Revised

Title. **Ovarian function following immunocontraceptive vaccination of mares using native porcine and recombinant zona pellucida vaccines formulated with a non-Freund's adjuvant and anti-GnRH vaccines**

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Abstract

An important determinant in the selection of any contraceptive agent is the impact on ovarian function, both in the short and longer term. In this study, ovarian activity was monitored in mares immunised with one of the following vaccine formulations; native porcine zona pellucida (pZP), recombinant zona pellucida proteins ZP3 and ZP4 (reZP), pZP and reZP

combined or a commercially available anti-GnRH vaccine. The ZP antigens were prepared in an adjuvant formulation consisting of 6% polymeric adjuvant (Montanide™ PetGel A, Seppic, France) and 500 µg polyinosinic-polycytidylic acid - TLR3-agonist (Poly(I:C) HMW VacciGrade™, Invivogen, USA). A vehicle-only control group was administered the adjuvant formulation without antigen. Ovarian activity was monitored using clinical observations (transrectal palpation and ultrasonography of the reproductive tract) in addition to blood sampling for serum progesterone and anti-Müllerian hormone (AMH) concentrations while employing a low sampling frequency. Treatments and measurements were initiated in December (southern hemisphere summer) and subsequent data collection was performed in January, February, March and May. Both reZP and anti-GnRH vaccination were associated with clinically evident ovarian suppression in the short term. Ovarian activity in mares administered a reZP or anti-GnRH vaccine was significantly different to adjuvant control and pZP treated mares. Serum AMH concentrations were different between pZP and anti-GnRH treated mares 3.5 months after the final vaccination. Serum AMH concentrations were significantly correlated with mare age, serum progesterone and ovarian volume.

Keywords

horse, immunocontraception, ovary, anestrus, anti-Müllerian hormone

1. Introduction

A number of antigens have been proposed as targets for fertility control via vaccination. These include peptide hormones, oocyte and sperm proteins and other molecules associated with fertilization and early embryonic development [1]. Two immunogens studied extensively in the horse and other species as potential contraceptive agents are gonadotrophin releasing hormone (GnRH) and native porcine zona pellucida (pZP) proteins [1].

An important determinant for the selection of a contraceptive agent is the effect on ovarian function, both in the short and long term [1]. The presumed immunocontraceptive mechanism of pZP in the horse involves antibody binding to the ZP sperm receptor sites and subsequent prevention of sperm-oocyte binding and fertilisation. Based on this supposition, pZP immunisation should not affect the hypothalamic-pituitary-gonadal axis, thereby permitting continuation of cyclical ovarian activity [2, 3] and associated behaviours [1]. However, pZP-based immunocontraception causes irreversible ovarian damage in some species [4, 5, 6]. By contrast, anti-GnRH vaccines trigger production of antibodies that neutralise endogenous GnRH, which prevents receptor binding and activation of pituitary gonadotrophs. The suppression of gonadotrophin secretion causes reproductive quiescence characterised by cessation of cyclical ovarian activity [7, 8]. Anti-GnRH vaccines therefore also suppress both physiological and behavioural oestrus in the immediate [7, 9, 10, 11] and longer terms [8]. Ovarian suppression onsets within three months of treatment [8-12] and is associated with decreased ovarian weight [12], length [9], volume [11, 12], and reductions in serum progesterone [9-12], oestradiol-17β [10], LH [12] and FSH [12] concentrations. Ovarian suppression has also been reported in mares subsequent to immunocontraception using pZP vaccine [13-15]. In this respect, apparent cessation of oestrous cyclicity and erratic cyclicity into the non-breeding season have been reported after long term treatment (> 3 years) with pZP vaccines [1, 16]. More recently, 93% of mares treated with a pZP vaccine ceased cyclical activity within four months of treatment [17]. Abrogated cyclicity was

associated with both consistently low serum progesterone and minimal ovarian activity as determined by clinical, macroscopic and histological examination of the ovaries.

Recombinant vaccines have been developed by the expression of porcine ZP3 and ZP4 in *Escherichia coli* (reZP) [18]. A recent report has described the intensively monitored ovarian function and fertility of pony mares subsequent to treatment with either a pZP or a reZP vaccine [19]. Control mares retained cyclical ovarian activity throughout the trial whereas six out of seven pZP treated mares and one reZP mare entered an extended (albeit reversible) anoestrus characterised by clinically apparent ovarian suppression and basal serum ovarian steroid concentrations. Pregnancy was established in 0%, 57% and 100% of pZP treated, reZP treated and control mares, respectively [19]. A recent vaccination trial in donkey jennies studied the effects of pZP and reZP on oestrous cyclicity and fertility [20]. The vaccines were similarly formulated to that of previous pZP and reZP vaccines administered in horses [19], using Freund's adjuvants. Seven of 9, 6/8 and 0/8 jennies entered anoestrus within three months after the final vaccination for the reZP, pZP and control jennies, respectively. No jennies in the two vaccinated groups became pregnant compared to 6/8 control jennies.

The advantages of a reZP (compared to pZP) vaccine include production efficiency and the avoidance of contamination with non-ZP proteins and heat-resistant microorganisms [19, 21, 22]. However, the efficacy of pZP or reZP as an immunocontraceptive agent relies on the inclusion of a strong adjuvant [23]. Freund's complete modified adjuvant (FCMA) is typically used for the primary inoculation followed by Freund's incomplete adjuvant (FIA) for booster inoculations. Freunds' adjuvants can cause undesirable side effects, which can be severe and persist for months [23]. The use of alternative adjuvants that produce a similar or better immune response with less severe side effects would be advantageous.

A more complete understanding of ovarian suppression subsequent to ZP-based immunocontraception will better define the mechanism of the contraceptive effect [19]. However, populations requiring contraception are typically managed under extensive

conditions and, therefore, practical methods with limited intervention opportunities are commonly required for monitoring effects [21]. Anti-Müllerian hormone (AMH) has been proposed as a tool for assessing ovarian function during ZP-based immunocontraception [24] as it is reportedly a consistent [25] and useful biological marker of ovarian function [26].

The current study aimed to describe ovarian function in mares managed under extensive conditions following treatment with pZP or reZP vaccines formulated using non-Freund's adjuvants or a commercially available anti-GnRH vaccine. In addition, AMH concentrations were compared between treatment groups.

We hypothesised that immunocontraception using pZP proteins formulated with non-Freund's adjuvants would have similar ovarian effects as anti-GnRH vaccination. Furthermore, immunocontraception using reZP proteins formulated with non-Freund's adjuvants were expected to elicit similar ovarian responses as pZP vaccination. Additionally, we anticipated changes in AMH concentrations in ZP immunocontracepted mares.

2. Materials and Methods

2.1 Mare selection, management and environment

A population of mixed breed mares (light body type: Arabian, Quarter Horse, Draught and Thoroughbred cross; age: 2 -10 y) were studied from November 2016 to May 2017. Inclusion criteria were non-pregnant, normal oestrous cyclicity, good physical and reproductive health and no previous immunocontraceptive treatment [19]. Fifty barren or maiden mares were initially screened for inclusion during a 30-day monitoring period. In this group, regular oestrous cyclicity was confirmed in 26 mares on the basis of periodic changes in the serum progesterone concentration [27]. Lactating mares at the same site were recruited following re-establishment of oestrous cycle activity (assessed by transrectal palpation and ultrasonography of the reproductive tract). Thirty-nine mares were ultimately enrolled (26 maiden or barren and 13 lactating). Mares were maintained on a single extensive

129 mountainous grassland site (3000ha) in pre-existing groups. The study site was located at
130 29 ° 51' 30.8664'' S 29 ° 20' 46.9068'' E. The study occurred during the physiological
131 breeding season [28]. The natural day length and environmental temperature range at the
132 beginning and end of the study period were 13 h 18 m, and 7 to 31 °C and 10 h 20 m, and -3
133 to 24 °C, respectively.

134 **2.2 Study design**

135 Horses (n=39) were stratified by body condition scores (BCS: 1-9) [29], parity and age
136 (Table 1) for random assignment to one of five treatment groups. Repeated measures data
137 were gathered *via* clinical observation and venous blood collection.

138 **2.3 Formulation of vaccines**

139 The same adjuvant formulation was used for each of the control, pZP-only, reZP-only and
140 combined pZP and reZP groups. Each vaccine dose (1 mL) was constituted by combining
141 the antigen, 6% polymeric adjuvant (Montanide™ PetGel A, Seppic, France) and 500 µg
142 polyinosinic-polycytidylic acid - TLR3-agonist (Poly(I:C) HMW VacciGrade™, Invivogen,
143 USA). The amount of antigen *per* treatment was as follows: 100 µg pZP (Trumpeter Farms
144 and Veterinary Service, Winters, California, USA) for pZP treatments; 250 µg recombinant
145 ZP3 (containing tetanus toxoid epitope) and 250 µg ZP4 (containing bovine RNase epitope;
146 reZP; supplied by BioSciences, CSIR, South Africa) for reZP treatments and no antigen for
147 the control group. Multi-dose vials of each vaccine formulation were prepared, lyophilised
148 and reconstituted with sterile water for injection.

149 **2.4 Vaccine administration**

150 The adjuvant control group (n=8) was treated on d=35 and again five weeks later (d=70).

151 The pZP-only group (n=7) received an initial vaccination at d=35 followed by an identical
152 booster vaccination after five weeks (d=70).
153 The reZP-only group (n=8) received an initial vaccination at d=0, followed by two identical
154 boosters at five week intervals (d=35 and d=70).

The pZP and reZP group (n=8) received an initial vaccination of pZP at d=35 followed by a booster vaccination of reZP after five weeks (d=70).

The anti-GnRH group (n=8) received an initial 2mL vaccination containing 400 µg GnRH-protein conjugate with an diethylaminoethyl (DEAE)-dextran adjuvant (Improvac®, Zoetis, South Africa) at d=35 followed by an identical booster vaccination five weeks later (d=70).

All vaccines were administered by deep intramuscular injection into the gluteal muscle mass; boosters were administered into the contralateral musculature.

2.5 Data collection

Animals were examined and samples collected in December (d=0), January (d=35), February (d=70), March (d=105) and May (d=175). Transrectal palpation and ultrasonography of the reproductive tract was performed at d=0, d=35, d=70, d=105 and d=175. During the examination, ovarian volume, presence of follicles ≥15mm diameter, presence of a CL (confirmed retrospectively by serum progesterone >1 ng/mL), uterine and cervical tone and the presence of uterine oedema were recorded for each mare. Oestrous cyclicity or activity was defined as the presence of follicles ≥15 mm and ovarian volume ≥25cm^3 (prolate ellipsoid formula) and the confirmation of an ovulation (if present) was confirmed by the presence of a previously unrecorded CL or corpus haemorrhagicum in conjunction with serum progesterone levels >1 ng/mL. In the absence of a CL, oestrous cyclicity was determined on the basis of observed tubular genital tract characteristics [30]. Ovarian inactivity was defined as bilaterally small ovaries (both <25 cm^3), the absence of a CL or any follicles ≥15 mm and basal (<1 ng/mL) serum progesterone [8, 11, 19, 31]. Blood samples were collected by jugular venipuncture at d=0, d=35, d=70, d=105 and d=175. Samples were centrifuged and serum stored at -20°C until assayed

2.6 Pasture breeding

Three mature, clinically-healthy and proven fertile stallions (6-8 years of age) were selected for pasture-based breeding. One stallion was randomly selected for each of the three breeding herds and introduced in March (d = 105). All stallions remained with the mares until

July. Foaling outcome was assessed at the end of the subsequent physiological breeding season based on available records.

2.7 Hormone assays

Serum progesterone was measured using a chemoluminescence technique (Immulite® 1000, Siemens, Germany) [32]. Serum AMH concentrations were determined using a commercially available ELISA according to the manufacturer's instructions (AMH Gen II ELISA; Beckman Coulter, Brea, CA, USA). This assay has been validated for use in mares [33] and the detection limit of the assay was 0.08 ng/mL. Intra- and inter-assay coefficients of variation were 3.7% and 4.4% respectively, for a low AMH concentration (3.82 ng/mL), and 3.4% and 4.0%, for a high AMH concentration (16.45 ng/mL).

2.8 Statistical analyses

Data concerning the presence/absence of individual measures of normal oestrous cyclicity were compared among treatment groups using mixed effects logistic regression. Quantitative data were log transformed and analysed using mixed effect linear regression. Regression models included fixed effect terms for treatment group, sampling time (categorical with five levels), a group by time interaction (AMH only) and age to adjust for potential confounding. Mare was included as a random effect and a first-order autoregressive correlation structure was used to account for repeated sampling. Post-hoc tests in the mixed-effects models were adjusted using the least significant differences (LSD) or Bonferroni method. Serum AMH concentrations at each sampling time were compared among groups using one-way ANOVA with multiple *post-hoc* comparisons adjusted using Bonferroni correction of P values. Pairwise correlations were estimated using Spearman's rho or Pearson's correlation coefficient as appropriate. Statistical testing was performed using commercially available software (IBM SPSS Statistics Version 25) and significance was set at $P \leq 0.05$.

3. Results

3.1 Ovarian activity

Treatment groups were comparable in respect to age, parity, and BCS (Table 1) and all mares had evidence of cyclic ovarian activity prior to treatment (Table 2). Treatment ($P = 0.001$) and time ($P < 0.001$) both had a significant effect on the presence/absence of normal ovarian activity. Mares in the control and pZP treated groups expressed normal cyclical ovarian activity most commonly followed by mares within the combined pZP and reZP, reZP-only, and GnRH treated groups in descending order of frequency. When summarized for all observation times, control mares were more likely to be cycling compared to reZP ($P = 0.005$) and GnRH ($P < 0.001$) treated mares. Similarly, pZP treated mares were also more likely to be cycling compared to reZP ($P = 0.002$) and GnRH ($P < 0.001$) treated mares. Five weeks after the first treatment and first booster for reZP-only (d=70), 8/8 control mares, 6/7 pZP-only mares, 5/8 pZP and reZP mares, 3/8 reZP-only mares and 2/8 anti-GnRH mares were demonstrating normal oestrus cyclicity. Five weeks after the final booster (d=105), 5/8 control mares, 4/7 pZP-only mares, 3/8 pZP and reZP mares, 1/8 reZP-only mares and 0/8 anti-GnRH mares were demonstrating normal oestrus activity. By the end of the active monitoring period (d=175), 3/8 control mares, 3/7 pZP-only mares, 0/8 pZP and reZP mares, 0/8 reZP-only mares and 0/8 anti-GnRH mares had evidence of normal ovarian activity. At the end of the subsequent breeding season the records for seven mares were available. Three control mares foaled and one pZP-only mare experienced a late-gestation abortion.

3.2 AMH

Treatment group had a significant effect on the serum AMH concentrations collected over the entire study ($P=0.030$). Furthermore, there were significant differences among treatment groups at d=105 ($P=0.037$) and d=175 ($P=0.019$). No post hoc pairwise comparisons were significant at d=105 but at d=175, mares treated with the anti-GnRH vaccine had higher concentrations compared to pZP-only treated mares ($P=0.029$). The difference between pZP-only treated and control mares at this time-point was not significant ($P=0.084$). Serum AMH concentrations changed over time in reZP-only mares with higher concentrations at

d=70 compared to d=105 (P=0.047) (Table 3). Serum AMH concentrations were positively correlated with ovarian volume (r=0.171, P=0.035) and mare age (ordinal categories; >3 y, 3-6 y, >6 y) (ρ=0.269, P<0.001) but negatively correlated with serum progesterone (r=-0.373, P=0.014).

4. Discussion

The immunocontraceptive method of action of pZP in the horse, and other species, has been proposed to involve the prevention of sperm–zona binding, with oestrous cyclicity presumed to continue undisturbed [2, 3]. However, the results of the present study demonstrate varying degrees of ovarian suppression across all treatment groups. Significantly more reZP-only and anti-GnRH mares stopped cycling sooner after vaccination than either the control or the pZP-only mares. A two-pronged treatment protocol utilising pZP as the primary inoculation and reZP for the booster had a protracted effect. Ovarian suppression following anti-GnRH vaccination has been reported and the mechanism (suppression of FSH and LH secretion) is well understood [7, 8, 11]. There is increasing evidence that, at least in some species, ovarian suppression is a contributory factor in the contraceptive efficacy of ZP vaccination [4, 5, 15, 16, 17, 19, 34]. However, more research is required to define the mechanism of ZP-associated ovarian suppression.

A previous study from our research group reported higher incidences of anoestrus and a superior contraceptive effect in mares treated with pZP compared to reZP [19]. However, the reZP vaccine [18] used in that study was manufactured in a different laboratory, was formulated with Freund's adjuvant and only a single booster treatment was administered. The current study is the first to report a non-Freund's adjuvant for ZP-based immunocontraception in the horse.

The reduction in ovarian activity in control mares was possibly an effect of season. A significant effect of time on ovarian activity was evident for all groups, and while this is

expected in seasonally breeding animals, it was a limitation of the current study. Reproductive activity is determined by season, primarily photoperiod, and to a lesser extent nutrition and environmental temperature [35]. The physiological breeding season in the southern hemisphere is October to March but variations have also been reported [28, 30]. Only 26 of the 50 mares initially assessed for the study were cyclic at the end of November and this delayed the initial administration of treatments and subsequent introduction of stallions for breeding. An additional limitation was the paucity of foaling records at the end of the following breeding season. Further investigations into the contraceptive efficacy, reversibility and safety of the novel formulation used in this study are warranted.

The AMH results of the present study were partially consistent with previous reports [24]. Mean AMH concentrations differed across groups at d=105, but differences between pZP-only and anti-GnRH mares were only evident at d=175. The reduced sampling frequency and relatively small group sizes in this study might have contributed to the absence of other significant differences. As a result, it is not clear whether AMH is a suitable indicator of the effect of ZP vaccination on ovarian follicular activity in mares managed under extensive conditions (i.e. sampled infrequently). However, AMH concentrations were correlated with ovarian volume and serum progesterone, suggesting that it can be useful under certain circumstances. Previous work by our research group noted the potential of serum AMH concentrations for monitoring ovarian function following immunocontraception in mares [24]. Samples were collected weekly from October to March in this previous work [19, 24] but only analysed for five strategic time periods. The results of the previous study served to inform the frequency of sampling and study design for the current project. Among other hypotheses, the current study investigated the premise that less intensive sampling for serum AMH would still be useful for monitoring ovarian function. This is important because most populations of horses that require immunocontraception are feral or semi-feral, greatly limiting the ease and frequency of interventions such as blood sampling. Sample collection coinciding with other interventions, such as inoculations, would enhance practicality in such circumstances.

Serum AMH has less cyclical variability in the horse [25] and may therefore be less influenced by season than clinical measures of cyclicity.

Serum AMH tended to be higher in older mares and there is a need to further investigate the dynamics of AMH concentrations in younger, albeit sexually mature, mares. This positive correlation between age and AMH has also been reported in Japanese Black cows [36].

In conclusion, a non-Freund's adjuvated reZP vaccine is a promising alternative for immunocontraception in the mare when ovarian suppression is an acceptable outcome. Serum AMH concentrations following ZP-based vaccination may be used to infer reductions in both ovarian volume and serum progesterone under extensive conditions.

Authorship

M.B. Nolan and M.L. Schulman contributed to the study design, data collection, data analysis and interpretation, preparation and final approval of the manuscript. H.J Bertschinger and G.T. Fosgate contributed to the study design, data analysis and interpretation, preparation and final approval of the manuscript. R. Roth and M. Crampton prepared the reZP and contributed to final approval of the manuscript. I.S. Martins contributed to the data collection and final approval of the manuscript. T.A.E. Stout contributed to data interpretation and preparation and final approval of the manuscript.

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Authors' declaration of interests

No competing interests to declare.

Ethical animal research

The study was approved by the University of Pretoria Animal Ethics Committee (V124-16)

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Table 1

Table 1. Treatment groups sub-divided on the basis of mare distribution by: age, median (range); parity, median (range); and BCS (1-9), median (range). P>0.05

Mare information	Treatment					P value
	Control (n=8)	pZP-only (n=7)	reZP-only (n=8)	pZP and reZP (n=8)	GnRH (n=8)	
Age (years)	4 (2, 9)	4 (2, 8)	4 (2, 10)	3 (2, 7)	4 (2, 7)	1.000
No. previous parities	1 (0, 3)	1 (0, 3)	1 (0, 5)	1 (0, 3)	1 (0, 4)	1.000
BCS (1-9)	5 (4, 7)	6 (4, 7)	5 (4, 7)	5 (3, 7)	6 (2, 8)	0.700

pZP, native porcine zona pellucida vaccine; reZP, recombinant porcine zona pellucida vaccine; GnRH, anti-GnRH vaccine (Improvac®)

Body condition score (BCS)

Table 2

Table 2. Number of mares displaying ovarian activity or inactivity at each time-point during anti-ZP or -GnRH vaccination or adjuvant-only (control) treatment

Time-point	Treatment									
	Control (n=8)		pZP-only (n=7)		reZP-only (n=8)		pZP and reZP (n=8)		GnRH (n=8)	
	Active	Inactive	Active	Inactive	Active	Inactive	Active	Inactive	Active	Inactive
d=0	8	0	7	0	8	0	8	0	8	0
d=35	8	0	7	0	8	0	8	0	8	0
d=70	8	0	6	1	3	5	5	3	2	6
d=105	5	3	4	3	1	7	3	5	0	8
d=175	3	5	3	4	0	8	0	8	0	8

pZP, native porcine zona pellucida vaccine; reZP, recombinant porcine zona pellucida vaccine; GnRH, anti-GnRH vaccine (Improvac®)

Table 3

Table 3. Mean (95% CI) serum anti-Müllerian hormone concentrations (AMH; ng/mL) in mares over a 6 month period during anti-ZP or -GnRH vaccination or adjuvant-only (control) treatment.

Time-point	Treatment					P value[†]
	Control (n=8)	pZP-only (n=7)	reZP-only (n=8)	pZP and reZP (n=8)	GnRH (n=8)	
d=0	1.21 (0.58, 2.56)	0.80 (0.36, 1.78)	0.88$^{\Delta e}$ (0.46, 1.70)	0.64 (0.29, 1.40)	0.89 (0.53, 1.50)	0.380
d=35[*]			0.70$^{\Delta e}$ (0.41, 1.19)			
d=70	1.04 (0.59, 1.84)	0.68 (0.39, 1.20)	0.96$^{\Delta}$ (0.50, 1.83)	0.64 (0.37, 1.09)	0.93 (0.39, 2.19)	0.730
d=105	0.80^{a} (0.33, 1.96)	0.47^{a} (0.38, 0.57)	0.44$^{a, e}$ (0.18, 1.07)	0.67^{a} (0.39, 1.18)	1.02^{a} (0.60, 1.72)	0.037
d=175	0.96^{ab} (0.48, 1.92)	0.45^{a} (0.21, 0.95)	0.67$^{ab,\Delta e}$ (0.33, 1.35)	0.90^{ab} (0.43, 1.87)	1.16^{b} (0.69, 1.96)	0.019
P value	0.180	0.330	0.049	0.140	0.690	

pZP, porcine zona pellucida vaccine; reZP, recombinant porcine zona pellucida vaccine; GnRH, anti-GnRH vaccine (Improvac®)

* reZP-only treatment group additional measurement

[†]Based on 1-way ANOVA comparing AMH among groups within each time-point. Means with different superscripts (letter) differ significantly after *post-hoc* testing incorporating Bonferroni correction

444 □Based on mixed effects linear regression comparing AMH over time within each treatment group including a random effect for mare to account
445 for the repeated sampling and fixed effects of age and time-point. Means with different superscripts (symbol) differ significantly after *post-hoc*
446 testing incorporating Bonferroni correction

447

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