Final Report:

Anti-Müllerian Hormone (AMH) as a potential predictive marker of fertility in mares

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Introduction

In a recent study we found that the average age of Thoroughbred broodmares in New Zealand was 10.4 years and that there was a significant reduction in the reproductive performance of mares older than 14 years (Hanlon *et al*, 2012). Increasing age is inversely related to fertility in all female species. Significant economic loss and frustration is associated with breeding older mares that fail to conceive. The ability to carry out a simple blood test to predict a mare's potential fertility would provide valuable information that could significantly improve the breeding efficiency of older mares.

Anti-Müllerian Hormone (AMH) is secreted by the granulosa cells of growing follicles and plays an important role in the regulation of the early stages of follicular development (La Marca *et al*, 2009). In women, AMH levels are used as a quantitative measure of ovarian reserve (number of remaining primordial follicles). Serum AMH levels progressively decline with age reflecting a decline in 'growing' follicle populations, with AMH levels eventually being undetectable in menopausal women (La Marca *et al*, 2009). It is impossible to directly measure the number of primordial follicles in the ovary, but measurement of AMH concentrations is an indirect method of determining an individual's remaining primordial follicle pool or 'ovarian reserve'. A strong correlation between declining AMH levels and the resting pool of primordial follicles has been demonstrated (Fanchin *et al*, 2005; Visser *et al*, 2006). Unlike other predictors of fertility, AMH levels are highly sensitive in the prediction of menopausal transition as it detects declining fertility before clinical signs of irregular cyclicity are observed or elevations in FSH levels are seen (Visser *et al*, 2006). Therefore, AMH levels may be used to predict the age of onset of decreased fertility in women. There would be significant benefits for the equine breeding industry if AMH levels in mares were similarly correlated to fertility as in humans thus allowing the detection of age related decreased fertility in mares.

The aims of this study were twofold:

1. To determine a "reference interval" for AMH levels in mares and,
2. To examine whether a correlation exists between AMH concentrations, age of the mare and fertility.

Materials and methods

Jugular venous blood samples were collected from 179 Thoroughbred mares on four different stud farms in the Waikato region, and 177 Standardbred mares on one stud farm in the Canterbury region during the 2014 breeding season. All procedures were performed with animal ethics approval (Kaiawhina AEC, protocol no. 014/14).

Measurement of AMH:

Each blood sample was collected into plain serum tubes. Serum was harvested within 2 hours of collection and frozen in sterile eppendorf tubes at -20°C for no longer than 3 months until analysed. In the initial stages of the project (2012/2013) we attempted to validate a commercially available human AMH assay (Generation II ELISA assay, Beckman-Coulter, California, USA), for the detection of equine AMH. Despite claims by the manufacturer that the assay had been standardized for horses, it appeared that this was not the case and we were unable to validate this assay for use in horses.

In 2013 we were able to obtain an equine-specific AMH assay (Equine AMH ELISA; AnshLabs, Texas, USA) and we were successful in validating this assay for use in horses. All blood samples were subsequently analysed for AMH using this assay.

Statistical analysis:

The relationship of mare age and breed on AMH concentrations was determined using multivariable regression analysis. The 95% reference interval (RI) was calculated using Cook's method of outlier detection and the 90% confidence intervals of the upper and lower limits were established (Friedrichs *et al*, 2012). Initially the RI was calculated for all mares in the sample population regardless of breed. However, there was a significant effect of breed on AMH, therefore separate RIs were calculated for Thoroughbred and Standardbred mares. Reference intervals for AMH were determined nonparametrically using the package "reference intervals" in R (Finnegan, 2012).

The reproductive outcomes of interest (dependent variables) were the first cycle pregnancy rate and the end of season pregnancy rate. The first cycle pregnancy rate was defined as the proportion of mares conceiving to their first service or insemination. The end of season pregnancy rate was defined as the proportion of mares that had a positive 42 day pregnancy test by the end of the breeding season. In both calculations, the denominator was the number of mares that had at least one service/insemination. Mares that were not inseminated or served, were excluded from these analyses although their AMH concentrations were used for the determination of the AMH reference interval. Multiple logistic regression was used to determine the effects of the independent variables, namely; AMH concentration, mare age, breed, and serving stallion on the first cycle and end of season pregnancy rates. All analyses were

performed using R Version 3.1.3 (R Development Core Team, 2014; R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org).

Results

The mean (± SD) age of mares in the study was 12 ± 5 years (range: 3 - 30 years). The mean AMH concentration was 17.2 ± 12.2 pmol/L and the range was 0.5 – 109 pmol/L. The reference interval for AMH for all mares in the study, regardless of breed was 2.3 – 37.1 pmol/L. The 90% CI for the lower limit was 1.9 – 2.9 pmol/L, and the 90% CI for the upper limit was 35.6 – 39.9 pmol/L. Standardbred mares had significantly higher mean AMH concentrations than Thoroughbred mares (18.7 ± 14.4 vs 15.7 ± 9.3 pmol/L respectively; P=0.02; Table 1). There was a significant effect of mare age on AMH concentrations, however this relationship was not linear, with AMH concentrations not declining significantly until 16 years of age. The mean AMH concentration in mares aged 16 years or less was 18.1 ± 13.3 pmol/L, whereas the mean AMH concentration in mares older than 16 years of age was 13.1 ± 10.4 pmol/L (P=0.003). In the multivariable model of AMH, both mare age and breed were significant (Table 2).

The mean first cycle pregnancy rate (FCPR) for all mares in the study was 45.4% and the end of season pregnancy rate (SPR) was 72.2%. Thoroughbred mares had significantly higher FCPR's and SPR's compared with Standardbred mares (Table 1). There were no significant interactions between AMH concentrations and reproductive outcomes. The mean AMH concentration of mares that conceived to their first service/insemination was not significantly different from those mares that failed to conceive to their first service/insemination (16.2 vs 16.8 pmol/L respectively; P=0.62). The mean AMH concentration of mares that conceived during the breeding season was not significantly different from those mares that failed to conceive during the breeding season (16.8 vs 16.5 pmol/L respectively; P=0.81). In both multivariable models for FCPR and SPR, the only significant exposure variables remaining in the model were mare age and breed (Table 3).

Table 1: Mean AMH concentrations and reproductive outcomes by breed

Breed	n	AMH (pmol/L) Mean ± SD	First cycle pregnancy rate (%)	End of season pregnancy rate (%)
Thoroughbred	179	15.7 ± 9.3[a]	50.6[a]	77.4[a]
Standardbred	177	18.7 ± 14.4[b]	38.5[b]	65.2[b]

[a,b] Values within the same column with different superscripts are significantly different P<0.05

Table 2: Multivariable linear regression model of the effect of mare age and breed on AMH concentrations based on samples taken from 179 Thoroughbred mares and 177 Standardbred mares in New Zealand

Variable	Coef	SE	P value
intercept	24.72	2.41	0.000
Age	-0.50	0.19	0.007
Breed (Thoroughbred)	-9.34	3.34	0.005
Age x breed interaction	0.52	0.26	0.04

Table 3: Multivariable logistic regression model of the effect of mare age, breed and AMH concentrations on the first cycle pregnancy rate (FCPR) and end of season pregnancy rate (SPR) of 179 Thoroughbred mares and 177 Standardbred mares in New Zealand

Variable	FCPR		SPR	
	Odds Ratio	95CI	Odds Ratio	95CI
AMH concentration	1.00	0.97 – 1.02	1.00	0.98 – 1.03
Age (years)	0.95	0.97 – 0.99	0.87	0.82 – 0.92
Breed				
Standardbred	Ref		Ref	
Thoroughbred	1.63[a]	1.1 – 2.68	2.04	1.2 – 3.63

[a] Interpretation: Thoroughbred mares were 1.63 times more likely to conceive to their first service than Standardbred mares

Conclusions

The first objective of this study was to determine the normal reference interval (RI) for AMH in mares. Using an equine-specific AMH assay we found that there was a significant effect of breed on AMH concentrations with Thoroughbred mares having lower AMH concentrations than Standardbred mares. Therefore, based on this study, the RI for AMH in Thoroughbred mares is 2.2 - 32.5 pmol/L and the RI for Standardbred mares is 2.2 - 39.9 pmol/L.

The second objective of this study was to examine whether AMH could be used as a predictor of fertility in mares. In agreement with findings in humans (Visser *et al*, 2006), we found a decline in AMH concentrations with advancing age in mares. This decline was non-linear, with AMH concentrations remaining stable until 16 years of age and then declining significantly. In women, a significant decline in fertility and correspondingly, AMH concentrations occurs at approximately 35 years of age (Steiner *et al*, 2011). The decline in AMH in women beyond 35 years of age is associated with a decrease in the number of primordial follicles present within the ovaries (Visser *et al*, 2006) and it is likely that the same occurs in mares that are older than 16 years of age. This age probably reflects the onset of ovarian senescence in mares.

Although there was a significant effect of mare age on AMH concentrations, we did not see a relationship between fertility and AMH concentrations. In the multivariable models of fertility (measured as first cycle pregnancy rate and end of season pregnancy rate), both breed and age were much stronger predictors of fertility than AMH. This finding is similar to the findings in recent human studies that showed that the correlation between AMH and pregnancy outcome is poor (Tal *et al*, 2015; Lin *et al*, 2013; Zarek *et al*, 2015) and the most important predictor of pregnancy is a woman's age. Put simply, young women with low AMH concentrations still have a higher pregnancy rate than older women with high AMH concentrations (Revelli, 2016). Likewise in our study, younger mares had higher fertility than older mares, regardless of their AMH concentration. Whilst AMH is a good indicator of ovarian reserve, it is likely that other factors such as uterine health, DNA integrity and oocyte age are far more important predictors of actual fertility, ie. the ability to conceive and carry a pregnancy to term.

Based on the findings of our study, AMH is not a useful predictor of fertility in mares.

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