines. In a 7-year review of GonaCon studies in various species (using AdjuVac as the adjuvant), Miller et al. (2008) state "The most common report is that there is no visible injection site reaction, or that there is a non-visible but palpable lump underneath the skin at the site of the injection." Two field studies using the same vaccine in white-tailed deer resulted in similar findings as well as "considerable injection-site reaction deep in the muscle" when the site was dissected (Gionfriddo et al. 2006). These findings are similar to ours where injection site reactions were more readily visible during necropsy. Neck injections tend to result in detectable reactions more often than do hip or gluteal injections in the mare (Lyda et al. 2005, Kirkpatrick et al. 2011), so the number of reactions that we observed were probably greater than they would have been, had we administered the vaccine in the gluteal region. Smaller injection volumes would likely also minimize localized reactions.

Ovaries collected from pZP-treated mares in this study were grossly smaller in size and lacked developing follicles compared to controls. Porcine ZP immunocontraception is believed to result from antibody occupation of the sperm binding receptors in the ZP of treated females' ova, which prevents fertilization; however, other mechanisms may involve non-specific immune responses to an ovarian cellular component in the vaccine, or immune-mediated disruption of folliculogenesis (Henderson et al. 1988).

Dunbar et al. (1980) first developed the mechanical pZP isolation technique used today, whereby oocytes are freed from the cellular matrix by sequential passages of ground tissue through a series of nylon screens of decreasing pore

size, and a tissue homogenizer then releases the ZP. They determined on a cellular basis that extractions were 95% pure and were 93–97% pure on an enzymatic basis, with cumulus cells being the major cellular contaminant. As a way to remove these cells, Henderson et al. (1988) recommended that after passage through a final 75-μm nylon mesh to trap eggs, the preparation should be centrifuged, applied to a discontinuous Percoll gradient, from which eggs are aspirated from the top and then gently homogenized to free the ZP. Our study used an isolation procedure similar to that used by Dunbar et al. (1980) to produce the SpayVac vaccines (Brown et al. 1997*b*). All pZP vaccines are made with partially purified porcine ZP, and other follicular proteins could potentially be involved in eliciting ovarian responses in vaccinates (Stoops et al. 2006). Western blot experiments have shown that immune responses to pZP vaccination in mares are against all of the pZP glycoproteins present in partially purified vaccine formulations (Liu et al. 2005), and the IHC staining in our study demonstrated an immune response; however, none of these tests eliminates the possible presence of other ovarian proteins, which could play a role in eliciting the ovarian effects seen.

Domestic ewes immunized with a non-liposomal pZP vaccine formulated with FCA developed ovaries characterized by an absence of growing follicles, a significant reduction in the number of primordial follicles, and abnormal granulosa cell clusters lacking oocytes (Stoops et al. 2006) —findings quite similar to those of our study. Specific functions for ZP proteins are proving more complex than originally expected and include a role in follicular development in addition to binding spermatozoa (Barber and Fayrer-Hosken 2000*b*). Zonae pellucidae contain 3 major glycoproteins (ZP1, ZP2, and ZP3) encoded by 3 gene families (ZPA, ZPB, and ZPC) that are highly conserved across species (Yurewicz et al. 1998), and a fourth glycoprotein has been identified in humans (Lefievre et al. 2005). Generally, ZP1 is believed to provide structure to the ZP matrix, ZP2 binds to acrosome-reacted sperm and prevents polyspermy, and ZP3 is thought to serve as the primary sperm ligand (Lefievre et al. 2005). High affinity sperm-binding sites are constructed by ZP proteins in different ways in the pig and mouse (Yurewicz et al. 1998), and differences in ZP structure and glycosylation likely contribute to species differences seen in response to pZP vaccination (Barber and Fayrer-Hosken 2000*b*, Prasad et al. 2000).

Location of ZP antigens also varies slightly among species. Immunolocalization of ZP antigens in the horse ovarian follicle using rabbit anti-pZP antibodies demonstrated staining in granulosa cells and ooplasm in addition to the ZP, similar to that seen in the dog (Barber et al. 2001). Vaccination with pZP in dogs results in permanent infertility due to extensive oocyte destruction through a process of autoimmune oophoritis (Brandon et al. 1998), likely because of the presence of T-cell epitopes on the ZP used (Barber and Fayrer-Hosken 2000*a*). Although our study did not find inflammation in any mare ovaries, the ZP is thought to play a role in granulosa cell differentiation and folliculogenesis (Prasad et al. 2000), so it is plausible that pZP antibodies interfered with ZP function and affected development of follicles in the mares we studied.

Other research also supports this idea. For example, Henderson et al. (1988) described how ZP antibodies bind to developing follicular ZP as well as the zonae of ovulated ova, and Skinner et al. (1984) stated that ZP antibodies may disrupt cellular communication between differentiating follicular cells and the developing oocyte, which may lead to the destruction of a portion or all of the oocyte pool. Earlier formulations of pZP vaccines required boosters and did not result in significant changes in ovarian endocrine parameters or estrous cyclicity (Kirkpatrick et al. 1992, 1995; Powell 2000); however, SpayVac's liposome-based adjuvant may have enhanced the immune response in treated mares potentiating ovarian dysfunction as seen in other species. The paucity of developing follicles seen in HE sections of ovaries from treated mares in our study could be an artifact of sampling, but when combined with the difficulty in finding follicles for IHC testing and the small size of ovaries in comparison to those of the controls, we speculate that the vaccine-induced response might result in follicular destruction as soon as ZP antigens become available on the follicle.

Serum concentrations of P_4 were significantly decreased in the 2 SpayVac treatment groups at 14 weeks post-vaccination compared to controls in our study. These results complement the ovarian changes (fewer developing follicles and corpora lutea) visualized by ultrasound imaging and gross and histologic examination of ovaries. Low serum concentrations of P_4 in pZP-treated mares were also reported by Kirkpatrick et al. (1992) and Killian et al. (2008). A few studies reported results not found in ours, such as persistent corpora lutea in mares vaccinated with pZP emulsified in Freund's Complete or Incomplete Adjuvant and pelletized with poly(lactide-co-glycolide) biodegradable polymers (Liu et al. 2005), and high rates of uterine edema and elevated serum concentrations of estradiol in mares vaccinated with pZP using AdjuVac as the adjuvant (Killian et al. 2008). The amount of pZP administered appears to affect estrous cyclicity and duration of infertility (Henderson et al. 1988), but other factors (like adjuvant) also play a role. Mares receiving the non-aqueous SpayVac formulation had a slightly more robust immune response compared to the aqueous treatment group as evidenced by greater antibody titers and earlier ovarian dysfunction. The reason for this is not known. Successfully contracepted squirrel monkeys (*Saimir sciureus*) treated with a total of 200 μg ZP3 immunogen (4 immunizations within a 15-week period) in Freund's adjuvant experienced a disruption in ovarian function that affected estrogen and P_4 secretion; however, despite high levels of antibodies that continued beyond 10 months post-vaccination, ovarian function appeared to stabilize (Sacco et al. 1987). Unfortunately, we were unable to test if the treated mares in this study would have regained normal ovarian function through time.

Gross and histological examinations of major organ systems at the end of the study did not reveal any abnormalities that could be tied to SpayVac vaccination

except possibly a decreased number of developing follicles in the ovaries of treated mares. These findings are consistent with those of other pZP studies (Barber and Fayrer-Hosken 2000*b*, Miller et al. 2001), and as Kirkpatrick et al. (2011) state, intuitively vaccines that exert their influence further downstream in the reproductive process will likely be less problematic. In fact, long-term studies with the horse population on Assateague Island demonstrated a significant increase in longevity of pZP-treated mares as compared to controls (Turner and Kirkpatrick 2002), because the energy demands of pregnancy and lactation in the controls leaves them with lower body condition scores and greater mortality rates (Kirkpatrick and Turner 2007).

In summary, the 2 SpayVac formulations tested in this study resulted in rapid production of pZP antibodies and significant decreases in serum concentrations of P_4, ovarian weights, and numbers of follicles compared to controls. The MFA non-aqueous formulation appeared to have had a slightly more pronounced effect than did the MFA aqueous formulation based on these parameters, but differences were not statistically significant. Vaccines should be given in the rump to minimize injection site reactions. The mechanisms of action whereby ovarian function is altered and subsequent contraception might be achieved are not clear. Complicating research in this field are the different pZP formulations being tested, which all elicit different immune responses. Henderson et al. (1988) and others (Skinner et al. 1990, Prasad et al. 1996) suggested that recombinant DNA technology could be used in future research to target specific ZP epitopes and optimize immunogenicity for production of antibodies that will only inhibit sperm binding and not result in ovarian dysfunction. Additional investigations focused on the mechanisms of action of ZP vaccines and the long-term consequences of immunization on a cellular level need to be conducted. This kind of information will clearly be important for successful field applications. A long-term study is currently being conducted by the United States Geological Survey (USGS) in collaboration with the BLM to assess contraceptive efficacy and antibody titers over a 5-year period in 90 mares treated with non-aqueous and aqueous MFA SpayVac vaccines, and research to further elucidate how SpayVac affects ovarian function in mares is being planned.

MANAGEMENT IMPLICATIONS

Feral horse populations can be managed by live removal of horses and/or by application of a contraceptive agent. SpayVac has great potential as a single-dose, multi-year immunocontraceptive vaccine because of its unique liposome formulation, which makes it both practical and economical for use in the field. Because ZP vaccines are tissue-specific, they cause fewer side effects compared to GnRH vaccines or other types of contraception, particularly if they require more frequent animal handling. SpayVac-treated mares were not cycling normally, which may result in different interactions with stallions in the wild; however, acyclic mares may be preferable to pZP-treated mares that continue to cycle and attract stallions' attention during an entire breeding season. Although contraceptive efficacy was not assessed in this study, treated mares were likely infertile as evidenced by an erosion of the follicle pool, lack of ovulation, and low serum concentrations of P_4. Long-term effects of SpayVac on the reproductive system are still unknown. Wildlife managers need to know how often and how many mares should be vaccinated in order to efficaciously apply contraceptive vaccines to free-ranging herds, potentially in conjunction with removals. If infertility is irreversible, a smaller number of mares would likely need to be contracepted, and the potential for mares to foal late in the season, after contraceptive efficacy declines (Nunez et al. 2010), would be minimized. Additional research to elucidate the vaccine's mechanism of action, actual contraceptive efficacy, and long-term effects is still needed.

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