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

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No competing interests to declare.

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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.

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 versus reZP was assessed.

• ***Study Design:*** Blinded, randomised, prospective clinical trial.

• ***Methods:*** Mares (n=21) were randomised into three groups of seven: Group I received the pZP vaccine, with a booster five weeks later; Group II received the reZP vaccine, with a booster five weeks later; and Group III (controls) received two treatments, five weeks apart, of saline and adjuvant alone. Mares underwent weekly monitoring via trans-rectal 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 four. Serum samples were obtained for antibody titres and ovarian steroid level analyses at seven day intervals. Cycling mares were bred via fresh semen artificial inseminations, over a maximum of two consecutive oestrous cycles, commencing five weeks post booster vaccination.

• ***Results:*** Control mares cycled throughout the trial. Post treatment, six of seven pZP mares (86%) and one reZP mare (14%) had extended anoestrus that correlated with basal serum oestradiol and progesterone levels. All mares resumed cyclicity by ten months post treatment. Pregnancies were diagnosed in all controls, four reZP- (57%) and none of the pZP- immunized 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.

Introduction

Investigation of porcine zona pellucida (pZP) as an immunocontraceptive in the mare began over twenty years ago [1]. In contrast to immunocontraceptive vaccines targeting gonadotrophin releasing hormone (GnRH), which cause 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 African elephant (*Loxodonta africana*) [4; 5]. Research on pZP as a human antifertility vaccine waned following evidence of ovarian dysfunction in non-human 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, one year of pZP treatment was found to have no effect on ovarian function [1], however longer periods of contraception, specifically ≥ three 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 four months of treatment with single-dose pZP vaccine formulations [13].

A recombinant zona pellucida vaccine may provide potential advantages when compared with native pZP vaccines that include production efficiency and the avoidance of contamination with non-ZP proteins [14]. Recently, recombinant vaccines based on the expression of porcine ZP3 and ZP4 in *Eschirichia 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

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

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 non-pregnant 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 25 (Table 1).

Vaccines

Native pZP vaccine was prepared according to standard methods [1; 11] and supplied by Trumpeter Farms and Veterinary Service[a]. Aliquots of 1 mg purified pZP in phosphate buffered saline (PBS) were 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 dialyzed separately in 20 mM 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 three treatment groups; the primary investigator was blinded to treatment assignment. Treatments were administered into the gluteal muscles, commencing in week four, 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 two 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, two 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.

Trans-rectal monitoring of the reproductive tract

Mares underwent examination by trans-rectal palpation and ultrasonography of the reproductive tract at seven 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* (CL), correlated to 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 three perpendicular planes. Ovarian volumes were calculated using the prolate ellipsoid formula (length x height x width x 0.523) [18]. Identifiable structures on each ovary were recorded and follicles ranked according to approximate diameter (< 15mm, 15 to 20mm, and 20 to 25mm). Follicles $\geq$ 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 two 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 $\leq$25 cm^3), scant follicular development and the absence of any follicles $\geq$15 mm in diameter [20].

Artificial inseminations

All cycling mares were bred by artificial insemination (AI) over a maximum of two consecutive oestrous cycles using fresh semen collected from a single stallion of proven fertility, commencing $\geq$ five weeks post V2. Inseminations were performed according to standard practices once a mare's ovulation was adjudged imminent, i.e. a pre-ovulatory follicle $\geq$ 35 mm together with maximal or decreasing endometrial oedema [21]. Semen doses consisted of $\geq$ 1 x 10^9 progressively motile spermatozoa, extended 1:1 in a pre-warmed 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[f]. Inseminations were repeated if a mare failed to ovulate within 72 h. Pregnancy diagnoses by trans-rectal 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 seven 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)[g] [22]. Assay sensitivities for progesterone and oestradiol were 0.06 nmol/L and 29 pmol/L respectively. For progesterone, intra- and inter-assay 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 inter-assay 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)[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_2CO_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 1:64000 for positive reference serum) of standard and test serum samples at 37°C for one hour. The positive reference serum consisted of pooled sera from all seven individuals in Group I at expected maximal antibody titre (four 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 one hour. After further washing, plates were developed with trimethylene blue (SureBlue™)[k]. The reaction was stopped by adding 50 µl 2M H_2SO_4 per well. Absorbance at 450 nm was measured using a microplate photometer (Multiskan™ FC)[m].
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 three 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 three and six months following the trial's completion to monitor reproductive activity. Teasing of mares continued after the study period as part of their routine management.

Statistical 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[n]. Categorical data were compared among treatment groups using chi-square or Fisher exact tests in available freeware[o]. The maximum oestradiol values and mean progesterone values pre and post 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[p]. Bonferroni adjustment was used to adjust for multiple post hoc testing and significance was set as $P < 0.05$.

Results

Trans-rectal 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 five 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 five weeks of V2, persisting until study completion. The remaining six mares cycled regularly throughout the study period.

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

By week 16 (seven 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 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 five weeks following booster vaccination (V2) are shown in Figs. 1 and 2, respectively. The mean progesterone concentrations pre- and post V2 for the three groups were: 20.4 versus 6.4 nmol/L (Group I), 20.8 versus 19.0 nmol/L (Group II) and 24.8 versus 25.3 nmol/L (Group III). There were no significant differences in average progesterone concentrations between groups prior to V2 ($P = 0.616$), thereafter the change in average concentrations were significantly different among groups ($P = 0.048$). Group I had the largest average difference in progesterone values but post hoc pairwise comparisons did not indicate significant differences with Groups II and III ($P = 0.149$ and $P = 0.068$, respectively).

The mean for the maximum oestradiol concentrations measured pre- and post V2 for the three groups were: 42.0 versus 6.8 pmol/L (Group I), 37.1 versus 19.8 pmol/L (Group II) and 51.5 versus 27.1 pmol/L (Group III). There were no significant differences in maximum oestradiol concentrations between groups prior to V2 ($P = 0.566$), thereafter the change in maximum concentrations in Group I was significantly lower than Group III ($P = 0.014$), but not between either Groups I and II ($P = 0.159$) or Groups II and III ($P = 0.794$).

Antibody response

Samples from Group I and II mares prior to the first vaccination and Group III mares at all 4 sampling times (pre-treatment, post primary and post booster treatments with FCMA and FIA, respectively, and end of season), effectively negative serum controls, showed a mean OD of 0.0841 (± SD 0.0218). This mean was statistically no different from the mean of all blank wells ($P = 0.209$; independent *t*-test). All samples following immunisation with pZP (Group I) or reZP (Group II) rendered ODs that were greater than this mean plus two standard deviations.

Anti-ZP antibody response varied by treatment group ($P < 0.001$) and time ($P < 0.001$) with the time effect also varying by treatment ($P<0.001$). Group I was significantly higher than Group II ($P < 0.001$), with Group II significantly higher than Group III ($P = 0.006$; Fig. 3).

Pregnancy outcome

In Group I, only four inseminations were performed due 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 nine 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 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 were detected in 20/21 mares post treatment. Overt, sterile abscessation occurred in three mares, all from Group II. Ultrasonography performed at the end of the study showed lesions affecting $\geq$ 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 $\leq$ 8 cm in width. A follow-up ultrasonographic examination three months later showed distinct improvement in the appearance of lesions in 11 of these mares.

287

288 **Reversibility**

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

291

292 **Discussion**

293 The traditionally-accepted mechanism of action of pZP in the equine involves, primarily, the
294 interference of anti-ZP antibodies in sperm-zona binding, leading to contraception with continued
295 oestrous cyclicity [1; 23]. The current study, however, demonstrated suppression of ovarian function
296 in six of seven pony mares following pZP treatment, characterised by small, inactive ovaries and
297 basal ovarian hormone levels. The discrepancy between our findings and that of an earlier report of
298 unaltered oestrous cyclicity during short-term treatment of mares with conventional pZP vaccine [1]
299 may be due to the higher pZP dose administered in our study (100 µg versus 65 µg pZP), selected to
300 reflect the current dose administered to feral horses [24-26]. Our findings confirm recently-reported
301 ovarian quiescence in mares treated with long-acting pZP vaccines [13], suggesting that suppressed
302 ovarian function is not unique to long-acting formulations.
303 Previous studies on the effects of pZP on behaviour and social structure in feral horse populations, at
304 the same dose of pZP, suggested that treated mares show decreased harem fidelity and increased
305 reproductive behaviours [27; 28]. These findings are inconsistent with the current study, in which the
306 majority of mares showed anoestrus following treatment, implying that there would be an opposite
307 change (albeit transiently) in reproductive behaviours. The absence of these behaviours can be
308 attributed to both the significant decrease in follicular number and sizes and oestradiol concentrations.
309 Follicle counts and maximum follicle diameters for Group II showed no statistically significant
310 differences to either Groups I or III in week 16, despite significant differences between the latter two
311 groups. This partial effect parallels Group II's intermediate antibody titres post V2. The pZP vaccine
312 comprises all three native porcine zona glycoproteins (ZP2, ZP3 and ZP4), whereas reZP comprises
313 only ZP3 and ZP4. The lower antibody titres post V2 in Group II as compared to Group I may be due
314 to the fact that the pZP vaccine will elicit an antibody response against ZP2 as well as ZP3 and ZP4,
315 and the ELISA read-outs using pZP antigen will reflect the summation of antibody titres against ZP2,

316 ZP3 and ZP4. Ideally, antibody titres against purified ZP3 and ZP4 should be assessed to determine
317 whether antibody titres are responsible for the intermediate ovarian response observed in Group II.
318 Further studies, involving either the administration of higher doses of the recombinant vaccine or
319 using native pZP as the primary injection followed by reZP booster injections, are warranted. A third
320 possibility would be to increase the number of booster vaccinations. Recently, it was shown that two
321 boosters of recombinant dog ZP3, instead of one, showed better contraceptive efficacy in female mice
322 [31].
323 An unexpected finding was the prevalence of injection site reactions, supporting Bechert *et al.* [13]
324 who reported injection site reactions in 43% of treated mares. Our findings failed to support anecdotal
325 reports of injection site reactions occurring less frequently when administered into the gluteal rather
326 than the neck musculature [32; 33]. The current findings, including the sterility of overt abscesses,
327 also contradict previous reports linking abscessation to remote vaccine delivery, presumed to result
328 from darts transferring dirt and bacteria into the subcutaneous tissues [34]. Reports of injection site
329 reactions in feral populations, described as abscessation, varied from 0-11.5% and were associated
330 with either Freund's Complete Adjuvant (FCA), FMCA or FIA [1; 33; 35-37]. Our use of domestic
331 mares enabled closer inspection than can be achieved in a feral horse population. Although
332 individuals from all groups showed lesions, Group II was particularly over-represented. This may be a
333 result of either or both the double volume of FCMA and FIA in their vaccination protocol or the tetanus
334 toxoid and bovine RNase linked to the ZP3 and ZP4 recombinant proteins, respectively.
335 The contraceptive efficacy of the pZP vaccine was confirmed in this study, however the absence of
336 oestrous cyclicity appears to be responsible for infertility to a larger extent than interference with
337 sperm-zona binding. In addition to species differences in response, contamination with non-zona
338 pellucida ovarian proteins has been proposed as a possible cause of ovarian malfunction in other
339 species [29]. The latter cannot be completely ruled out for the pZP vaccine, although such
340 contamination is impossible with the use of recombinant vaccines. Apart from oophoritis, a possible
341 mechanism of ovarian suppression could be an interference with cellular communications between
342 the developing oocyte and its surrounding granulosa cells, as a result of immune-mediated alterations
343 to the zona pellucida. A family of proteins known as connexins is involved in oocyte-granulosa cell
344 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 ten months of V2 and confirms the reported reversibility of pZP vaccines [38]. In the current study, follow-up examinations coincided partially with winter, thus the effect of seasonal anoestrous in biasing resumption of cyclicity remains undefined.

Conclusion

The current study demonstrates the reversible suppression of ovarian function in six of seven (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.

Manufacturers' addresses

[a]Winters, California, USA

[b]National Institute of Immunology, New Delhi, India

[c]Pierce, Rockford, Illinois, USA

[d]Sonoscape, Shenzhen, China

[e]Section of Reproduction, University of Pretoria, Onderstepoort, South Africa

[f]Spermacue; Minitube International, Tiefenbach, Germany

[g]Siemens Healthcare Diagnostics, Los Angeles, California, USA

[h]Thermo Fisher Scientific, Roskilde, Denmark (Cat: NUN430341)

[i]LTC Tech South Africa, Johannesburg, South Africa

[k]Kirkegaard & Perry Lab Inc, Gaithersburg, Maryland, USA (Cat: 52-00-03)

[m]Thermo Fisher Scientific, Waltham, Massachusetts, USA

[n]MINITAB Statistical Software, Release 13.32, Minitab Inc, State College, Pennsylvania, USA

[o]Epi Info, version 6.04, C DC, Atlanta, Georgia, USA

[p]IBM SPSS Statistics Version 22, International Business Machines Corp., Armonk, NY, USA

Figure legends

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

Fig 2. Graph showing mean weekly serum progesterone levels (SE bars) for cycling mares in each study group over three consecutive oestrous cycles $\geq$ 5 weeks post V2, where days 0, 7 and 14 of each cycle have been synchronised in time. Weekly data is depicted for non-cycling mares.

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

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525 **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

526 pZP = porcine zona pellucida vaccine, reZP = recombinant zona pellucida vaccine

527

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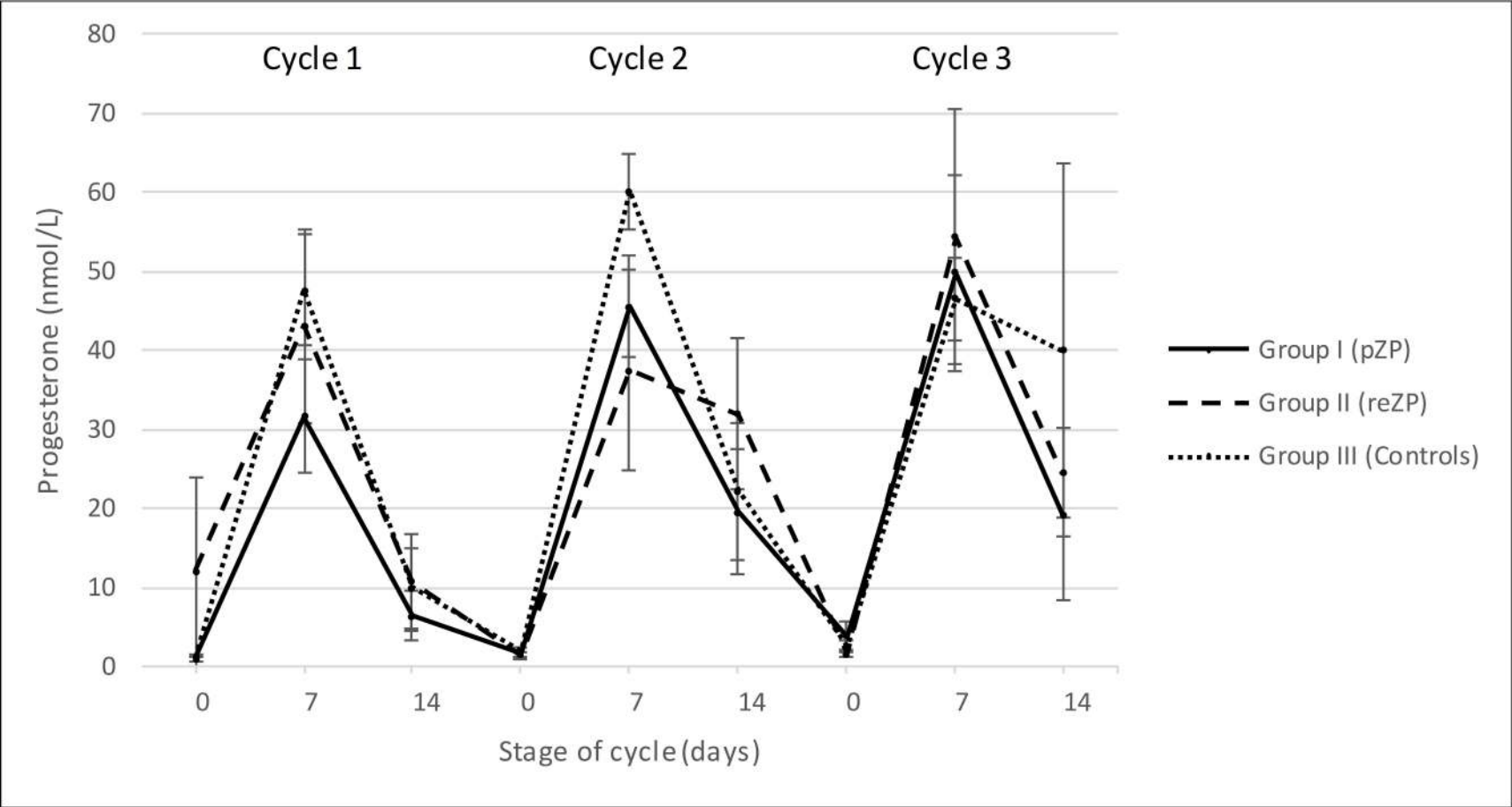
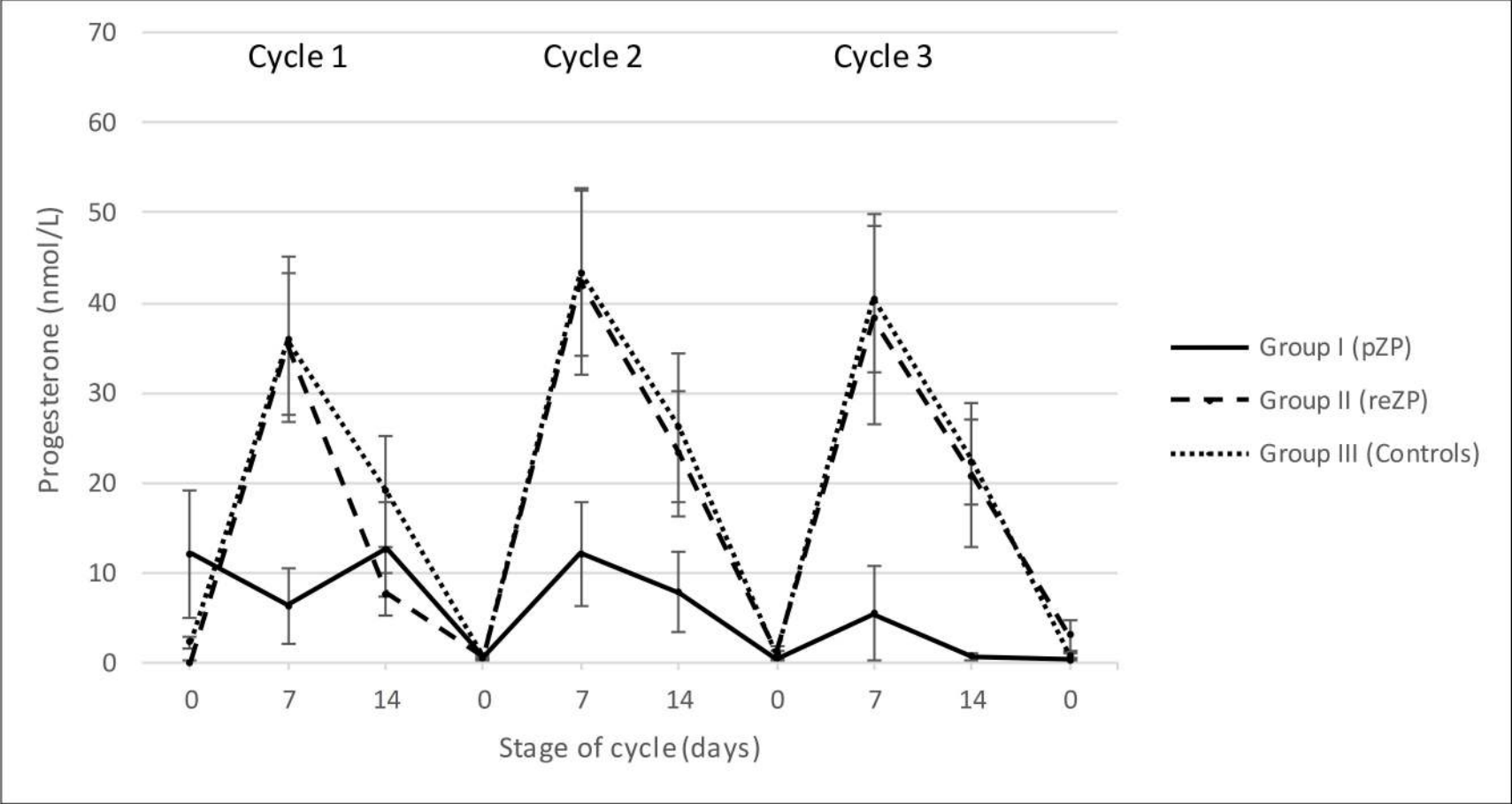
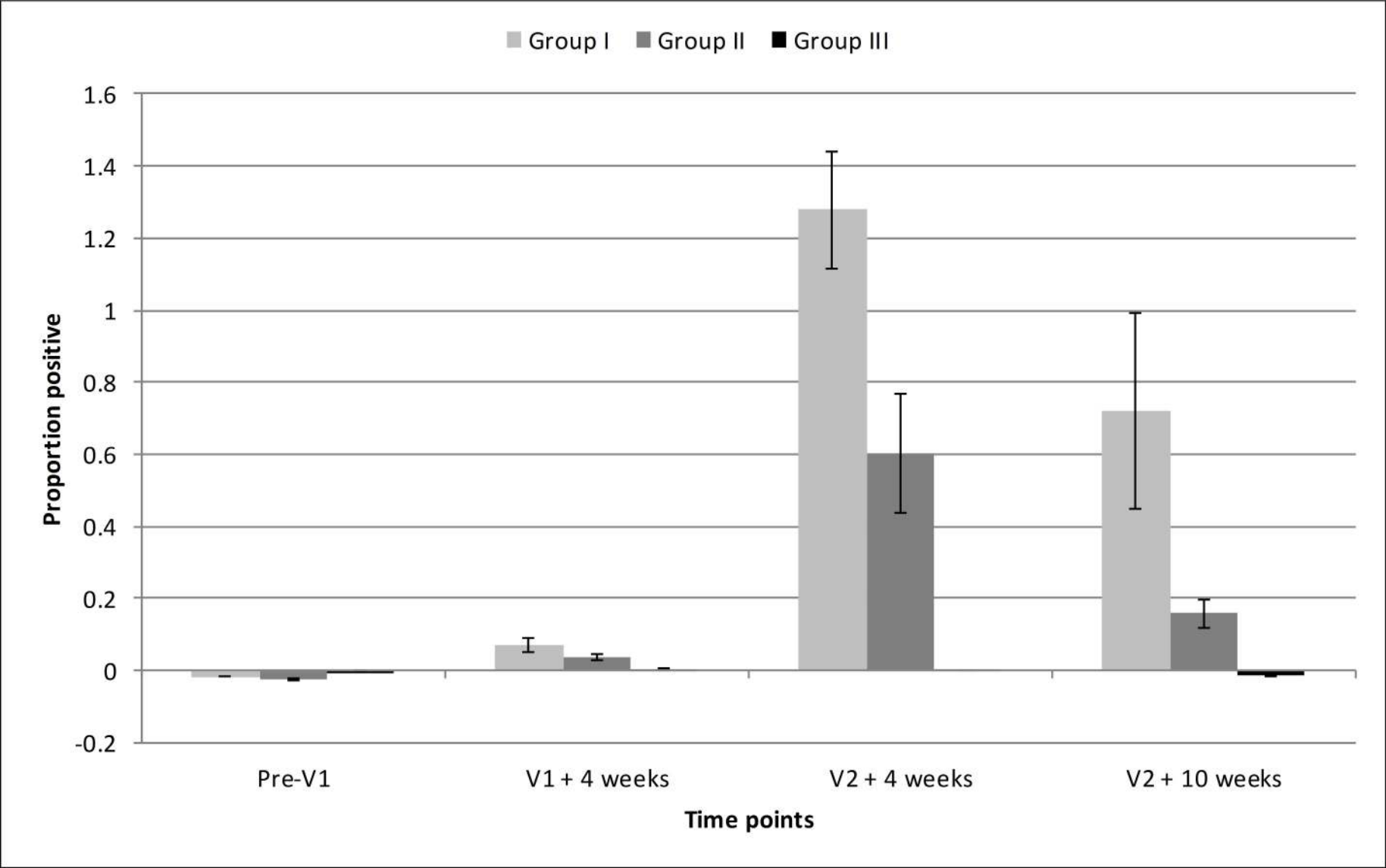
Table 2. Results of trans-rectal monitoring of the reproductive tract for 21 pony mares prior to and following treatment with either pZP (Group I), reZP (Group II) or saline (Group III)

			Group I (n = 7)	Group II (n = 7)	Group III (n = 7)	P values[c]
Pre-treatment[a]	Week 1[b]	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[b]	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[b]	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[a]	Week 14[b]	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[b]	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[b]	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

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


Cycle 1
Cycle 2
Cycle 3
80
70
60
50
40
30
20
10
0
Progesterone (nmol/L)
0
7
14
0
7
14
0
7
14
Stage of cycle (days)
Group I (pZP)
Group II (reZP)
Group III (Controls)


Cycle 1
Cycle 2
Cycle 3
70
60
50
40
30
20
10
0
Progesterone (nmol/L)
0
7
14
0
7
14
0
7
14
0
Stage of cycle (days)
Group I (pZP)
Group II (reZP)
Group III (Controls)


Group I
Group II
Group III
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
Proportion positive
Pre-V1
V1 + 4 weeks
V2 + 4 weeks
V2 + 10 weeks
Time points