



Serum anti-Müllerian hormone dynamics in mares following immunosuppression with anti-zona pellucida or -GnRH vaccines

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ABSTRACT

Circulating anti-Müllerian hormone concentration (AMH) is positively correlated to the number of small growing follicles in the mare and may reflect ovarian function. Dynamics of AMH during immunosuppression have not previously been investigated. This study aimed to compare serum AMH in mares following treatment with native porcine zona pellucida (pZP), recombinant pZP3 and pZP4 (reZP) or gonadotrophin releasing hormone (GnRH) vaccines, and saline-treated controls. Stored sera collected during two previous studies examining ovarian activity in mares during zona pellucida (ZP) or GnRH immunosuppression were analysed for serum AMH. Data were compared among treatment groups using mixed-effects linear regression and one-way ANOVA with post hoc testing. Correlations between AMH and previously reported clinical variables were estimated using Spearman's rho. Mares immunized against GnRH showed variable but detectable AMH throughout successive breeding and non-breeding seasons that were not significantly different to unvaccinated control mares. Mares treated with pZP demonstrated marked, reversible suppression of AMH. Mares immunized using reZP showed an intermediate effect. In the ZP study, AMH was positively correlated to serum progesterone concentrations, mean ovarian volumes and antral follicle counts, whereas no correlations between AMH and serum progesterone concentrations, mean ovarian volumes, or the presence of one or more follicles ≥ 20 mm in diameter were detected in the GnRH study. In conclusion, marked suppression of AMH during pZP immunosuppression, but not during GnRH immunosuppression, suggested enhanced suppression of ovarian follicular development and, or follicular function during pZP immunosuppression. Serum AMH concentrations may provide a novel tool for the assessment of ovarian function during ZP-based immunosuppression.

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1. Introduction

The porcine zona pellucida (pZP) and gonadotrophin releasing hormone (GnRH) vaccines are effective forms of immunosuppression in the mare [1]. The pZP vaccine induces an immune response to endogenous zona pellucida (ZP) glycoproteins surrounding the mammalian oocyte. This has been the preferred vaccine for population control in species with complex social

structures, such as the feral horse (*Equus caballus*), due to pZP's association with the maintenance of reproductive cyclicity [2]. In contrast, GnRH antibodies deplete effective GnRH within the hypothalamic-pituitary portal blood vessels, inhibiting GnRH-induced release of LH and FSH from the anterior pituitary and culminating in reproductive quiescence. This vaccine, therefore, shows promise as a means of preventing unwanted reproductive behaviours in the domestic horse [3].

The ovarian effects of pZP immunosuppression in the mare have been assessed clinically (transrectal palpation and ultrasonography), histologically (ovarian histology) and endocrinologically (measurements of ovarian steroids or their metabolites)

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[4–8]. Initial reports observed no ovarian effects following short-term pZP treatment [4]. Longer intervals (up to 7 years) of anti-pZP immunisation resulted in decreased ovarian oestrogen production and ovulation rates [5,9]. More recently, ovarian inactivity was detected in $\geq 86\%$ of mares within four months of treatment with both the conventional pZP vaccine [8] and a single-dose formulation of pZP [7]. The latter two studies highlighted a need for further study of the mechanism of action of ZP-based vaccines in the mare.

The ovarian effects of GnRH-based immunocontraception in the mare have similarly been assessed both clinically and endocrinologically [10–14]. Immunisation against GnRH was significantly associated with decreased ovarian weight [10], length [12] and volume [10,14]; decreased ovarian activity (the absence of corpora lutea and follicles ≥ 10 mm) [10–15]; suppressed or variable oestrus expression [10,12,13,15,16]; and suppressed progesterone [10,12–15], oestradiol-17 β [13,15], LH [10,11,13] and FSH secretion [10].

Measurement of serum anti-Müllerian hormone concentration (AMH) may represent a novel method for the assessment of ovarian function during immunocontraception in mares and other species. This homodimeric glycoprotein hormone, a member of the TGF β family of growth and differentiation factors, is expressed by granulosa cells of developing follicles in the post-natal ovary [17]. In the mare, follicles between 6 and 20 mm in diameter have the greatest influence on AMH [18]. Positive correlations between AMH, antral follicle count (AFC) and primordial follicle count are reported in mice [19], cattle [20] and women [21]. Similarly, AMH is positively correlated with AFC in mares over eight years of age and this correlation strengthens with increasing age [18].

This study aimed to investigate AMH in mares following their active immunisation against one of GnRH, native pZP or a combination of recombinant pZP3 and pZP4 (reZP; [22]) vaccines. Measured AMH was then compared to previously-published clinical variables, including mare age, serum progesterone concentrations, mean ovarian volumes and AFC or the presence of follicles ≥ 20 mm in diameter [8,14,23].

2. Materials and methods

2.1. Experimental design

Specimens were obtained from two previously-reported controlled studies in the mare that described the ovarian effects and reversibility of native pZP, recombinant porcine ZP3 and ZP4 [8] and GnRH vaccines [14,23]. Briefly, native pZP (Trumpeter Farms and Veterinary Services, Winters, California, USA) was prepared according to standard methods [4]. The reZP vaccine (Dr. Satish Gupta; Reproductive Cell Biology Laboratory, National Institute of Immunology, New Delhi, India) consisted of pZP3 and pZP4 expressed by *Escherichia coli* as chimeric fusion proteins linked to promiscuous T-cell epitopes of tetanus toxoid (TT-KK-ZP3) or bovine RNase (bRNase-KK-ZP4), respectively [22]. The GnRH vaccine is commercially available (Improvac; Pfizer Animal Health, Sandton, South Africa).

For the ZP vaccine study, mares were stratified according to age and randomly assigned to one of three study groups: pZP, reZP and saline-treated controls ($n = 7$ per group) [8]. All treatments commenced during the southern hemisphere summer, consisting of a primary vaccination incorporating Freund's modified complete adjuvant (V1) and a single booster with Freund's incomplete adjuvant (V2) 35 days later. Mares were monitored by transrectal palpation and ultrasound examinations of their internal reproductive tracts. Recorded variables included ovarian volumes, AFC (grouped according to size) and the presence of corpora lutea.

Blood samples were collected weekly, centrifuged and sera stored at -20 °C until analysed. Follow-up gynaecological examinations and blood sampling occurred during the following winter and again at the start of the subsequent breeding season. Seven and four mares became pregnant in the control and reZP groups respectively.

For the GnRH study, mares were stratified according to age and randomly assigned to either GnRH vaccine ($n = 9$) or saline-treated control ($n = 3$) groups [14,23]. Treatments similarly commenced during the southern hemisphere summer and consisted of a primary vaccination (V1) followed by a booster (V2) 35 days later. Mares underwent three similar gynaecological examinations (to the ZP vaccine study) at V1, V2 and five weeks post-V2. Recorded variables included ovarian volumes and the presence of corpora lutea and follicles ≥ 20 mm in diameter. Blood samples were similarly acquired individually until resumption of cyclic ovarian activity, with sera being stored at -20 °C until analysed.

2.2. Data comparison between ZP and GnRH studies

Treatments for both ZP and GnRH studies commenced within a similar 6-week period of the year (12 November – 22 December). In order to compare data across studies while minimising temporal effects, data were normalised according to vaccination status within the following periods: period I correlated to pre-V1 to within one month post V1 (3–22 December), period II correlated to between V2 and within one month post-V2 (2–29 January), period III correlated to between one and two months post-V2 (12 February – 6 March), period IV correlated to the first winter post-treatment with a vaccination status of four to six months post-V2 (3 May – 26 June). Period V correlated to the approximate commencement of the following breeding season (29 Sep 2007, GnRH study; 6 October 2014, ZP study), or later, depending on the date of resumption of cyclicity (up to 3 October 2008, GnRH study) [12]. Anoestrus was defined as the period when mares had small ovaries (< 45 cm³) with follicles ≤ 20 mm in diameter and a progesterone concentration below 6 nmol/l. For the ZP study, anoestrus was further qualified by the absence of an ultrasonographically visible corpus luteum.

2.3. Anti-Müllerian hormone assay

Anti-Müllerian hormone concentrations were determined by means of a commercially available ELISA according to the manufacturer's instructions (AMH Gen II ELISA; Beckman Coulter, Brea, CA, USA). This assay has been previously validated for use in mares [24]. Briefly, standard dilutions, controls and samples were incubated in an anti-AMH antibody coated microtitration plate. Samples were sequentially incubated with biotin-labelled anti-AMH antibody, streptavidin-horseradish peroxidase and tetramethylbenzidine. An acidic stop solution halted further colour development. Absorbance was measured at 450 nm using a microplate photometer (Multiskan™ FCd; Thermo Fisher Scientific, Waltham, Massachusetts, USA), and AMH concentrations determined by comparison to a calibration curve. 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% respectively, for a high AMH concentration (16.45 ng/ml).

2.4. Statistical analysis

AMH concentrations below the assay detection limit of 0.08 ng/ml were assigned values of 0.07 ng/ml for statistical modelling. The normality assumption was assessed by calculating descriptive statistics, creating histograms, and performing the Anderson-Darling

test for normality in commercial software (MINITAB Statistical Software, Release 13.32; Minitab Inc, State College, Pennsylvania, USA). Measured AMH values violated the normality assumption and were transformed using the natural logarithm prior to statistical analysis. The AMH values were compared among treatment groups using mixed-effects linear regression. Regression models included fixed effect terms for treatment group, sampling time (periods I to V), the interaction between treatment group and sampling time, and age categorized as <5, 5–9, and >9 years of age to adjust for potential confounding. Horse identity was included as a random effect to account for the repeated measures sampling design. The complete model was followed by independent models for each treatment group to evaluate changes in AMH over time using a linear mixed model incorporating a random effect for horse to account for the repeated sampling. One-way ANOVA was used to compare AMH among treatment groups within each sampling time. Post-hoc testing was adjusted for multiple comparisons using Bonferroni correction of P values. Correlations between AMH and previously reported variables, including mare age, serum progesterone concentrations, mean ovarian volumes, AFC (ZP study) or the presence of one or more follicles ≥ 20 mm in diameter (GnRH study), were estimated using Spearman's rho, across all time periods. Statistical testing was performed using commercially available software (IBM SPSS Statistics Version 23; International Business Machines Corp., Armonk, NY, USA) and results were interpreted at the 5% level of significance.

3. Results

According to the linear mixed model, measured AMH differed among treatment groups ($P < 0.001$) and there was a significant treatment by time interaction ($P < 0.001$). The pZP group was lower than all other groups ($P < 0.04$). The reZP group had lower AMH values than the GnRH-treated group ($P = 0.004$) but neither of the control groups ($P > 0.73$). The AMH values of the GnRH-treated group showed no difference to either control group ($P > 0.63$).

Data were further analysed within each time period (Table 1). Median AMH in the pZP group was below the detection limit of the assay, and significantly lower than that of control mares, at time periods II, III and IV. The reZP group demonstrated an intermediate effect between the pZP and control groups at time periods II, III and IV.

At time period V, AMH differed between the four groups ($P = 0.02$). Descriptively, the pZP and GnRH groups had similar concentrations that appeared different from the reZP and control groups, which also appeared similar to one another. However, no

pairwise differences were significant after adjusting P values for the multiple post hoc testing.

Finally, data were analysed over time, within treatment groups (Table 1). Anti-Müllerian hormone concentration in the control group changed significantly over time ($P = 0.04$). Although no pairwise comparisons to time period I were significant, AMH tended to be lower at time period II ($P = 0.07$; Fig. 1a). In the pZP group, AMH showed a marked change over time ($P < 0.001$), characterised by marked suppression of AMH at time periods II to IV. Furthermore, AMH increased significantly at time period V in this group, with AMH exceeding 6 ng/ml in two mares (Fig. 1b). In contrast, the GnRH group showed a gradual increase in AMH over time ($P = 0.001$; Fig. 1c). The reZP group showed no significant change in AMH over time.

No correlation between AMH and age was detected in either vaccine study alone or in combination ($P > 0.2$). In the ZP study (pZP and reZP), AMH was positively correlated to serum progesterone concentrations (Spearman's rho = 0.244, $P = 0.03$; Fig. 2) and mean ovarian volumes (Spearman's rho = 0.391, $P < 0.001$; Fig. 3). Moreover, AMH had a strong positive correlation to AFC (Spearman's rho = 0.713, $P < 0.001$; Fig. 4). In the GnRH study, AMH showed no correlation to previously reported serum progesterone concentrations ($P = 0.9$), mean ovarian volumes ($P = 0.2$), or the presence of one or more follicles ≥ 20 mm in diameter ($P = 0.7$).

4. Discussion

This is the first report describing variations in AMH in mares following their immunocontraception with either pZP, reZP or GnRH vaccines. Marked differences in AMH of ZP-immunocontracepted mares, particularly pZP, in comparison to GnRH vaccine-treated mares, were observed.

Post-treatment, AMH in pZP-treated mares was markedly suppressed and significantly lower than GnRH-treated and control mares. Clinically, six of seven pZP-treated mares demonstrated intermittent to persistent anoestrus during this time, characterised by bilaterally small ovaries, baseline serum progesterone concentrations and low AFC [8]. Decreased AMH during pZP-immunocontraception may be due to decreased AFC and, or downregulation of AMH expression by granulosa cells. The strong correlation between AMH and AFC in the ZP study supported the hypothesis that decreased AFC is a component of suppressed AMH.

Ovarian suppression following anti-pZP vaccination in the mare has been ascribed to immune-mediated destruction of developing follicles or inhibited folliculogenesis following antibody-mediated interference with oocyte-granulosa cell communication [7]. In the

Table 1
Mean (95% confidence interval)* serum anti-Müllerian hormone concentrations (AMH; ng/ml) in mares over five consecutive time periods during anti-ZP or -GnRH vaccination or saline (control) treatment.

Time period	Treatment					P value [†]
	pZP (n = 7)	reZP (n = 7)	GnRH (n = 9)	Control 1 (n = 7)	Control 2 (n = 3)	
I	0.80 (0.48, 1.35)	0.57 (0.26, 1.29)	0.70 (0.29, 1.67)	1.01 (0.56, 1.82)	0.82 (0.28, 2.45)	0.8
II	0.08 ^a (0.05, 0.13)	0.29 ^b (0.14, 0.61)	0.80 ^c (0.46, 1.38)	0.33 ^{b,c} (0.19, 0.56)	0.93 ^{b,c} (0.17, 5.06)	<0.001
III	0.07 ^{a,**}	0.29 ^b (0.14, 0.63)	1.07 ^c (0.74, 1.53)	1.26 ^c (0.66, 2.41)	0.50 ^{b,c} (0.12, 2.07)	<0.001
IV	0.10 ^a (0.04, 0.21)	0.29 ^{ab} (0.10, 0.81)	1.54 ^c (0.96, 2.47)	0.54 ^{b,c} (0.19, 1.51)	0.48 ^{a,c} (0.01, 29.8)	<0.001
V	1.86 ^a (0.72, 4.84)	0.67 ^a (0.22, 1.99)	1.81 ^a (1.53, 2.14)	0.55 ^a (0.22, 1.36)	NA	0.02
P value [‡]	<0.001	0.1	0.001	<0.001	0.763	

pZP, porcine zona pellucida vaccine; reZP, recombinant porcine zona pellucida vaccine; GnRH, anti-GnRH vaccine. NA = no animals.

*Mean and confidence interval calculated on the natural logarithm transformed data and then back-transformed into the natural scale for presentation.

[†]Based on 1-way ANOVA comparing AMH among groups within each sampling time. Medians without superscripts in common are significantly different after post-hoc testing incorporating Bonferroni correction of P values.

[‡]Based on mixed effects linear regression comparing AMH over time within each treatment group independently including a random effect for horse to account for the repeated sampling.

**All values below the detection limit and not possible to estimate a confidence interval.

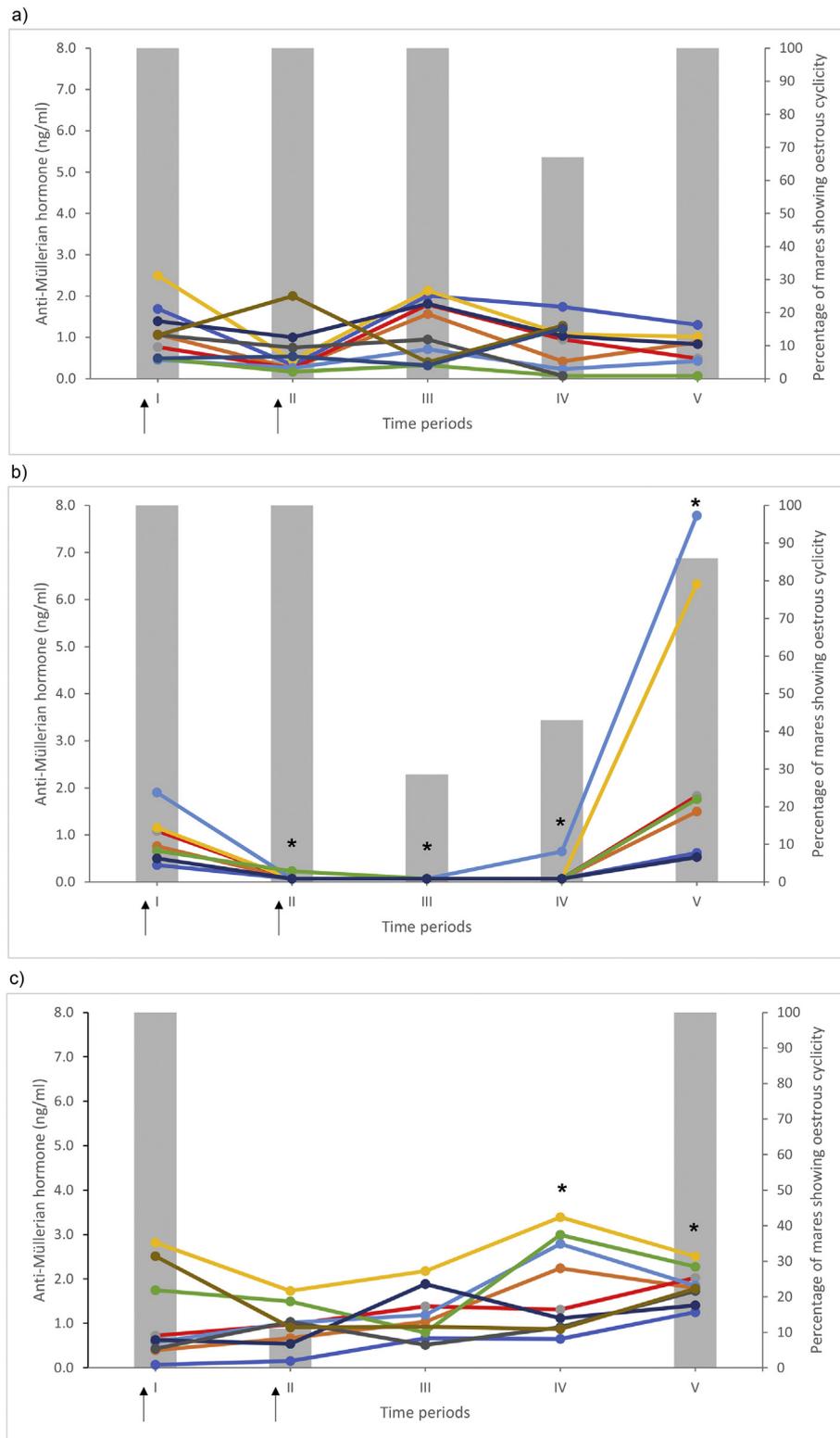


Fig. 1. Line graphs of serum anti-Müllerian hormone concentrations in a) saline-treated control (n = 10), b) native pZP-treated (n = 7), and c) GnRH vaccine-treated (n = 9) mares, over five consecutive time periods. A bar graph depicting the percentage of mares showing oestrous cyclicity at each time period, derived from data reported previously (for graph (a), n = 3 at time periods IV and V), is superimposed. Arrows depict approximate treatment times (V1 and V2). Asterisks indicate time periods at which AMHs are significantly different to time period I.

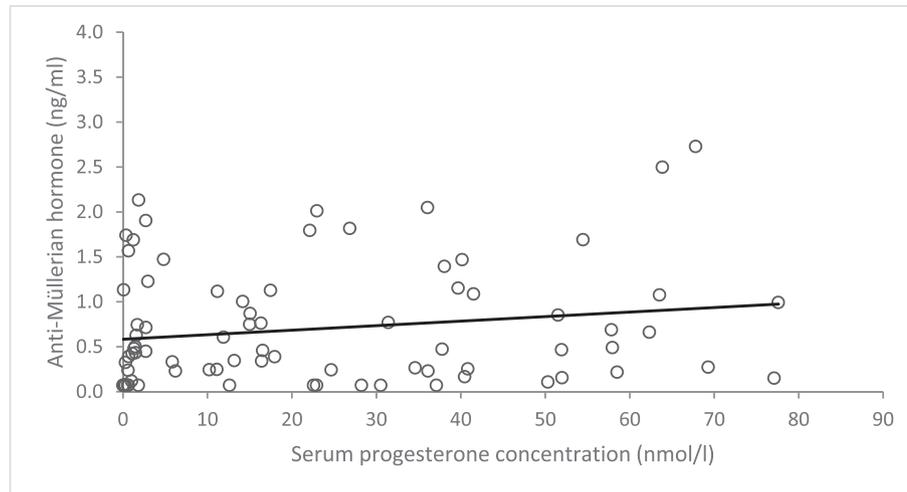


Fig. 2. Correlation between serum anti-Müllerian hormone concentration (AMH) and serum progesterone concentration in mares from the ZP study. Serum progesterone concentration was positively correlated (Spearman's rho = 0.244, $P = 0.03$) to AMH.

latter hypothesis, aberrant communication between the oocyte and its surrounding granulosa cells may lead to compromised competence of the developing follicle and diminished AMH expression. Expression of genes involved in steroidogenesis and follicular development, in relation to AMH, has been investigated in equine follicles. Mares with low AFC or circulating AMH were found to show decreased granulosa cell expression of AMH and AMH- and FSH-receptors [25]. A further study to examine molecular and hormonal changes within follicles during pZP immunocontraception is warranted.

In the mare, contraception as an outcome of anti-pZP vaccination has been shown to be reversible in the short term, associated with decreased antibody titres below a threshold level [4]. In feral populations however, prolonged treatment (up to 7 years) has been associated with prolonged intervals to reversal [5,26]. Interestingly in the current study, in pZP-treated mares, AMH was markedly elevated in two mares at time period V, suggestive of a rebound effect of heightened folliculogenesis approximately concurrent with their reversal. Whether a similar resurgence in AMH occurred in the remaining five pZP-treated mares before or after the date of

sampling is unknown. In other species, particularly women, AMH has shown promise as an indicator of ovarian reserve, defined as the pool of primordial follicles [21,27]. Assuming that a similar correlation between AMH and ovarian reserve exists in the mare, pZP immunocontraception may represent one instance where this correlation is rendered invalid.

The reZP-treated group showed an effect intermediate between the pZP-treated and control mares in terms of antibody titres, AFC and maximum follicle diameters [8]. Similarly, their AMH values were intermediate between pZP and control groups between one and 6 months post-V2. This finding encourages further research aimed at improved efficacy of reZP in mares, such as the administration of additional booster vaccinations [28] or alternative formulations such as the incorporation of liposomes [7].

Anoestrus during GnRH immunocontraception has previously been compared to winter anoestrus in the mare [10,15]. Both are characterised by low bioavailability of GnRH associated with near-complete suppression of LH secretion. In contrast to LH, serum FSH levels are suppressed but detectable during both anti-GnRH vaccination [10] and winter anoestrus [29,30], suggesting a non-

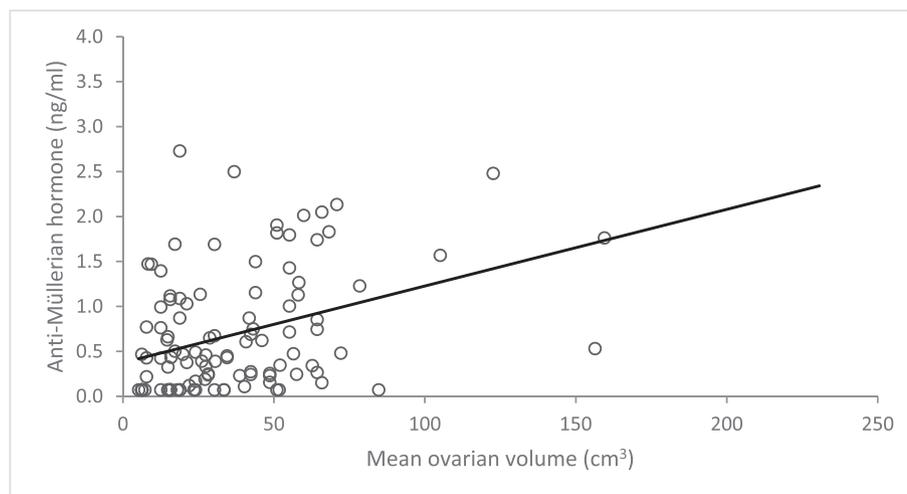


Fig. 3. Correlation between serum anti-Müllerian hormone concentration (AMH) and mean ovarian volume in mares from the ZP study. Mean ovarian volume was positively correlated (Spearman's rho = 0.391, $P < 0.001$) to AMH.

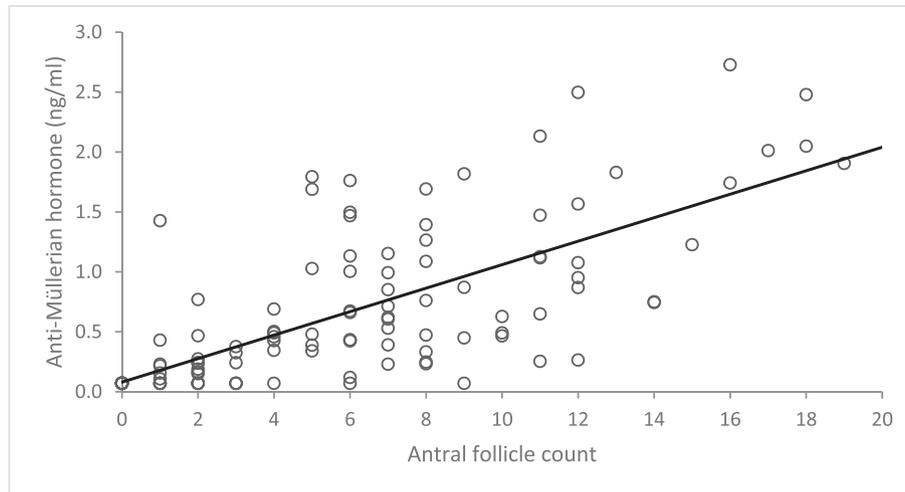


Fig. 4. Correlation between serum anti-Müllerian hormone concentration (AMH) and antral follicle count in mares from the ZP study. Antral follicle count was positively correlated (Spearman's rho = 0.713, $P < 0.001$) to AMH.

GnRH dependent component of FSH secretion. Moreover, the ongoing presence of small antral follicles has been reported during anoestrus, whether induced via season [30] or anti-GnRH vaccination [11,12]. In the current study, AMH values in GnRH-treated mares were similar to those in controls at each successive time period, likely due to continued AMH secretion by small antral follicles. However, AMH in GnRH-treated mares increased gradually over time, a pattern not detected in control mares. Increased AMH in anti-GnRH treated mares over time could be due to increased AFC or more efficient AMH production by granulosa cells. Increased AFC could result from either decreased atresia of growing follicles or increased recruitment of growing follicles from the ovarian reserve. Anti-Müllerian hormone was shown to play an important inhibitory role in primordial follicle recruitment, demonstrated using AMH *null* mice in which depletion of the primordial follicle pool occurred at a faster rate than wild type mice [31]. Increased primordial follicle recruitment as a hypothesis for increased circulating AMH concentrations in anti-GnRH-treated mares is therefore unlikely. In goats [32] and women [33], transient decreases in AMH following exogenous FSH administration was suggested in association with FSH-induced development of large antral and preovulatory follicles, temporarily decreasing the number of smaller follicles. A reversal of this process possibly occurs during anti-GnRH vaccination in the mare, where anoestrous periods are characterised by the absence of follicular development to preovulatory stages, resulting in decreased depletion of the population of preantral and small antral follicles. Unfortunately, clinical data regarding AFC within the GnRH study included only the presence or absence of follicles ≥ 20 mm in diameter. Since populations of follicles smaller than 20 mm in diameter appear to correlate better to circulating AMH concentrations [18], limited conclusions can be drawn. More detailed and frequent AFC assessments during GnRH immunocontraception are required. Interestingly, a novel role for AMH as a stimulator of GnRH secretion was recently demonstrated in mice [34]. Whether or not lowered GnRH levels during anti-GnRH vaccination affects AMH over time due to a disrupted feedback mechanism between AMH and GnRH warrants further research.

Mares show great individual variation in AMH [18,35]. However, AMH values are repeatable within mares, showing minimal changes throughout the oestrous cycle and gestation [35]. In the current study, AMH in the control group were similarly variable and

comparable to previously reported AMH values in cycling and, or pregnant mares [18,35]. Moreover, AMH in control mares, including four mares pregnant at time periods IV and V, did not change significantly over time. The non-significant decrease in AMH at time period II is of uncertain significance. Anti-Müllerian hormone concentrations during the non-breeding season in the mare have not previously been reported. In goats, a similarly seasonal breeder, AMH remains stable across seasons [32]. Although the findings of the current study suggested minimal variation in AMH throughout the breeding and non-breeding seasons in mares, both the unavailability of clinical data in controls from the GnRH study and pregnancies in several mares from the ZP study at time periods IV and V resulted in a small sample of relevant mares at these time periods, limiting valid conclusions. An important limitation of the results presented here is the relatively small number of horses studied and the potential for low power for the statistical tests to identify clinically important differences in AMH concentrations.

5. Conclusion

In mares, pZP immunocontraception caused a profound, temporary suppression of AMH. In contrast, AMH values were maintained and rose gradually during GnRH immunocontraception. Maintenance of AMH during seasonally-induced anoestrus, although suggested by current findings, requires further study. The analysis of AMH provided a novel modality for the assessment of ovarian function during ZP-based immunocontraception of domestic mares, potentially valuable in wildlife and feral species where repeated gynaecological examinations are not feasible.

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