Ovarian function and pregnancy outcome in pony mares following immunocontraception with native and recombinant porcine zona pellucida vaccines

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Summary

Reasons for performing study: Few studies have investigated ovarian function in the mare undergoing porcine zona pellucida (pZP) immunocontraception despite reported ovarian dysfunction in other species.
Objectives: This study aimed to describe ovarian function and oestrous cyclicity in pony mares following treatment with either the conventional pZP vaccine or a novel recombinant form of the vaccine derived from porcine ZP3 and ZP4 (reZP). In addition, the contraceptive efficacy of pZP vs. reZP was assessed.
Study design: Blinded, randomised, prospective clinical trial.
Methods: Mares (n = 21) were randomised into 3 groups of 7: *Group I* received the pZP vaccine, with a booster 5 weeks later; *Group II* received the reZP vaccine, with a booster 5 weeks later; and *Group III* (controls) received 2 treatments, 5 weeks apart, of saline and adjuvant alone. Mares underwent weekly monitoring via transrectal palpation and ultrasound examination of the reproductive tract, with daily monitoring during oestrus. Data were collected over a 24 week period coinciding with the physiological breeding season; treatments commenced in Week 4. Serum samples were obtained for antibody titres and ovarian steroid level analyses at 7 day intervals. Cycling mares were bred via fresh semen artificial inseminations over a maximum of 2 consecutive oestrous cycles, commencing 5 weeks post booster vaccination.
Results: Control mares cycled throughout the trial. After treatment, 6 of 7 pZP mares (86%) and one reZP mare (14%) had an extended anoestrus that correlated with basal serum oestradiol and progesterone levels. All mares resumed cyclicity by 10 months post treatment. Pregnancies were diagnosed in all controls, 4 reZP- (57%) and none of the pZP-immunised mares.
Conclusions: The current study demonstrates the reversible suppression of ovarian function in pony mares following treatment with pZP. The effect of the reZP vaccine on pregnancy outcome requires further investigation.

Keywords: horse; ovarian function; anoestrus; progesterone; oestradiol; artificial insemination

Introduction

Investigation of porcine zona pellucida (pZP) as an immunocontraceptive in the mare began over 20 years ago [1]. In contrast to immunocontraceptive vaccines targeting gonadotrophin releasing hormone (GnRH), which causes reproductive quiescence [2], pZP has traditionally been associated with continued oestrous cyclicity [3]. The maintenance of reproductive behaviours has made the pZP vaccine the preferred immunological method of population control in species with complex social structures, such as the feral horse (*Equus caballus*) and the African elephant (*Loxodonta africana*) [4,5].

Research on pZP as a human antifertility vaccine waned following evidence of ovarian dysfunction in nonhuman primates [6]. Similar effects are reported in the rabbit, dog and sheep [7–9]. In contrast, little evidence for interference with ovarian function has been reported in the mare. In a previous study, 1 year of pZP treatment was found to have no effect on ovarian function [1]; however, longer periods of contraception, specifically ≥3 years, were associated with declining oestradiol levels and ovulation rates in feral horses of the USA [10,11]. A subsequent study in the same population reported no association between the incidence of ovulatory failure in mares and their duration of treatment [12]. Recently, investigators demonstrated ovarian inactivity in 13 of 14 mares within 4 months of treatment with single-dose pZP vaccine formulations [13].

A recombinant zona pellucida vaccine may provide potential advantages compared with native pZP vaccines that include production efficiency and the avoidance of contamination with non-ZP proteins [14]. Recently, investigators

recombinant vaccines based on the expression of porcine ZP3 and ZP4 in *Escherichia coli*, hereafter referred to as reZP, were developed [15].

The current study aimed to describe ovarian function and oestrous cyclicity in pony mares following treatment with either native pZP or reZP vaccines. In addition, the contraceptive efficacy of reZP in the mare was investigated.

Materials and methods

Mare management

Twenty-one Nooitgedacht pony mares, aged between 3 and 14 years and of variable parity, were studied from October 2013 to March 2014, coinciding with the physiological breeding season in the southern hemisphere [16]. Inclusion criteria were nonpregnant status, good physical and reproductive health and no previous immunocontraceptive exposure. Ponies were housed in outdoor grass paddocks, with free access to water and *Eragrostis tef* grass hay. Clinical examinations were performed weekly and mares were weighed using an electronic scale during Weeks 1, 8 and 24 (Table 1).

Vaccines

Native pZP vaccine was prepared according to standard methods [1,11][a]. Aliquots of 1 mg purified pZP in phosphate buffered saline (PBS) were

TABLE 1: Mare distribution according to parity, age (median (range)), and body weight (median (range)) for each study group

Mare information	Group I: pZP (n = 7)	Group II: reZP (n = 7)	Group III: controls (n = 7)	P value
Nulliparous	4	3	5	0.6
Foaled within last 3 years	3	2	1	0.5
Foaled >3 years ago	0	2	1	0.3
Age (years)	7 (4, 10)	8 (3, 13)	6 (3, 14)	0.8
Body weight (kg) at Week 1	416 (360, 503)	396 (329, 433)	436 (360, 473)	0.2
Body weight (kg) at Week 8	396 (352, 495)	405 (333, 433)	405 (361, 433)	0.6
Body weight (kg) at Week 24	435 (382, 515)	439 (359, 467)	445 (369, 472)	0.7

pZP, porcine zona pellucida vaccine; reZP, recombinant zona pellucida vaccine.

transferred to glass vials and lyophilised, sealed and stored at 4°C. Before vaccination, each vial was reconstituted with 5 ml sterile injection water with a final protein concentration of 200 μg/ml.

The reZP vaccines, TT-KK-ZP3 and bRNase-KK-ZP4, were supplied by Dr Satish Kumar Gupta (Reproductive Cell Biology Laboratory)[b]. Porcine ZP3 (amino acid (aa) residues 20–344) was expressed as a chimeric fusion protein encompassing a promiscuous T-cell epitope of tetanus toxoid (TT; aa residues 830–844) at its N-terminus and separated from ZP3 by a dilysine linker (TT-KK-ZP3) in *E. coli* [15]. Similarly, porcine ZP4 (aa residues 22–462) was expressed in *E. coli* as a chimeric fusion protein incorporating a promiscuous T-cell epitope of bovine RNase (bRNase; aa residues 94–104; bRNase-KK-ZP4) [15]. Recombinant proteins were purified from inclusion bodies followed by refolding, as described previously [17]. Recombinant TT-KK-ZP3 and bRNase-KK-ZP4 were dialysed separately in 20 mmol/l Tris pH 6.0 and the respective protein concentration estimated using a BCA Protein Estimation Kit[c] and adjusted to 500 μg/ml.

Study design

Mares were stratified by age and randomly assigned to one of 3 treatment groups; the primary investigator was blinded to treatment assignment. Treatments were administered into the gluteal muscles, commencing in Week 4, as follows.

Group I (n = 7) received a primary vaccination (V1) consisting of 100 μg (0.5 ml) pZP emulsified with 0.5 ml Freund's modified complete adjuvant (FMCA). Five weeks later, a booster (V2) consisting of 100 μg pZP emulsified with 0.5 ml Freund's incomplete adjuvant (FIA) was administered into the contralateral hindquarter.

Group II (n = 7) received 2 primary vaccinations (V1), one on each side of the hindquarters, consisting of 250 μg (0.5 ml) recombinant ZP3 and ZP4 proteins, respectively, each emulsified with 0.5 ml FMCA. Five weeks later, 2 boosters (V2) consisting of the same doses of recombinant ZP3 and ZP4 emulsified with 0.5 ml FIA, were similarly administered.

Group III (n = 7, control group) received an initial treatment (V1) consisting of 0.5 ml sterile saline emulsified with 0.5 ml FMCA. Five weeks later, a second treatment (V2) consisting of 0.5 ml sterile saline emulsified with 0.5 ml FIA was administered into the contralateral hindquarter.

Transrectal monitoring of the reproductive tract

Mares underwent examination by transrectal palpation and ultrasonography of the reproductive tract at 7 day intervals. In cycling mares, examinations coincided with Days 7 and 14 of consecutive oestrous cycles, with daily monitoring from Day 14 until ovulation (Day 0). Day 0 was defined by the ultrasonographic detection of a *corpus luteum*, correlated with the absence of a dominant follicle identified on the previous day. Ultrasound examinations were performed using a portable ultrasound machine (A6V)[d] and a 3–8 MHz linear array rectal probe.

Ovarian dimensions were estimated digitally and recorded in 3 perpendicular planes. Ovarian volumes were calculated using the prolate ellipsoid formula (length × height × width × 0.523) [18]. Identifiable structures on each ovary were recorded and follicles ranked according to approximate diameter (<15 , 15–20 and 20–25 mm). Follicles ≥25 mm in diameter were individually measured from the ultrasonographic image of the follicle at its maximum using the electronic calliper function of the ultrasound machine. The average of 2 perpendicular diameter measurements, one of which represented the widest diameter of the follicle, was recorded as the follicle diameter [19]. Anoestrus was defined as bilaterally small ovaries (both ≤25 cm^3), scant follicular development and the absence of any follicles ≥15 mm in diameter [20].

Artificial inseminations

All cycling mares were bred by artificial insemination over a maximum of 2 consecutive oestrous cycles using fresh semen collected from a single stallion of proven fertility, commencing ≥5 weeks post V2. Inseminations were performed according to standard practices once a mare's ovulation was adjudged imminent, i.e. a preovulatory follicle ≥35 mm together with maximal or decreasing endometrial oedema [21]. Semen doses consisted of $\geq 1 \times 10^9$ progressively motile spermatozoa, extended 1:1 in a prewarmed skim milk (MCT) medium[e]. Semen motility was evaluated subjectively under light microscopy. Semen concentration was quantified using a photometer calibrated for use with equine semen (Spermacue)[f]. Inseminations were repeated if a mare failed to ovulate within 72 h. Pregnancy diagnoses by transrectal ultrasound examination were performed 14 days post ovulation. If pregnant, mares were excluded from further breeding and sampling.

Blood samples for hormonal assays and antibody titre determination

Blood samples from all mares were collected by jugular venipuncture at 7 day intervals. In cycling mares, sampling coincided with Days 0, 7 and 14 of the mares' oestrous cycles. Samples were centrifuged and serum stored at -20°C until required.

Serum progesterone and oestradiol assays

Serum progesterone and oestradiol levels were determined by means of radioimmunoassay (Coat-A-Count progesterone and oestradiol kits)[g] [22]. Assay sensitivities for progesterone and oestradiol were 0.06 nmol/l and 29 pmol/l, respectively. For progesterone, intra- and interassay coefficients of variation were 6.1%, 3.5% and 4.7% and 10.3%, 4.3% and 5.2% for low, medium and high concentrations, respectively. For oestradiol, intra- and interassay coefficients of variation were 7.0%, 4.3% and 4.0%, and 8.1%, 6.8% and 4.2% for low, medium and high concentrations, respectively.

Antibody response

Anti-ZP antibody response was measured by enzyme immunoassay (EIA), using a modification of a method previously described [2]. Briefly, 96-well plates (MaxiSorp, cat. no. NUN430341)[h] were incubated at 2–8°C for 16 h with 1 μg purified pZP in 100 μl coating buffer (2.94% NaHCO$_3$, 1.59% Na$_2$CO$_3$, pH 9.6) per well. Plates were washed with PBS containing 0.05% Tween 20 and then blocked with 0.03% BSA in PBS for 16 h at 2–8°C. Plates were then incubated with serial dilutions (1:1000 to 1:8000 for test samples and 1:1000 to 1:64,000 for positive reference serum) of standard and test serum samples at 37°C for 1 h. The positive reference serum consisted of pooled sera from all 7 individuals in *Group I* at expected maximal antibody titre (4 weeks post V2). Blank wells were used as negative controls. After washing, antibodies were detected by incubating plates with recombinant protein G-horseradish peroxidase[i] at 37°C for 1 h. After further washing, plates were developed with trimethylene blue (SureBlue™; cat. no. 52-00-03)[j]. The reaction was stopped by adding 50 μl of 2 mol/l H$_2$SO$_4$ per well. Absorbance at 450 nm was measured using a microplate photometer (Multiskan™ FC)[k].

Antibody response was measured as the mean sample absorbance (minus blank) expressed as a proportion of the mean absorbance (minus blank) of the positive reference sample at the same dilution for each plate (1:2000; 1:4000; 1:8000). The overall proportion positive (PP) was calculated as the average value over the 3 dilutions.

Monitoring injection sites

All mares were monitored daily for visible lesions, including heat and swelling, and weekly by palpation of the approximate injection sites. Transcutaneous ultrasonography of the injection site area was performed when indicated by clinical findings. Monitoring continued following completion of the study as part of the routine care of experimental animals.

Reversibility

All mares underwent examinations at 3 and 6 months following the trial's completion to monitor reproductive activity. Teasing of mares continued after the study period as part of their routine management.

Data analysis

Data were assessed for normality through the plotting of histograms, calculation of descriptive statistics, and the Anderson–Darling test for normality, which was performed in commercially available software (MINITAB Statistical Software, Release 13.32)[l]. Categorical data were compared among treatment groups using Chi-square or Fisher exact tests in available freeware (Epi Info, version 6.04)[m]. The maximum oestradiol values and mean progesterone values before and after V2 were extracted for each mare and used for the statistical comparison among groups. Quantitative data satisfying the normality assumption were subsequently compared among groups using one-way ANOVA. Non-normal data were compared using Kruskal–Wallis tests followed by pairwise Mann–Whitney U tests with correction of P values for multiple *post hoc* tests. A linear mixed model was used to estimate the effect of treatment group and time on antibody responses measured as proportion of the positive control. 'Horse' was included as a random effect to account for the repeated sampling design. Mixed effects models were analysed in commercially available statistical software (IBM SPSS Statistics Version 22)[n]. Bonferroni adjustment was used to adjust for multiple *post hoc* testing and significance was set at P≤0.05.

Results

Transrectal monitoring of the reproductive tract

All mares demonstrated cyclic ovarian activity prior to V2, although one mare in *Group II* showed a period of anoestrus between normal oestrous periods prior to commencing treatment.

In *Group I* (pZP), one mare cycled regularly throughout the study period. Four mares demonstrated anoestrus within 5 weeks of V2 that persisted until the end of the study. One showed anoestrus from 12 weeks post V2 until study completion, while another cycled erratically, characterised by one brief period of oestrus between prolonged periods of anoestrus.

In *Group II* (reZP), one mare entered anoestrus within 5 weeks of V2, persisting until study completion. The remaining 6 mares cycled regularly throughout the study period.

In *Group III* (controls), 6 mares demonstrated regular cyclic activity throughout the study. One developed a persistent *corpus luteum* of unknown cause, which resolved spontaneously.

By Week 16 (7 weeks post V2, prior to any positive pregnancy diagnoses), left and right ovary follicle counts and maximum follicle diameters in *Group I* were significantly lower than in *Group III*. There were no significant differences in *Group II* between either *Group I* or *Group III* for these data points, suggesting an intermediate effect (Table 2).

Serum progesterone and oestradiol profiles

Mean progesterone profiles of *Groups I, II* and *III* mares prior to, and more than 5 weeks following, booster vaccination (V2) are shown in Figures 1 and 2, respectively. The mean progesterone concentrations before and

after V2 for the 3 groups were: 20.4 vs. 6.4 nmol/l (*Group I*), 20.8 vs. 19.0 nmol/l (*Group II*) and 24.8 vs. 25.3 nmol/l (*Group III*). There were no significant differences in average progesterone concentrations between groups prior to V2 (P = 0.6); thereafter the change in average concentrations was significantly different among groups (P = 0.05). *Group I* had the largest average difference in progesterone values but *post hoc* pairwise comparisons did not indicate significant differences compared with *Groups II* and *III* (P = 0.2 and P = 0.07, respectively).

The mean for the maximum oestradiol concentrations measured before and after V2 for the 3 groups were: 42.0 vs. 6.8 pmol/l (*Group I*), 37.1 vs. 19.8 pmol/l (*Group II*) and 51.5 vs. 27.1 pmol/l (*Group III*). There were no significant differences in maximum oestradiol concentrations between groups prior to V2 (P = 0.6); thereafter the change in maximum concentrations in *Group I* was significantly different to that in *Group III* (P = 0.01), with no significant differences, in terms of the change in maximum oestradiol concentrations, between either between either *Groups I* and *II* (P = 0.2) or *Groups II* and *III* (P = 0.8).

Antibody response

Samples from *Group I* and *II* mares prior to the first vaccination and *Group III* mares at all 4 sampling times (pre-V1, four weeks post V1, four weeks post V2 and ten weeks post V2), effectively negative serum controls, showed a mean (± s.d.) optical density (OD) of 0.0841 (± 0.0218). This mean was statistically no different from the mean of all blank wells (P = 0.2; independent *t* test). All samples following immunisation with pZP (*Group I*) or reZP (*Group II*) rendered ODs that were greater than this mean plus 2 standard deviations.

Anti-ZP antibody response, expressed as proportion positive values as described in materials and methods, varied by treatment group (P<0.001) and time (P<0.001), with the time effect also varying by treatment (P<0.001). Proportion positive values for *Group I* were significantly higher than those of *Group II* (P<0.001), and those of *Group II* were significantly higher than those of *Group III* (P = 0.006; Fig 3) post V2.

Pregnancy outcome

In *Group I*, only 4 inseminations were performed owing to the paucity of oestrous cycles available. In *Group II*, one mare showed anoestrus throughout and could not be bred. A total of 11 and 9 inseminations were performed in *Groups II* and *III*, respectively. The proportion of pregnancies achieved in *Groups I, II* and *III* were 0%, 57% and 100%, respectively. Comparison of these proportions for *Groups I* and *III* was significant (P<0.001), with no significant difference detected between *Groups I* and *II*, nor between *Groups II* and *III* (P = 0.07 and 0.2, respectively).

Injection site reactions

No lameness or pyrexias were recorded. Swelling and/or palpable changes in muscular density at injection sites was detected in 20/21 mares post treatment. Overt, sterile abscessation occurred in 3 mares, all from *Group II*. Ultrasonography performed at the end of the study showed lesions affecting ≥one hindquarter in 17 of the 18 remaining mares. Lesions varied from mild changes in muscular architecture to poorly marginated areas of complex echogenic pattern ≤8 cm in width. A follow-up ultrasonographic examination 3 months later showed a distinct improvement in the appearance of lesions in 11 of these mares.

Reversibility

All mares that had demonstrated anoestrus following treatment had resumed oestrous cyclicity by 10 months post V2, based on follow-up oestrous monitoring or teasing records.

Discussion

The traditionally accepted mechanism of action of pZP in the equid involves, primarily, the interference of anti-ZP antibodies in sperm–zona binding, leading to contraception with continued oestrous cyclicity [1,23]. The current study, however, demonstrated suppression of ovarian function in 6 of 7 pony mares following pZP treatment, characterised by small,

TABLE 2: Results of transrectal monitoring of the reproductive tract for 21 pony mares prior to and following treatment with either pZP (*Group I*), reZP (*Group II*) or saline (*Group III*)

			Group I (n = 7)	*Group II* (n = 7)	*Group III* (n = 7)	P values[‡]
Pretreatment*	Week 1[†]	Left ovary volume (cm^3)	150.6 (52.3, 401.7)	78.5 (4.2, 153.8)	83.7 (28.2, 627.6)	0.5
		Right ovary volume (cm^3)	28.2 (6.3, 83.7)	6.3 (1.6, 94.1)	100.4 (9.4, 585.8)	0.1
		Left ovary follicle count	3 (0, 6)	3 (0, 6)	4 (2, 8)	0.4
		Right ovary follicle count	3 (1, 6)	3 (0, 3)	5 (1, 7)	0.3
		Maximum follicle diameter (mm)	30.3 (15.0, 56.1)	46.5 (0.0, 48.9)	45.1 (22.4, 55.8)	0.2
	Week 2[†]	Left ovary volume (cm^3)	58.6 (18.8, 150.6)	18.8 (4.2, 205.0)	50.2 (14.1, 131.8)	0.7
		Right ovary volume (cm^3)	28.2 (18.8, 78.5)	15.7 (4.2, 52.3)	18.8 (6.3, 418.4)	0.6
		Left ovary follicle count	4 (3, 7)	2 (2, 4)	3 (1, 9)	0.3
		Right ovary follicle count	4 (2, 6)	3 (2, 4)	3 (1, 6)	0.4
		Maximum follicle diameter (mm)	33.2 (20.0, 56.4)	28.1 (15.0, 48.9)	31.8 (12.0, 66.9)	0.8
	Week 3[†]	Left ovary volume (cm^3)	62.8 (9.4, 267.8)	18.8 (2.1, 205.1)	33.5 (6.3, 267.8)	0.6
		Right ovary volume (cm^3)	18.8 (6.3, 78.5)	15.7 (6.3, 78.5)	25.1 (18.8, 179.4)	0.3
		Left ovary follicle count	4 (0, 9)	2 (0, 4)	4 (1, 13)	0.9
		Right ovary follicle count	3 (1, 8)	3 (0, 6)	7 (3, 10)	0.09
		Maximum follicle diameter (mm)	42.1 (15.0, 49.2)	36.1 (15.0, 52.5)	34.5 (21.2, 49.1)	0.7
Post treatment*	Week 14[†]	Left ovary volume (cm^3)	23.5 (6.3, 78.5)	78.5 (18.8, 179.4)	78.5 (6.3, 94.1)	0.3
		Right ovary volume (cm^3)	12.6 (6.3, 78.5)	28.2 (18.8, 94.1)	18.8 (6.3, 50.3)	0.5
		Left ovary follicle count	1 (0, 6)	1 (0, 4)	3 (1, 6)	0.5
		Right ovary follicle count	1^a (0, 5)	2^{a,b} (0, 6)	6^b (2, 9)	0.008
		Maximum follicle diameter (mm)	15.0 (0.0, 36.5)	18.0 (0.0, 47.6)	30.2 (15.0, 46.5)	0.8
	Week 15[†]	Left ovary volume (cm^3)	18.8 (4.2, 131.8)	23.5 (6.3, 150.6)	58.6 (8.4, 153.8)	0.5
		Right ovary volume (cm^3)	9.4 (6.3, 28.2)	18.8 (6.3, 205.0)	25.1 (9.4, 153.8)	0.1
		Left ovary follicle count	1^a (0, 2)	3^b (0, 8)	2^{a,b} (0, 4)	0.02
		Right ovary follicle count	1^a (0, 4)	2^{a,b} (0, 4)	5^b (2, 7)	0.006
		Maximum follicle diameter (mm)	10.0 (0.0, 48.3)	15.0 (10.0, 49.2)	25.0 (15.0, 42.3)	0.5
	Week 16[†]	Left ovary volume (cm^3)	23.5 (6.3, 50.2)	41.8 (18.8, 205.0)	131.8 (2.1, 205.0)	0.2
		Right ovary volume (cm^3)	12.6 (6.3, 31.4)	28.2 (6.3, 205.0)	25.1 (18.8, 153.8)	0.1
		Left ovary follicle count	0^a (0, 3)	2^{a,b} (0, 4)	3^b (0, 7)	0.04
		Right ovary follicle count	0^a (0, 2)	2^{a,b} (0, 5)	5^b (3, 8)	0.001
		Maximum follicle diameter (mm)	0.0^a (0.0, 22.0)	20.0^{a,b} (0.0, 53.1)	40.3^b (16.0, 53.4)	0.006

*All data are reported as median (range). [†]If more than one data point for a mare existed during a particular week, the data point exhibiting the greatest follicle diameter was included. [‡]For data points with superscripts, medians without superscripts in common are statistically different at P≤0.05 after adjustment for multiple *post hoc* testing.
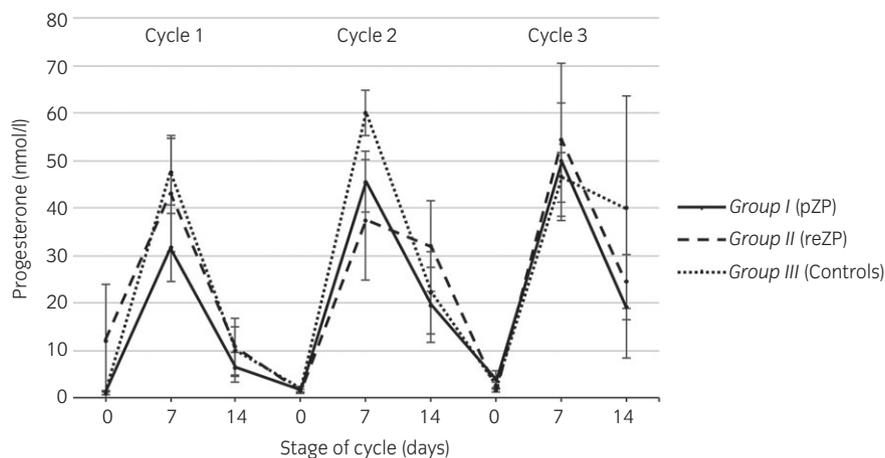

Fig 1: Graph showing mean weekly serum progesterone levels (s.e. bars) for each study group over 3 consecutive oestrous cycles prior to V2, where Days 0, 7 and 14 of each cycle have been synchronised in time.

inactive ovaries and basal ovarian hormone levels. The discrepancy between our findings and that of an earlier report of unaltered oestrous cyclicity during short-term treatment of mares with conventional pZP vaccine [1] may be due to the higher pZP dose administered in our study (100 μg vs. 65 μg pZP), selected to reflect the current dose administered to feral horses [24–26]. Our findings confirm recently reported ovarian quiescence in mares treated with long-acting pZP vaccines [13], suggesting that suppressed ovarian function is not unique to long-acting formulations.

Previous studies on the effects of pZP on behaviour and social structure in feral horse populations, at the same dose of pZP, suggested that treated
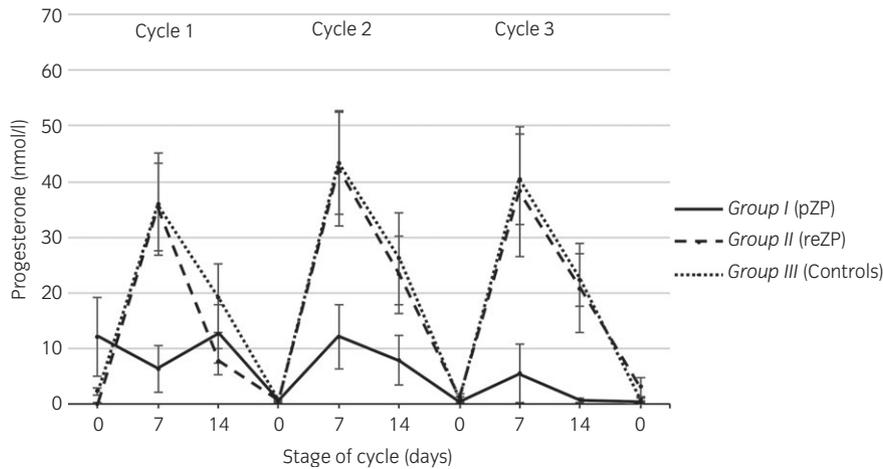
Fig 2: Graph showing mean weekly serum progesterone levels (s.e. bars) for cycling mares in each study group over 3 consecutive oestrous cycles ≥5 weeks post V2, where Days 0, 7 and 14 of each cycle have been synchronised in time. Weekly data are depicted for noncycling mares.
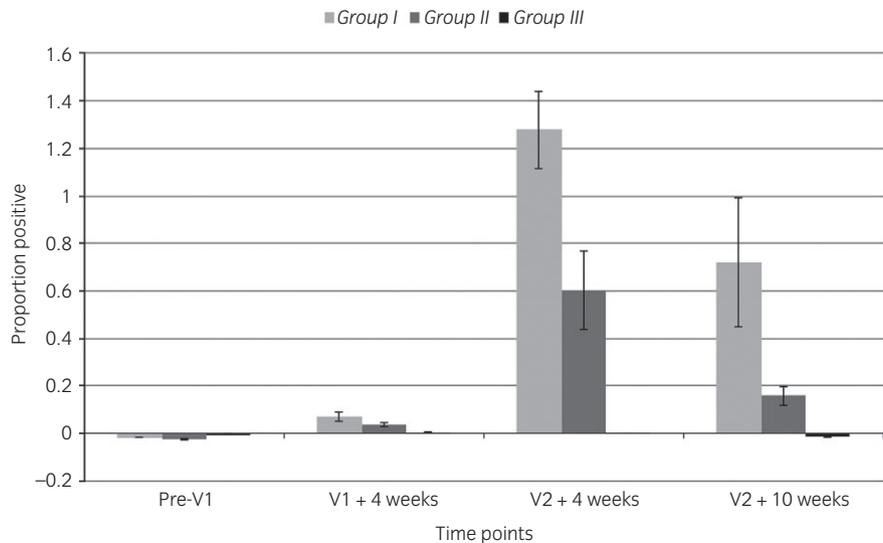

Fig 3: Mean anti-ZP antibody response expressed as a proportion of the positive control (with s.e.) for each treatment group at 4 successive time points.

mares show decreased harem fidelity and increased reproductive behaviours [27,28]. These findings are inconsistent with the current study, in which the majority of mares showed anoestrus following treatment, implying that there would be an opposite change (albeit transiently) in reproductive behaviours. The absence of these behaviours can be attributed to the significant decrease in follicular number and sizes, associated with decreased oestradiol concentrations.

Follicle counts and maximum follicle diameters for Group II showed no statistically significant differences from either Group I or Group III in Week 16, despite significant differences between the latter 2 groups. This partial effect parallels Group II's intermediate antibody titres post V2. The pZP vaccine comprises all 3 native porcine zona glycoproteins (ZP2, ZP3 and ZP4), whereas reZP comprises only ZP3 and ZP4. The lower antibody titres post V2 in Group II compared with Group I may be due to the fact that the pZP vaccine will elicit an antibody response against ZP2 as well as ZP3 and ZP4, and the EIA read-outs using pZP antigen will reflect the summation of antibody titres against ZP2, ZP3 and ZP4. Ideally, antibody titres against purified ZP3 and ZP4 should be assessed to determine whether antibody titres are responsible for the intermediate ovarian response observed in Group II.

Further studies, involving either the administration of higher doses of the recombinant vaccine or using native pZP as the primary injection followed by reZP booster injections, are warranted. A third possibility would be to increase the number of booster vaccinations. Recently, it was shown that 2 boosters of recombinant dog ZP3, instead of one, showed better contraceptive efficacy in female mice [31].

An unexpected finding was the prevalence of injection site reactions, supporting Bechert et al. [13] who reported injection site reactions in 43% of treated mares. Our findings failed to support anecdotal reports of injection site reactions occurring less frequently when administered into the gluteal rather than the neck musculature [32,33]. The current findings, including the sterility of overt abscesses, also contradict previous reports linking abscessation to remote vaccine delivery, presumed to result from darts transferring dirt and bacteria into the subcutaneous tissues [34]. Reports of injection site reactions in feral populations, described as abscessation, varied from 0 to 11.5% and were associated with either Freund's complete adjuvant (FCA), (FMCA) or (FIA) [1,33,35–37]. Our use of domestic mares enabled closer inspection than can be achieved in a feral horse population. Although individuals from all groups showed lesions, Group II was particularly over-represented. This may be a result of either or both the double volume of FMCA and FIA in their vaccination protocol or the tetanus toxoid and bovine RNase linked to the ZP3 and ZP4 recombinant proteins, respectively.

The contraceptive efficacy of the pZP vaccine was confirmed in this study; however, the absence of oestrous cyclicity appears to be responsible for infertility to a larger extent than interference with sperm–zona binding. In addition to species differences in response, contamination with non-zona pellucida ovarian proteins has been proposed as a possible cause of ovarian malfunction in other species [29]. The latter cannot be completely ruled out for the pZP vaccine, although such contamination is impossible with the use of recombinant vaccines. Apart from oophoritis, a possible mechanism of ovarian suppression could be an interference with cellular communications between the developing oocyte and its surrounding granulosa cells, as a result of immune-mediated alterations to the zona pellucida. A family of proteins known as connexins is involved in oocyte–granulosa cell communication. Connexin gene-knockout mice were found to demonstrate suppressed ovarian activity with a lack of tertiary follicular development, reminiscent of the findings of the current study in mares [30].

All mares exhibiting anoestrus following treatment showed evidence of cyclic activity within 10 months of V2, which confirms the reported reversibility of pZP vaccines [38]. In the current study, follow-up examinations coincided partially with winter, thus the effect of seasonal anoestrus in biasing resumption of cyclicity remains undefined.

Conclusion

The current study demonstrates the reversible suppression of ovarian function in 6 of 7 (86%) pony mares following treatment with the native pZP vaccine. No significant contraceptive effect was produced by the (reZP) vaccine; however, further investigation of recombinant ZP vaccines, as an alternative contraceptive in the mare, is warranted.

Authors' declaration of interests

No competing interests to declare.

Ethical animal research

The study was approved by the University of Pretoria's Animal Ethics Committee (V051-13).

Authorship

C.J. Joonè, H.J. Bertschinger and M.L. Schulman contributed to the study design, study execution, data analysis and interpretation, preparation and final approval of the manuscript. S.K. Gupta contributed to study design and preparation of the manuscript. A.P. Arukha and V. Minhas prepared the recombinant vaccines and were involved in final approval of the manuscript. E. Dieterman contributed to the acquisition of data. G.T. Fosgate is an epidemiologist who contributed to study design, data analysis and interpretation, and preparation of the manuscript.

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gSiemens Healthcare Diagnostics, Los Angeles, California, USA.
hThermo Fisher Scientific, Roskilde, Denmark.
iLTC Tech South Africa, Johannesburg, South Africa.
jKirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland, USA.
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lMinitab Inc., State College, Pennsylvania, USA.
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