

# Reduced anti-Müllerian hormone (AMH) in mares with hemorrhagic anovulatory follicles

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## Summary

Anti-Müllerian hormone (AMH) has been observed to decrease with the development of hemorrhagic anovulatory follicles (HAFs) in mares. Two studies were conducted to evaluate AMH concentration in mares with HAFs compared to seasonally anoestrous and cyclic mares, and to elucidate changes of AMH with the development of luteinised unruptured follicles (LUFs). In study 1, AMH and progesterone were evaluated in seasonally anoestrous, anovulatory (with HAF) and cyclic mares (at mid luteal phase). In study 2, mares in control and LUF groups were treated with 1500 IU/case hCG when they had a  $\geq 32$ -mm follicle and an endometrial oedema score of three (day 0). Mares in the control group received no further treatment. Mares in the LUF group received 1.7 mg/kg flunixin meglumine at the time of hCG administration, and 12, 24 and 36 h after it. Ultrasonography and blood collection for AMH and progesterone measurement were performed on days 0, 1, 2, 4, 6 and 8. In study 1, AMH concentration was lower in seasonally anoestrous and HAF mares than cyclic mares ( $P < 0.05$ ). Progesterone concentration did not differ between HAF and cyclic mares ( $P > 0.05$ ). In study 2, AMH was not different between LUF and control mares ( $P > 0.05$ ); however, progesterone had a lower concentration and a delayed rise after hCG administration in LUF mares compared with the control group ( $P < 0.05$ ). The results indicated that similar to seasonally anoestrous mares, AMH concentrations decreased in mares with HAFs. LUFs were also found to be functionally different from HAFs.

**Key words:** Mare, Anti-Müllerian hormone, Hemorrhagic anovulatory follicle, Luteinised unruptured follicle

## Introduction

In equine, blood extravasation into preovulatory follicles together with the failure of the follicles to ovulate leads to the development of hemorrhagic anovulatory follicles (HAFs) which are mostly luteinised (McCue and Squires, 2002; Ginther *et al.*, 2007; Cuervo-Arango and Newcombe, 2010). HAFs result in behavioural anoestrus and prolonged inter-ovulatory intervals, thereby decreasing fertility and causing breeding management problems (McCue and Squires, 2002; Ginther *et al.*, 2007). Ovulation failure is thus detrimental to the equine industry. Nevertheless, little is known about the pathogenesis of HAFs (Arango and Newcombe, 2010), which necessitates studies to investigate its pathogenesis.

Using a combination of flunixin meglumine (to block ovulation) and hCG (to induce luteinisation), Cuervo-Arango and Domingo-Ortiz (2011) succeeded in inducing luteinised unruptured follicles (LUFs) in mares. Comparing LUFs with HAFs, Cuervo-Arango and Newcombe (2012) found that they shared similar ultrasound characteristics, suggesting that LUFs could serve as a model to unravel mechanisms underlying the pathogenesis of ovulation failure in mares.

In mares, the anti-Müllerian hormone (AMH) is

mainly expressed by granulosa cells of secondary and small antral follicles (Ball *et al.*, 2008). It has been observed recently that serum AMH concentration decreases with the development of HAFs and increases after HAFs disappear (Gharagozlou *et al.*, 2013). Accordingly, it can be inferred that reduced serum AMH concentrations in anovulatory mares might result from ovarian inactivity imposed by HAFs. Ovarian quiescence is well-established in seasonally anoestrous mares (Newcombe, 1998; Williams *et al.*, 2012). The present study aimed to compare AMH concentrations in mares with HAFs to seasonally anoestrous mares (with inactive ovaries) and cyclic mares (with active ovaries). To elucidate whether LUFs resemble the diminishing effect of HAFs on AMH concentration, another study was also designed to evaluate AMH concentration in mares with LUFs and ovulatory follicles.

## Materials and Methods

### Study 1

Blood samples were collected from three categories of mares (thoroughbred and mixed breeds; age =  $7.1 \pm 0.47$  years), including seasonally anoestrous ( $n=7$ ), anovulatory (with HAF;  $n=8$ ) and cyclic ( $n=8$ ) mares. Seasonal anoestrus was determined based on the

presence of follicles  $\leq 20$  mm in either ovary in serial ultrasonographic examinations and progesterone concentrations of  $< 1$  ng/ml during the non-breeding season of 2012 (Mumford *et al.*, 1994). HAFs in anovulatory mares were diagnosed based on the presence of large unovulated follicles with intrafollicular strands and particles in ultrasonography and elevated progesterone concentrations during the breeding season of 2012 and 2013 (McCue, 2006). In cyclic mares, blood samples were collected 8 days after spontaneous ovulation at mid luteal phase.

## Study 2

Thirteen healthy cyclic Caspian mares (age =  $6.2 \pm 0.54$  years) were studied during May to July 2013. They were fed with alfalfa and grass hay and had *ad libitum* access to water. Induction of LUF and ovulation was done as previously described (Cuervo-Arango *et al.*, 2011). In brief, mares were subjected to daily transrectal ultrasonography 14 days after ovulation and treated with hCG (1500 IU/case; IVF-C<sup>®</sup>; LG Life Sciences, South Korea) when they had a  $\geq 32$ -mm follicle and an endometrial oedema score of three (maximum degree of endometrial oedema; Hayes *et al.*, 1985). Mares were randomly assigned to two experimental groups at the time of hCG administration (day 0). Those in the control group (n=6) received no further treatment while those in the LUF group (n=7) received 4 administrations of flunixin meglumine (1.7 mg/kg; Vetafluxin<sup>®</sup>; Aburaihan Pharmaceutical Co., Iran) concomitant with the hCG administration, and 12, 24 and 36 h afterwards.

## Ultrasonography

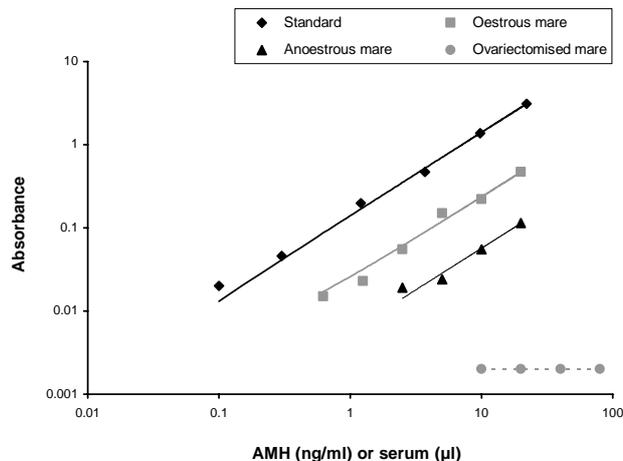
Transrectal ultrasonography in study 1 and 2 (on days 0, 1, 2, 4, 6, 8 of the experiment) was implemented using an ultrasound scanner (Medison Sonovet 600, Medison, Seoul, South Korea) equipped with a linear array 5.0 MHz transducer. To determine the diameter of ovarian structures, sonograms were frozen when ovarian structures were observed at their maximum size. The structure size was then determined by averaging two perpendicular measurements.

## Blood collection and hormonal assay

For both studies, blood samples were collected from the jugular vein using venipuncture tubes (on days 0, 1, 2, 4, 6, 8 of the experiment). The samples were centrifuged (for 15 min at  $1500 \times g$ ) within 2 h after collection. Serum was stored at  $-20^\circ\text{C}$  until hormonal assay.

Human MIS/AMH ELISA kit (Beckman Coulter Inc., CA, USA) was used to measure serum AMH concentration. The detection limit was 0.08 ng/ml, and inter- and intra-assay coefficients of variation were 5.6% and 5.4%, respectively. To validate equine serum AMH assay, parallelism of dilutions of sera from oestrous (n=1), anoestrous (n=1) and ovariectomised (n=1) mares with an AMH standard curve was assessed. Dilution curves of cyclic and anoestrous mares were parallel with the AMH standard curve (Fig. 1). Moreover, AMH was

undetectable in dilutions of sera from the ovariectomised mare (Fig. 1).



**Fig. 1:** Parallelism of sera from cyclic and anoestrous mares with the AMH standard curve

Progesterone concentration was measured using radioimmunoassay (Izotop, Budapest, Hungary). The detection limit was 0.1 ng/ml, and inter- and intra-assay coefficients of variation were 9.1% and 6.7%, respectively.

## Statistical analysis

Data associated with AMH and progesterone in study 1 were analysed using the GLM procedure. For study 2, data associated with the diameter of ovarian structures, AMH and progesterone were analysed using MIXED procedure including RANDOM and REPEATED statements in the model to specify covariation between and within mares, respectively (Littell *et al.*, 1998). LSMEANS was used to perform multiple comparisons. All analyses were conducted in SAS (2008). Differences were considered statistically significant at  $P < 0.05$ .

## Results

### Study 1

AMH concentration was higher in cyclic mares (8 days after spontaneous ovulation at the mid luteal phase) than the seasonally anoestrous and HAF mares ( $P < 0.05$ ). However, serum AMH concentration did not differ between seasonally anoestrous and HAF mares ( $P > 0.05$ , Table 1). Progesterone concentration was higher in HAF and cyclic mares than seasonally anoestrous mares ( $P < 0.0001$ ); however, it was not different for HAF and cyclic mares ( $P > 0.05$ , Table 1).

### Study 2

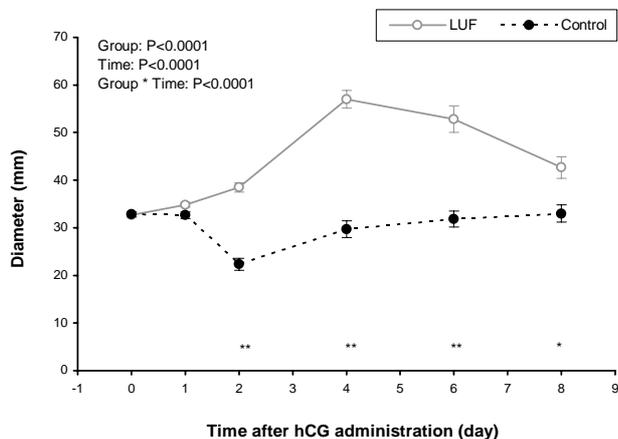
In the LUF group, data associated with one mare was excluded from the study due to lack of response to flunixin meglumine and ovulation.

From day 1 onward, the diameter of the LUFs increased ( $P = 0.037$ ), reached their maximum size ( $57.2 \pm 1.99$  mm) on day 4. Thereafter, diameter of LUFs

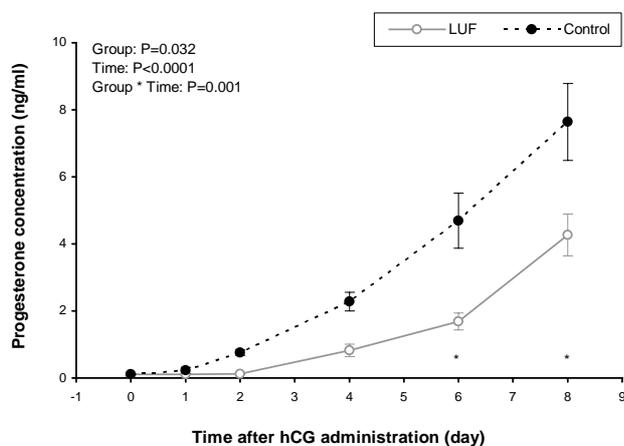
**Table 1:** AMH and progesterone concentrations in seasonally anoestrous, cyclic (8 days after spontaneous ovulation at mid luteal phase) and HAF mares. Data are presented as means ± SEM

Parameter	Seasonally anoestrous mares (n=7)	HAF mares (n=8)	Cyclic mares (n=8)
AMH (ng/ml)	0.3 ± 0.05 <sup>a</sup>	0.4 ± 0.09 <sup>a</sup>	1.1 ± 0.24 <sup>b</sup>
Progesterone (ng/ml)	0.1 ± 0.02 <sup>a</sup>	8.8 ± 1.01 <sup>b</sup>	10.7 ± 0.75 <sup>b</sup>

<sup>a, b</sup> Values with different superscripts within rows differ (P<0.05)



**Fig. 2:** Means ± SEM for diameter of ovarian structures in LUF and control groups on days 0, 1, 2, 4, 6 and 8 of experiment (study 2). Asterisks (\* P<0.001, \*\* P<0.0001) indicate differences between LUF and control groups at the specified day

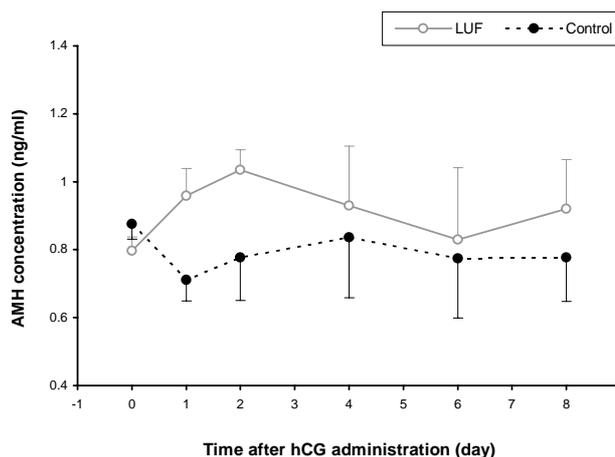


**Fig. 3:** Means ± SEM for progesterone concentration in LUF and control groups on days 0, 1, 2, 4, 6 and 8 of experiment (study 2). Asterisks (\* P<0.01) indicate differences between LUF and control groups at the specified day

decreased (P=0.008; Fig. 2). The diameter of corpora lutea (CLs) increased between days 2 and 4 (P<0.0001), but did not change between days 4 and 8 (P>0.05, Fig. 2). The diameter of the LUFs was greater than that of the CLs on days 2, 4, 6 and 8 (P<0.001, Fig. 2).

Regardless of group, progesterone concentration increased over time (P<0.0001); however, the commencement of the increase in progesterone concentration occurred from day 6 in the LUF group (P<0.0001), and from day 2 in the control group (P=0.001, Fig. 3). On days 6 and 8, progesterone concentration was higher in the control than the LUF group (P<0.01, Fig. 3).

Group, time and interaction of group by time did not influence AMH concentration (P>0.05, Fig. 4).



**Fig. 4:** Means ± SEM for AMH concentration in LUF and control groups on days 0, 1, 2, 4, 6 and 8 of experiment (study 2)

### Discussion

Study 1 demonstrated that reduced follicular development in seasonally anoestrous mares (Newcombe, 1998; Williams *et al.*, 2012) was reflected in serum AMH concentrations. Moreover, similar to seasonally anoestrous mares, low concentrations of AMH in HAF mares, showed that the latter also experienced diminished follicular activity. Our previous report implies that reduced AMH concentration that reflects diminished follicular activity originates from the HAFs themselves rather than the physiologic conditions leading to development of HAFs (Gharagozlou *et al.*, 2013). Given that AMH is primarily produced by granulosa cells (Ball *et al.*, 2008), disruption in the cells' function might be responsible for diminished follicular activity in mares with HAFs. Therefore, it seems that behavioural anoestrus in mares with HAFs (McCue and Squires, 2002; Ginther *et al.*, 2007) stems from the diminished granulosa cells' function and the corresponding reduced folliculogenesis imposed by HAFs.

Nevertheless, AMH concentrations were not different between mares with luteinised anovulatory cycles and those with ovulatory cycles in study 2. Almeida *et al.* (2011) found no change in concentrations of AMH throughout the oestrous cycle in normal cyclic mares. It appears that LUFs do not impose the diminished granulosa cells' function and follicular activity as do HAFs.

Not only were progesterone concentrations lower in flunixin meglumine-treated than untreated mares, but rise in progesterone after hCG administration was also delayed in flunixin meglumine-treated compared with untreated mares. Likewise, Cuervo-Arango *et al.* (2011) found lower concentrations of progesterone in flunixin meglumine-treated mares as compared to those untreated. Flunixin meglumine is a cyclooxygenase inhibitor that blocks the production of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  (Campbell and Blikslager, 2000). In bovine,  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  have been recognized to stimulate progesterone production and release in early luteal CL (Miyamoto *et al.*, 1993; Kobayashi *et al.*, 2001a; Bah *et al.*, 2006).  $\text{PGF}_{2\alpha}$  has also been reported to stimulate progesterone secretion from bovine luteinized granulosa cells (Meidan *et al.*, 1992; Okuda *et al.*, 1998). Additionally, growth factors such as growth hormone, insulin-like growth factor-1, basic fibroblast growth factor and vascular endothelial growth factor have been observed to increase  $\text{PGF}_{2\alpha}$  which supports progesterone production and release in early luteal CL (Kobayashi *et al.*, 2001a, b). Overall, it seems that  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  enhance progesterone production in early luteal CL (Miyamoto *et al.*, 2009). Therefore, blockage of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  production by flunixin meglumine could have partly contributed to lower and delayed progesterone production in mares with LUFs in study 2.

Progesterone concentration in mares with HAFs did not differ from that of cyclic mares, which is consistent with the study conducted by Ginther *et al.* (2006). This finding indicates that the HAFs' influence on AMH may not be attributed to progesterone. Furthermore, progesterone concentration was higher in mares with HAFs than anoestrous mares, suggesting that follicular quiescence imposed by HAFs was different from that imposed by the decrease of daylight.

In conclusion, the present research indicated reduced concentration of AMH in mares with HAFs, which might have resulted from diminished follicular development. In addition, the results implied that despite being ultrasonographically comparable, LUFs and HAFs were functionally different (Cuervo-Arango and Newcombe, 2012). Therefore, LUFs may not serve as a model for investigating the pathogenesis of ovulation failure in mares.

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