Efficacy and safety of native and recombinant zona pellucida immunocontraceptive vaccines in donkeys

Hilari French[a], Erik Peterson[a], Martin Schulman[c], Robyn Roth[d], Michael Crampton[d], Anne Conan[a], Silvia Marchi[a], Darryn Knobel[ag], Henk Bertschinger[c*]

[a]Ross University School of Veterinary Medicine, PO Box 334, Basseterre, St Kitts, West Indies, HFrench@rossu.edu

[a]Ross University School of Veterinary Medicine, PO Box 334, Basseterre, St Kitts, West Indies, ErPeterson@rossu.edu

[c]Veterinary Population Management Laboratory, Section of Reproduction, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, South Africa, martin.schulman@up.ac.za

[a]Ross University School of Veterinary Medicine, PO Box 334, Basseterre, St Kitts, West Indies, AConan@rossvet.edu.kn

[d]Council for Scientific and Industrial Research, Pretoria, South Africa, rroth@csir.co.za

[d]Council for Scientific and Industrial Research, Pretoria, South Africa, mcrampton68@gmail.com

[e]Ross University School of Veterinary Medicine, PO Box 334, Basseterre, St Kitts, West Indies, SMarchi@rossu.edu

[a]Ross University School of Veterinary Medicine, PO Box 334, Basseterre, St Kitts, West Indies, DKnobel@rossvet.edu.kn

[g]Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa

[c]Veterinary Population Management Laboratory, Section of Reproduction, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, South Africa, henkbert@tiscali.co.za;

*Corresponding author: Tel.: +27128043312
Email address: henkbert@tiscali.co.za (H.J. Bertschinger)

ABSTRACT

Feral and semi-feral donkeys are recognized as a problem in some world regions. The main problem associated with uncontrolled donkey populations is habitat degradation and competition for feed resources, especially in arid climes. Controlling population numbers would reduce the impact of donkeys and other species. While removal by various means is effective, it has been shown to stimulate reproductive rate. Probably the most effective and humane solution is reducing reproduction using minimally invasive methods including immunocontraception. This study tested the immunocontraceptive efficacy and safety of zona pellucida (ZP) vaccines, both recombinant (reZP; three treatments) and native porcine (pZP; two treatments) vaccines formulated with Freund's modified complete (primary) and Freund's incomplete (boosters) adjuvants in donkey jennies. Control jennies received adjuvants only (two treatments). Twenty-five non-pregnant jennies were randomly assigned to reZP (n=9), pZP (n=8) or control (n=8) groups. Weekly monitoring of the reproductive tract and ovaries via transrectal palpation and ultrasound and inspection of injection sites was conducted and anti-pZP antibody titers were measured. Five weeks after last treatment, one donkey jack was introduced to each group and rotated every 21 days. By 232 days after last treatment the number pregnant and median days to pregnancy was 2/9 and 214 (reZP group), 1/8 and 196 (pZP group) and 8/8 and 77 (control group). Median time to ovarian shut-down was 77 (9/9) and 56 (7/8) days for reZP and pZP groups, respectively. This was observed in association with a distinct reduction in mean uterine diameter. The antibody response was equally good for both ZP-treated groups. Incorporation of Freund's adjuvants initially produced a high incidence of side effects from local swelling and intermittent lameness followed weeks later by sterile abscesses (reZP, 9/9; pZP, 7/8; control, 3/8). Both ZP vaccines effectively controlled reproduction in jennies, albeit with a high incidence of adjuvant-associated side effects.

Keywords: recombinant and native zona pellucida vaccines, immunocontraception, donkeys, fertility, ovarian suppression, anti-pZP antibody titers.

1. INTRODUCTION

Feral and semi-feral donkey populations are described as an important emerging global human-animal conflict scenario [1,2]. These perceived conflicts are reported in various countries (Australia, India, Northern Ireland, South Africa, USA, Zimbabwe) and include deleterious environmental impacts such as erosion, damage to crops, competition for resources with livestock or wildlife [3], road and air traffic hazards and as a perceived nuisance in urban areas. Currently, however, there is a paucity of evidence-based reports supporting these perceptions and the available data are often contradictory [4,5,6,7]. Methods reportedly employed in attempting to manage these populations to mitigate their impacts include fencing, fertility control, and lethal approaches such as shooting, immobilization followed by lethal injection, and mustering or trapping for transport and slaughter at abattoirs. These methods vary in their humaneness, efficacy, cost-efficacy and impact on non-target species [8].

Fertility control has proved effective in certain herbivore populations, including African elephants (*Loxodonta africana*), that experience density-dependent increases in reproductive rates following short-term removal programs [9]. In these populations, reducing reproductive rates through fertility control may be more sustainable in the long-term. Current methods of fertility control include surgical sterilization, hormonal (chemical) methods and immunocontraception. In the latter, injection of formulations containing antigenic epitopes results in the production of circulating antibodies or immune effector cells that interrupt reproductive processes, causing variable periods of infertility in treated animals [10]. One target is the zona pellucida (ZP) capsule, a complex glycoprotein matrix surrounding the mammalian oocyte [11]. Zona pellucida proteins are weak antigens requiring inclusion of strongly immunogenic adjuvants in vaccine formulations in order to elicit an effective immune response. For immunocontraception, native porcine zona ZP (pZP) proteins are typically emulsified with modified Freund's complete adjuvant (MFCA) for primary and Freund's incomplete adjuvant (FIA) for booster immunizations, respectively [12]. These formulations have been used successfully for contraception in more than 90 species [13], most notably feral equids in the USA [14,15,16,17,18] and African elephants in South Africa [19,20]. In donkeys, to our knowledge, a single study

reported the successful use of pZP immunocontraception [21]. Recombinant ZP vaccines based on the expression of porcine ZP3 and ZP4 in *Escherichia coli* have also been developed [22]. Their potential advantages over vaccines using native pZP include production efficiency and avoiding contamination with non-ZP proteins.

The aims of the current study were to evaluate the contraceptive efficacy, ovarian effects and safety of pZP and reZP vaccines, both formulated with MFCA and FIA, in female donkeys (jennies). Results of this study will help to inform choice of method for humane and sustainable reproductive management of feral and semi-feral donkey populations.

2. MATERIALS AND METHODS

The study was reviewed and approved by Ross University School of Veterinary Medicine Institutional Animal Care and Use Committee (protocol no. 15.12.032).

2.1 Animals and management

The study was conducted at Ross University School of Veterinary Medicine (RUSVM) on the island of St Kitts in the federation of St Kitts and Nevis. Twenty-five feral jennies were caught on the island of Nevis. On capture, all appeared to be in good body condition and estimated to be in late gestation which was confirmed by transcutaneous transabdominal ultrasound (US). No additional history was available. Jennies were subsequently transported to St Kitts via water ferry. On arrival at RUSVM, all were microchipped and individually identified with numbered collars. Individual ages in y varied from three to 13, estimated according to the appearance of the incisors on dental inspection [23]. Jennies were housed in outdoor grass paddocks with free access to water and freshly-cut New Guinea grass (*Megathyrsus maximus*). They were monitored twice daily for signs of impending parturition and weekly by transcutaneous transabdominal US until they had foaled. Three clinically healthy jacks between the ages of three to six y (estimated by dental inspection) with two apparently normal intrascrotal testes assessed by inspection and palpation, were chosen for this study. Jacks were housed in a separate outdoor grass paddock with similar access to water and feed.

2.2 Study design

Inclusion criteria for the study were good physical condition, non-pregnant status, a normal reproductive tract and active ovaries on clinical examination. After foaling, all 25 jennies met these criteria and were randomly assigned to one of three treatment groups (Fig. 1) stratified according to age. The groups were housed in separate, adjacent outdoor grass paddocks. The study commenced after all eligible jennies had shown at least one complete estrous cycle. Day 0 of the study was defined as the day of last treatment. On Day 35, one jack was introduced into each group of jennies; jacks were rotated between groups every 21 days. The primary endpoint for the study was a pregnancy diagnosed by transrectal palpation and US of the reproductive tract. The secondary endpoint was 'ovarian shutdown', defined as the presence of bilaterally inactive ovaries with only small follicles (<10mm) and no corpus luteum (CL) visible on transrectal US [24,25]. Jennies remained in the study until diagnosed pregnant or Day 232, the last day of the study.

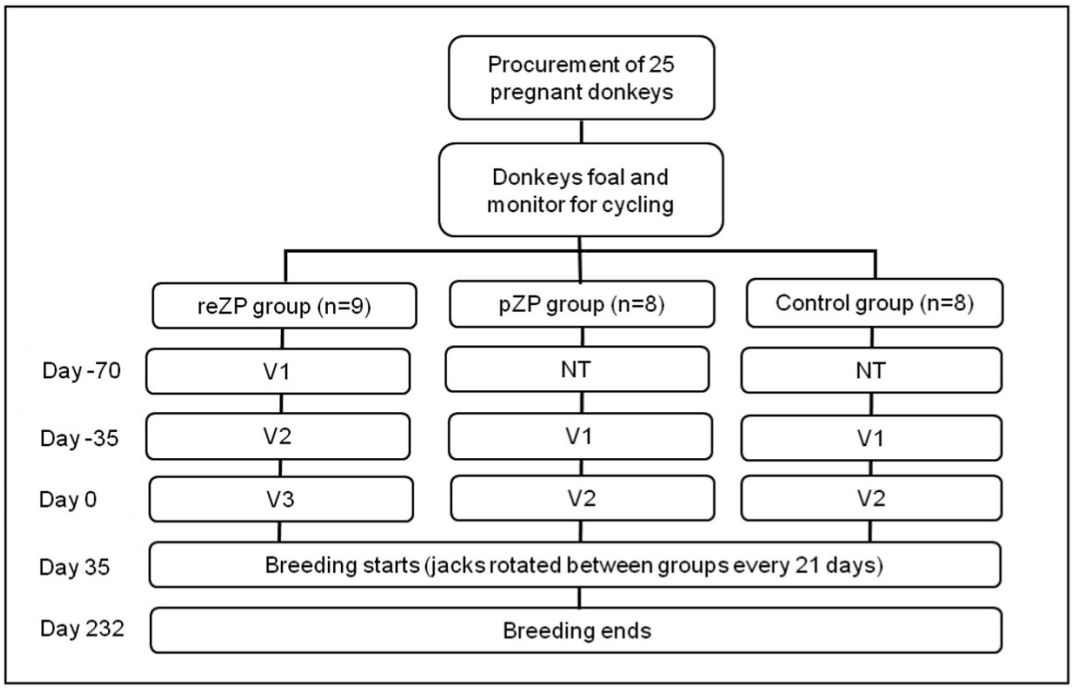

Fig. 1. Overall timeline and treatment protocol for the 25 jennies. NT = no treatment; V1 = primary vaccination; V2 = 1st booster; V3 = 2nd booster.

2.3 Treatments

2.3.1 Preparation of vaccines

All three vaccine formulations were prepared less than 30 min before administration.

Recombinant ZP_3 and ZP_4 proteins, supplied by the Council for Scientific and Industrial Research, South Africa, were expressed in *E. coli* according to Gupta et al. [22] with several modifications. The reZP vaccine used in the current study comprised recombinant ZP_3 and recombinant ZP_4, containing the promiscuous T-cell tetanus and bovine RNase epitopes at the N-terminus, respectively. The recombinant ZP_3 and ZP_4 proteins were analyzed via SDS-PAGE gels (Fig. 2) and confirmed by LC-MS peptide mapping. ~~p~~ZP3 with the TT epitope had 93.3% coverage at 95% confidence, while ~~p~~ZP4 with the bRNase epitope had 92.6% coverage at 95% confidence, analysed with ProteinPilot Software (AB Sciex). The resultant reZP product was lyophilized in vials, each containing 2 mg of each protein. Before administration, individual vials were reconstituted with 5 mL sterile water for injection providing a final concentration of 400 µg/mL of each protein. For the primary dose (V1), 0.5 mL reZP (200 µg per protein) was mixed with 0.5 mL MFCA to provide a stable emulsion. For the booster doses (V2 and V3), 0.5 mL recZP was mixed with 0.5 mL FIA to provide a stable emulsion.

Native pZP was prepared according to a standard method [14] and was supplied lyophilized in 1 mg vials by Trumpeter Farms and Veterinary Service (Winters, CA, USA). Each vial was reconstituted with 5 mL sterile water for injection providing a final protein concentration of 200 µg/mL. For the primary dose (V1), 0.5 mL pZP (100 µg) was mixed with 0.5 mL MFCA to provide a stable emulsion. For the booster dose (V2), 0.5 ml pZP (100 µg) was mixed with 0.5 mL FIA to provide a stable emulsion.

An adjuvant control consisted of 0.5 mL sterile saline emulsified with 0.5 mL MFCA for the primary dose (V1) and 0.5 mL sterile saline emulsified with 0.5 mL FIA for the booster dose (V2).

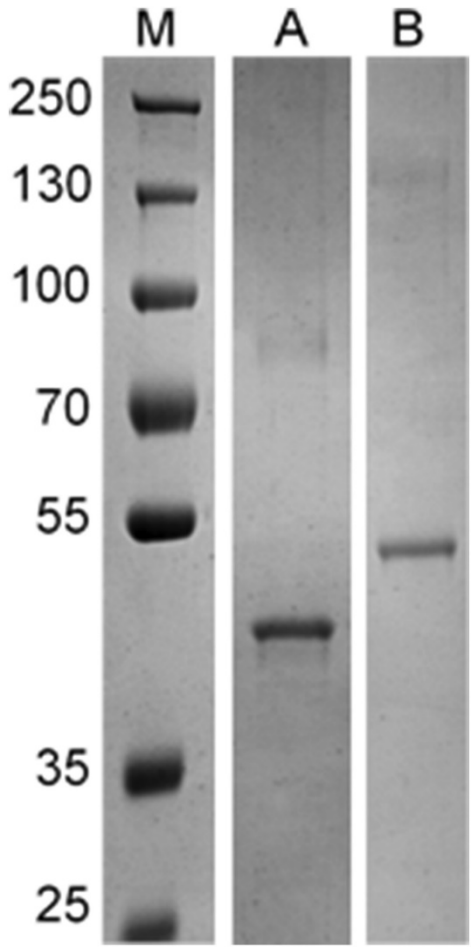

Fig. 2. 10% SDS-PAGE gel of purified (A) ZP3 with the TT epitope and (B) ZP4 with the bRNase epitope. M = PageRuler plus prestained protein ladder (Thermo).

2.3.2 Vaccinations

reZP group (n=9; mean age 6.1 y; age range 3-13 y): the primary dose (1 mL; V1) was administered by injection into the gluteal muscle mass on Day -70, the first booster (1 mL; V2) was similarly administered into the contralateral gluteal muscle mass on Day -35 and the final booster (1 mL; V3) was administered into the pectoral muscles on Day 0;

pZP group (n=8; mean age 6.5 y; range 3-13 y): the primary dose (1 mL; V1) was injected into the gluteal muscle mass on Day -35 and the booster (1 mL; V2), into the contralateral gluteal muscle mass on Day 0;

Control group (n=8; mean age 5.9 y; range 4-10 y): the primary dose (1 mL; V1) was injected into the gluteal muscle mass on Day -35 and the booster (1 mL; V2), into the contralateral gluteal muscle mass on Day 0.

2.4 Observations and sample collection

2.4.1 Transrectal monitoring of the reproductive tract

Clinical examination of the reproductive tract by transrectal palpation and US was performed weekly from Day -105 using a linear 5mHz probe (Sonosite, Universal). The left and right uterine horn diameters were measured at their base using US. Ovarian findings were recorded as the number of visible follicles (approximate resolution threshold of 5-10 mm) on each ovary and either the presence or absence of a CL. Following introduction of jacks, the jennies were monitored weekly until Day 232 for pregnancy via transrectal US.

2.4.2 Monitoring of injection sites

Jennies were monitored daily for five days after each vaccination for signs of swelling and pain. Thereafter injection sites were monitored weekly until the end of the study.

2.4.3 Serum collection and antibody titer assays

Blood samples from all jennies were collected with plain, evacuated tubes (Terumo™ VENOJECT™ II, Fischer Scientific, Waltham, Massachusetts, USA) by jugular venipuncture at 5-weekly intervals from Day -70 to Day 70 for assessment of anti-pZP antibody titers. Samples were allowed to coagulate at room temperature and serum was separated and stored at -80°C until assayed. Anti-pZP antibody response was measured by enzyme immunoassay (EIA), using a modification of a method previously described [24]. Positive reference serum consisted of pooled samples collected from the pZP group (n=8) on Day 35, when maximal antibody responses were anticipated for use as reference standards. Briefly, 96-well plates (MaxiSorp, cat. no. NUN430341) were incubated at 2-8°C for 16h with 1µg purified pZP in 100µL coating buffer (2.94% $NaHCO_3$, 1.59% Na_2CO_3, pH 9.6) per well. Plates were washed four times with phosphate buffered saline (PBS) containing 0.05% Tween 20 and then blocked with 0.03% bovine serum

albumin in PBS for 16h at 2–8°C. Plates were then incubated with serial dilutions of reference standards (1:500–1:64,000) and test samples (1:250-1:32,000) in duplicate at 37°C for 1h. Wells containing PBS served as blanks. After washing four times, antibodies were detected by incubating plates with 100µL/well of a 1:10,000 solution (diluted with assay buffer) of a Protein G-horse radish peroxidase conjugate concentrate (1 mg/mL; EMD Millipore Corporation, 28820 Single Oak Drive Temecula, CA 92590 USA) at 37°C for 1h. After washing four times, plates were developed with trimethylene blue (SureBlue™, KPL, Gaithersburg, Maryland, USA). The reaction was stopped by adding 50µL of 2 mol/L H_2SO_4 per well. Absorbance was measured at 450nm using a microplate photometer (BioTeck ELx800, Winooski, Vermont, USA). Sample antibody response (titer) was expressed as the serum dilution rate which achieved an absorbance 1.

2.5 Data analysis

The proportion of pregnancies and of ovarian shutdowns by Day 232, mean of antibody titers and 95% confidence intervals (95% CI) were computed by treatment group. Comparisons of shutdown and pregnancy proportions were done using a permutation test. Threshold of significance was set at 0.05. Comparisons of antibodies titers were done using Wilcoxon rank-sum test. To take in account multiple comparison, P-value threshold was set up at 0.0056 for these statistical tests (Bonferroni correction). Number of follicles and uterine horn diameter were plotted over time until pregnancy diagnosis or the end of the study (Day 232) using local polynomial regression fitting (‘loess’) smoothing with 95% CI. Mean of antibodies titers were plotted with an error plot (95% CI). The effect of antibody titers at Day 35 on time to ovarian shutdown was evaluated in the pZP and reZP groups combined using a Cox proportional hazards model. The threshold effect was identified using a penalized spline term. All data analyses were performed in R software [25] using the packages coin, ggplot2 and survival [26,27,28].

3. RESULTS

3.1 Effects of treatment on pregnancy rate, ovarian function and the uterus

Table 1 summarizes the results for the primary and secondary endpoints for the study. The proportion of pregnant jennies differed across the three treatment groups (P =

0.0004). All in the control group were confirmed pregnant by Day 119. One in the pZP group (Day 196) and two the reZP group (Days 196 and 232) were diagnosed pregnant. The proportion of jennies fulfilling the criteria for ovarian shutdown also differed across the three treatment groups ($P < 0.00054$). None in the control group, all in the reZP and all but one in the pZP group showed shutdown. The median time (days from Day 0) to shutdown was 56 days for the pZP and 77 days for the reZP group. In the pZP group, the jenny not showing ovarian shutdown was diagnosed pregnant on Day 196. A second jenny in this pZP group had resumed ovarian cyclicity on Day 232. In the reZP group, two jennies resumed ovarian cyclicity after shutdown, by Days 165 and 210, respectively. Both were subsequently diagnosed as pregnant on Days 196 and 232, respectively.

Table 1: Summary of reproductive outcomes observed in jennies following the final administration (Day 0) of native porcine zona pellucida vaccine (pZP), recombinant zona pellucida vaccine (reZP) or saline control, all formulated with Freund's adjuvants.

Observation	control group (n=8)	reZP group (n=9)	pZP group (n=8)
number (%) pregnant by Day 232	8 (100%)	2 (22.2%)	1 (12.5%)
median time to pregnancy (days)	77 (n=8)	214 (n=2)	196 (n=1)
median time to shutdown (days)	-	77 (n=9)	56 (n=7)
Jennies with temporary shutdown - days to resumption of cyclicity (%)	0 (0%)	1 - 165 (11.1%) 1 - 210 (11.1%)	1 – 232 (12.5%)
Number of jennies with prolonged shutdown	0 (0%)	7 (77.8%)	6 (75 %)

Jennies in both pZP and reZP groups showed a post-treatment reduction in their total ultrasonographically-visible follicle counts compared with those in the control group (Fig. 3). In addition, their mean uterine horn diameter was smaller compared to those in the control group (Fig. 4).

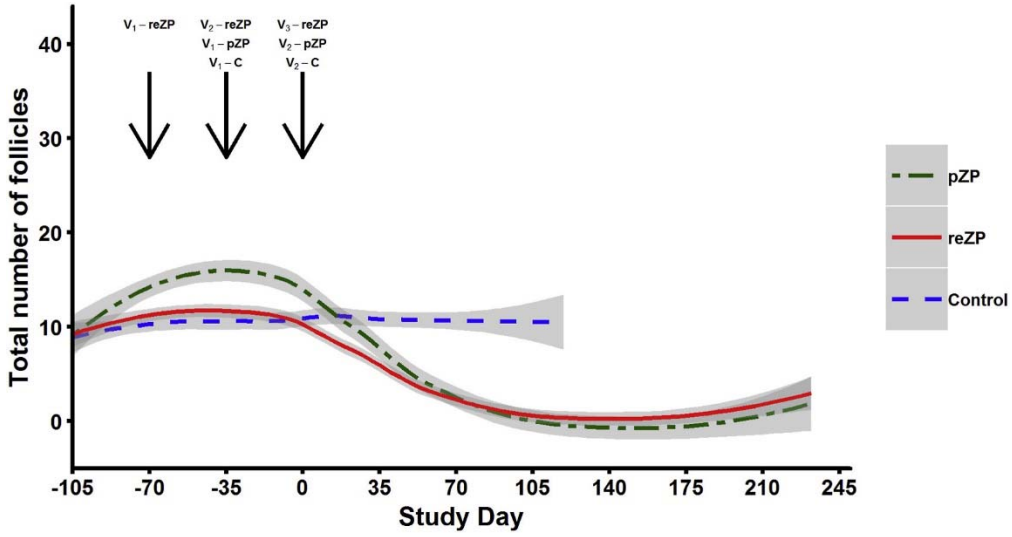

Fig. 3. Total mean follicle counts (≥5–10 mm) of pZP, reZP and control groups from Day −105 (prior to vaccination) to the end of the study (Day 232) after 2 or 3 vaccinations. V_1 = primary vaccination; V_2 = 1st booster vaccination; V_3 = 2nd booster vaccination. Curves were smoothed using loess regression. Grey shading indicates the 95% confidence intervals.

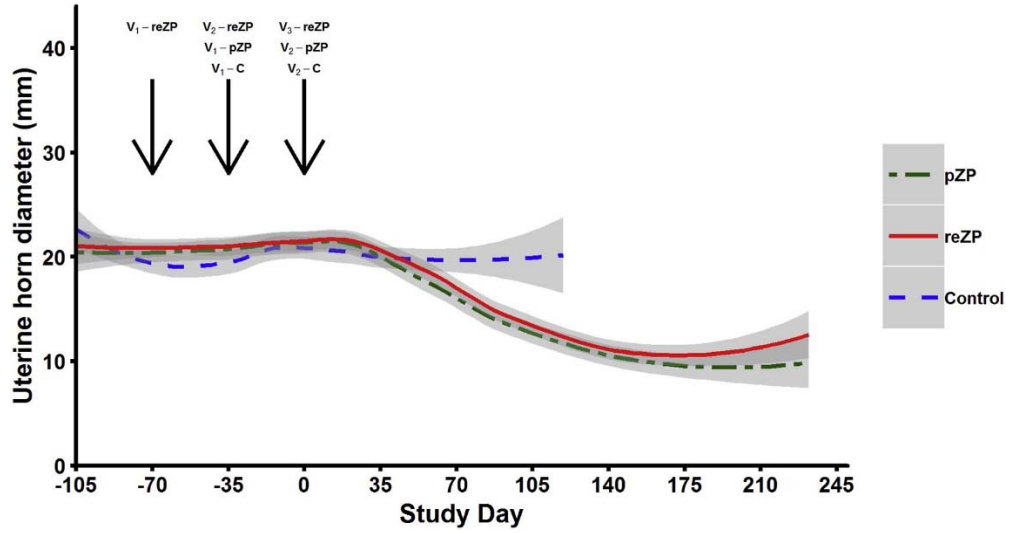

Fig. 4. Mean uterine diameter at the base of the uterine horn in jennies from pZP, reZP and control groups from Day −105 (prior to vaccination) to the end of the study (Day 232) after 2 or 3 vaccinations. V_1 = primary vaccination; V_2 = 1st booster vaccination; V_3 = 2nd booster vaccination. Curves were smoothed using loess regression. Grey shading indicates the 95% confidence intervals.

3.2 Injection site reactions

Post-treatment injection site reactions were more frequently observed in ZP-treated jennies than in the controls. Reactions ranged from localized swelling, sometimes

accompanied by intermittent lameness during the first five days post vaccination, to abscesses which appeared several weeks later. Subjectively assessed, the swelling and pain exhibited following the primary and first booster treatments was more pronounced in the reZP than the pZP and even more so, the control group. Abscesses were observed in 9/9, 7/8 (87.5%) and 3/8 (37.5%) jennies treated with reZP, pZP and control formulation, respectively. Bacteriological cultures of these abscesses were negative. As a result of the more severe injection site reactions observed after the first booster in the reZP group, a decision was made to substitute FIA with physiological saline for the second booster. As a result, only minor injection site reactions were observed.

3.3 Post treatment antibody titers

The mean antibody titers of each group over time are shown in Fig. 5. Groups pZP and reZP differed significantly from the control group on Days 0, 35 and 70 ($P<0.001$ in all cases). There were no significant differences between the mean titers of the pZP and reZP groups on days 0 ($P=0.24$), 35 ($P=0.37$) and 70 ($P=0.42$). Table 2 shows the antibody titers (Day -35 to Day 70) of the jennies that were diagnosed pregnant in the reZP (#10, Day 232; D #23, Day 196) and pZP (#4, Day 196) groups, respectively. It also shows the mean group titers and titers of jennies that were lower (at least on the last sampling day), than jennies that became pregnant during the observation period. There was a statistically significant negative association between titer at Day 35 and time to shutdown. The “hazard” (instantaneous potential for shutdown) was increased by 13% (95% CI: 2-26%) for every 1,000 unit increase in Day 35 titer ($p=0.02$). This effect varied non-linearly, with a threshold of effect identified at a Day 35 titer of ~17,000 (Fig. 6). This figure excludes the one subject in the pZP group with a very low titer that did not shut down, as the effect of that one subject masked the above threshold effect to a large extent. Donkeys categorized as “high responders” on the basis of this threshold (titers>17,000; n=10) showed shut down significantly faster than “low responders” (titers<17,000; n=7), with a median time to shutdown of 59.5 days in high responders and 84 days in low responders (log-rank test p-value = 0.03).

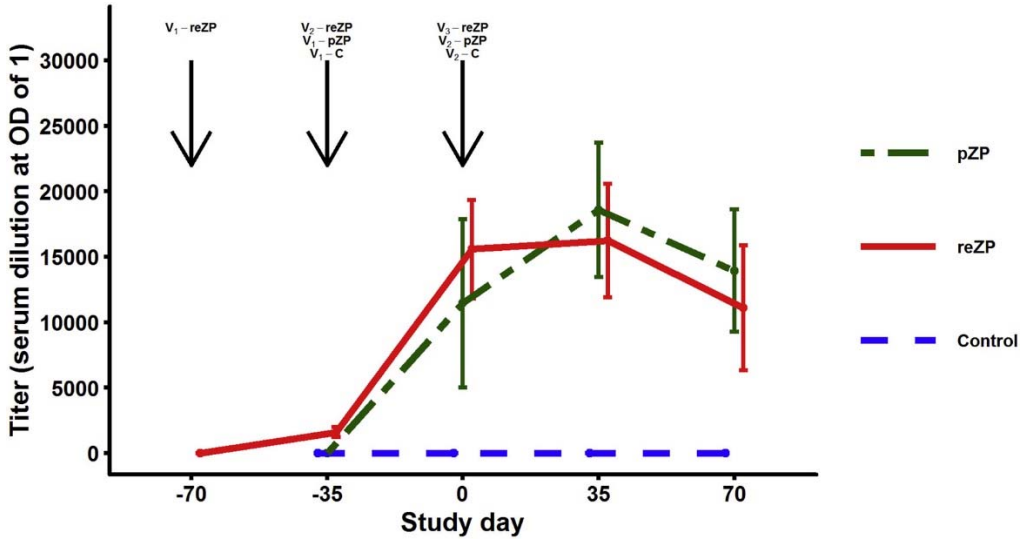

Fig. 5. Mean antibody titers expressed as serum dilution rates of pZP, reZP and control samples which achieved an absorbance of 1 from Day −70 (prior to vaccination) to Day 70 (after 2 or 3 vaccinations). V_1 = primary vaccination; V_2 = 1st booster vaccination; V_3 = 2nd booster vaccination. Vertical bars represent 95% confidence intervals.

Table 2: Antibody titers expressed as serum dilution rates which achieved an absorbance of pregnant and non-pregnant jennies with low Day 70 titers, and the group means during the study.

Group	ID	Titer			
		Day -35	Day 0	Day 35	Day 70
reZP group	#10 pregnant	2600	8461	19549	9978
	#23 pregnant	1300	13763	12988	6537
	#19 Low not pregnant	1539	8935	7559	4503
	#18 Low not pregnant	1363	18744	8268	4593
	reZP group mean	1604	15577	16229	11103
pZP group	#4 pregnant		7065	7081	9912
	#6 Low not pregnant		1556	13435	7016
	#15 Low not pregnant		18939	19582	7016
	pZP group mean		11437	18574	13941

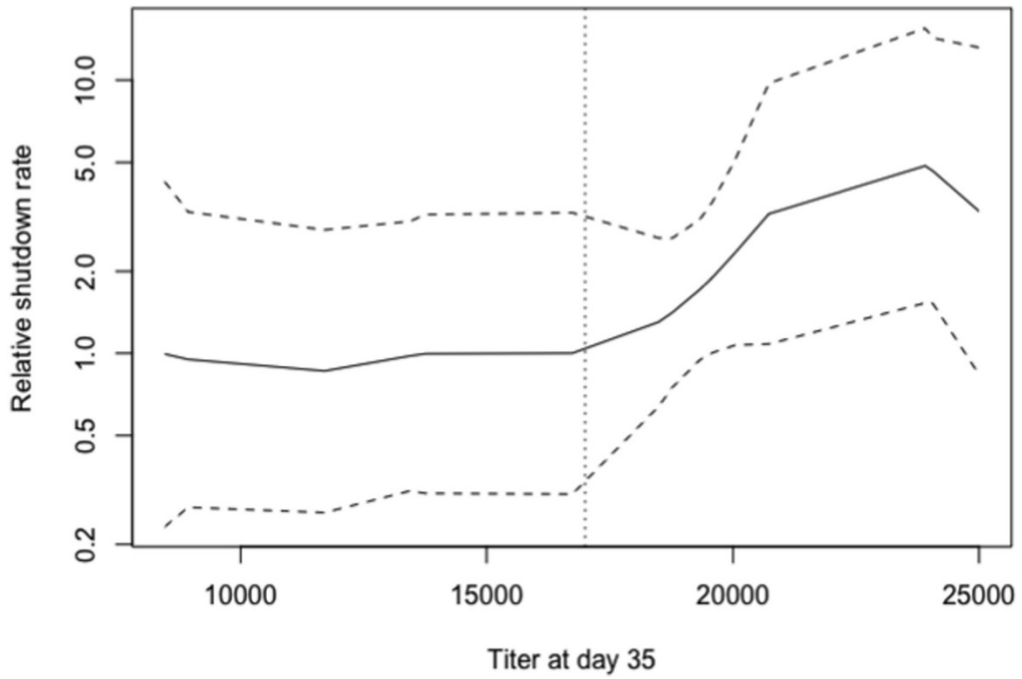

Fig. 6. Effect of antibody titer at Day 35 on time to ovarian shutdown in the pZP and reZP groups combined, excluding one subject with low titers which did not shut down. The solid line shows the shutdown rate relative to the mean antibody titer as the reference. The dashed lines show the 95% confidence intervals. The proposed threshold value of 17,000 is shown by the vertical dotted line.

4. DISCUSSION

4.1 Contraceptive efficacy

The most important finding of the study was the contraceptive efficacy of both ZP vaccines. On the last day of the trial (Day 232), 7/9 and 7/8 jennies in the reZP and pZP groups were not pregnant. Compared to the controls, the first conceptions were delayed by more than 100 days. The median time to pregnancy diagnosis from the final treatment was 77 days for the controls, whereas conceptions in the reZP and pZP groups were recorded on Days 196 and 232, and Day 196, respectively. Given the weekly examination of the jennies, estimated time of conception would have been 2-3 weeks earlier. The contraceptive efficacy was similar to those reported in mares treated using pZP formulated with Freund's adjuvants [14,15,17,29]. In the only other study reporting the immunocontraception of donkeys with pZP in the Virgin Island National Park [21], 16 feral jennies were treated and 11 served as controls. Thirteen jennies were treated by remote delivery of two injections of 65 μg pZP in Freund's complete adjuvant (FCA; primary) and

FIA (booster) 3 weeks apart. Another three jennies were treated once with a combination of a 65 µg pZP formulated in a slow-release pellet and 65 µg pZP in FCA. All jennies received another booster in FCA 10-12 months later. The jennies were monitored for pregnancy using a fecal progestogen and total estrogen assays. Of the 16 treated, one was pregnant 12-24 months after the final booster whereas 6/11 control animals were found to be pregnant during the same period. Other than providing definite proof of efficacy, especially during the 12-24 month period, the two studies are difficult to compare. Our jennies were captive whereas theirs were feral. All our females were non-pregnant whereas 4/27 (3 control and 1 treatment) were already pregnant at the commencement of their study. The authors did not report on post-treatment injection site reactions or changes to the reproductive organs.

The current study is novel in that it describes the first successful application of reZP to control fertility in an equid species. This supports reZP as an alternative to native pZP vaccination with its added potential benefits as a non-animal derived product of enhanced production efficacy, antigen protein purity and microbiological safety.

4.2 Ovarian and uterine effects

The previously-presumed mechanism of pZP contraception was largely ascribed to blocking of sperm receptor sites by antibodies in response to ZP_3 protein. Some reports did suggest a limited disturbance in ovarian function, including ovulation failure [15,17] and decreased total urine estrogen concentrations [16]. These consequences were mainly associated with repeated (annual) administration of pZP. It is possible, however, that studies may have overlooked ovarian shut-down because animals were first monitored following a post-treatment delay of $\geq$3-4 months by which time the presumed ovarian status may have reversed [14,15,17]. Studies in feral mares either did not monitor ovarian function [14,15,29] or did so only indirectly via the measurement of fecal or urinary steroids, and then only for short periods [30].

Recent reports have however associated administration of pZP vaccines in mares with temporary ovarian suppression. Bechert et al. [31] compared two formulations of a single-

injection pZP vaccine in MFCA in mares and reported that 93% of mares were non-cyclic 3-4 months after injection. This was associated with basal progesterone concentrations and significantly smaller ovaries with multiple small follicles and no CLs on trans-rectal US. Similarly, Joonè et al. [24] reported that 86% of mares receiving a pZP vaccination protocol similar to this study experienced a period of prolonged anestrus five to 12 weeks post vaccination. Anestrus was characterized by basal serum estradiol and progesterone concentrations and scanty, small follicles on transrectal US. These mares all resumed ovarian activity and reproductive cyclicity by 10 months post-booster injection. On further investigation, it was found that T-helper and cytotoxic cells were associated with the ovarian changes [32]. The current study reports similar findings of ovarian shut-down, thus confirming the findings in the two horse studies. By day 77, all reZP-treated and 88% of the pZP-treated jennies had ceased cyclic ovarian activity. Two reZP- and one pZP-treated jenny had resumed ovarian activity by Day 165, 210 and 232, respectively. The pZP jenny diagnosed pregnant on Day 196 had cycled throughout the study.

Although the precise mechanism leading to suppression of ovarian function is currently undefined, these observations suggest that this phenomenon may play an important role in ZP immunocontraception in equids and other species [32]. It would seem that ovarian suppression following ZP immunocontraception is not confined to vaccine formulations using FCMA and FIA, having also been observed in varying levels in mares treated with reZP and pZP formulated with Pet Gel A and Poly(I:C) [33].

The reduction in uterine horn diameter is an interesting hitherto unreported observation in pZP-immunocontracepted mares. These changes were associated with reductions in ovarian size, follicular and luteal activity. We suggest that this change was associated with decreased stimulation of the endometrium by ovarian steroids.

4.3 Anti-pZP antibody titers and infertility

Although good anti-pZP antibody titers are achieved in response to pZP immunocontraception in most species, they vary considerably [35,36]. In general, antibody titers within a species are closely related to infertility [14], but between

individuals there is considerable variation. Also, comparison between laboratories is difficult unless universal standards are used. Roelle et al. [37] reported that mean anti-pZP titers of non-pregnant mares treated with a slow release pZP formulation (SpayVac) were 33.7–91.9% higher than those of pregnant mares. However, several mares that developed high maximum titers became pregnant whereas one mare with a maximum titer of only 60.11% failed to conceive. A study by Willis et al. [38] may shed some light on these inconsistencies. Three mares were immunized with pZP (primary and booster after 4.5 weeks). The polyclonal antibodies of each mare were characterized by immunoblots against pZP and equine ZP (eZP). All three mares recognised pZP bands of approximately 90 and 55 kDa. For eZP, one mare reacted to the 75-90 and 40-50 kDa bands while the two others mainly reacted to the 90-106 kDa band, but also the 45-55 kDa band. The differing response may explain why anti-pZP antibody titers are not always accurate in predicting contraceptive efficacy in mares. Speculatively, the same may be true for other species including donkeys. The two pregnant jennies in the reZP group had lower final antibody titers than the group mean. However, there were two other jennies with lower titers on Day 70 that did not fall pregnant within the study period. Similarly, the jenny that was pregnant in the pZP group had a higher titer than two non-pregnant jennies on Day 70. The titers of all three were lower than the group mean on Day 70 (Table 2). From this it would seem that anti-pZP titers can be used as a guideline to predict infertility, however, there is considerable as yet unexplained variation between individuals. On the other hand, antibody titers were found to be significant predictors of ovarian shutdown in mares treated with either pZP or reZP [39]. Similarly, an exploratory analysis in the present study, revealed an apparent distinct threshold of effect at a Day 35 titer of ~17000.

4.4 Injection site reactions

The formulation of reZP and pZP with MFCA for primary and FIA for booster immunizations produced excellent antibody titer responses in this study. The inclusion of Freund's adjuvants in the vaccine formulations was unfortunately also associated with undesirable injection site reactions. The localized swellings and lameness were a common finding in all groups, the tendency increasing after the second treatment. Subjectively assessed, swelling and pain was more pronounced in the reZP group.

Subsequently, sterile abscesses developed in many controls, all reZP and most of the pZP jennies. Similar injection site reactions were reported in mares treated with two single-injection pZP formulations [31] and with pZP and reZP formulated with FCMA and FIA [24]. Similarly, these were more common with reZP than pZP treatment [24]. Speculatively, this difference may be partly attributable to the promiscuous T-cell epitopes for tetanus toxoid and bovine RNase in reZP in combination with the Freund's adjuvants. Given the more severe reactions to V1 and V2 in the reZP-group, FIA was substituted with saline for V3. The minor reactions associated with the incorporation of saline may also explain the smaller rise in mean antibody titer following V3.

5. CONCLUSIONS

Both immunocontraceptive vaccines successfully reduced pregnancy rates in treated jennies. Significantly, this is the first study reporting the successful application of reZP for fertility control in any equid species. Both vaccines induced ovarian shut-down which may be partly responsible for their observed contraceptive effect. In our opinion the injection site reactions associated with inclusion of Freund's adjuvants with both reZP and pZP are an untenable feature of these formulations. Alternative similarly immunogenic adjuvant formulations without the associated severe local reactions are an essential prerequisite for future application. The recently-reported use of the commercially-available adjuvants Pet Gel A and Poly (I:C) with pZP and reZP in mares producing antibody titer responses equivalent to those observed with Freund's adjuvants [33,34] may be a suitable alternative for donkey jennies.

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