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Serum anti-Müllerian hormone levels during estrus and diestrus in mares[1)]

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Summary

Anti-Müllerian hormone (AMH) is a homodimeric glycoprotin secreted by granulosa cells in postnatal females. AMH plays an important role in follicle recruitment. The aim of the study was to analyse AMH concentrations under the guidance of circulating estradiol and progesterone levels in mares. The study was conducted on 25 non-lactating mares with regular estrous cycles. Blood samples were collected during estrus and diestrus to analyse estradiol, progesterone and AMH concentrations. Estradiol and progesterone concentrations were significantly different ($P < 0.001$) between estrus and diestrus. AMH concentrations in estrus were significantly higher than those in diestrus ($P < 0.05$). According to the authors' knowledge, this is the first report to suggest that the sexual cycle stage affects AMH concentrations in mares. Further investigations are needed to reveal factors affecting the AMH production and secretion pattern throughout the sexual cycle.

Keywords: anti-Müllerian hormone, equine sexual cycle, mare

Anti-Müllerian hormone (AMH) is a homodimeric glycoprotein secreted by Sertoli cells, which is responsible for fetal sex discrimination by regression of Müllerian ducts. AMH is also expressed in granulosa cells of preantral and small antral follicles in postnatal females (4, 19, 22). AMH is a member of the transforming growth factor-β superfamily, which plays an important role in follicle recruitment (4, 19). AMH reduces the sensitivity of growing follicles to the follicle-stimulating hormone and limits the number of actively growing follicles (4, 19, 22).

AMH measurement has become a routine diagnostic tool for reproductive function in women, especially for predicting ovarian reserve and monitoring menopausal transition. AMH levels show a reduction as ovarian reserve decreases with age. Eventually, AMH is undetectable after menopause (10, 22). Since AMH is produced by follicles in the common-growth phase, it is a valuable marker both for prediction of poor ovarian response and for prognosis in patients undergoing assisted reproductive procedures. In addition, AMH measurement is also used as a marker for ovarian dysfunctions, such as polycystic ovary syndrome, and for neoplasies, such as granulosa cell tumors (22).

Although research on AMH in mares has gained momentum in recent years, literature knowledge is relatively limited, unlike for AMH in women. Immunohistochemical studies performed on the mare's ovary show AMH expression in granulosa cells of small antral follicles, which are surrounded by more than one layer of granulosa cells (4). Several studies have been published on AMH reference intervals; possible associations between AMH levels, age and fertility; and the role of AMH measurement in diagnosis of granulusa cell tumors (2-6, 9, 11, 13, 17, 25).

The aim of the present study was to analyse AMH concentrations under the guidance of circulating estradiol and progesterone levels. It was hypothesized that circulating AMH concentrations may have different reference values during estrus and diestrus in mares.

Material and methods

Animal procedures, experimental design. The animal procedures for the study were approved by the Istanbul University Local Committee on Animal Research Ethics (permit no. 142). The study was conducted during the breeding season in the northern hemisphere (40°59'N 28°43'E). The mares were selected according to their general health and gynaecological conditions. Twenty-five non-lactating mares with regular estrous cycles were used in the study [21 Thoroughbred and 4 Arabian mares with a mean age of 13 ± 5 years and a body condition score of 5-6 according to Henneke (15)].

The animals received a complete diet prepared according to their nutritional requirements. It consisted of 1 kg of roughage and 1 kg of concentrate feed per 100 kg bwt. The access

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to water was *ad libitum*. The mares were kept in an outdoor paddock during the day and in standard-sized equine boxes during the night.

Blood samples collected during the estrus and diestrus of the second ovulatory cycle of the breeding season constituted the study material. No pharmacological drugs were used to hasten the transition period or to synchronise the estrous cycles.

Follicular activities of the mares were monitored by a real-time B mode ultrasound (Medison SA60V, Medison Co. Ltd., Seoul, South Korea) equipped with a 5 MHz linear probe. The mares were considered to be in estrus when the preovulatory follicle reached a diameter > 35 mm with an accompanying teasing score of 5/5 and ultrasonically detectable uterine edema of UE4 (24). Subsequently, blood samples were collected from the jugular vein into silicone-coated tubes to analyse estradiol, progesterone and AMH concentrations during estrus (day 0). Blood sampling was repeated on day 7 to determine hormone levels during diestrus. The blood samples were centrifuged (NF 800R, Nüve, Ankara, Turkey) at 3000 × g for 15 min, and the supernatants were stored at –20°C until analysis.

Analysis of blood samples. Commercial radioimmunoassay (RIA) kits were used for determining estradiol (Ultrasensitive Estradiol RIA, DSL4800, Beckman Coulter, Inc., Brea, CA, USA) and progesterone (RIA for progesterone, IM1188, Immunotech, a Beckman Coulter Company, Monrovia, CA, USA). The sensitivity of the kits were 2.2 pg/mL for estradiol and 0.05 ng/mL for progesterone. The intra- and inter-assay precision of the kits were, respectively, ≤ 8.9% and ≤ 12.2% for estradiol; and ≤ 5.8% and ≤ 9.0% for progesterone. Enzyme-linked immunosorbent assay (ELISA) was used for analysing AMH concentrations (AMH Gen II ELISA, A79765, Beckman Coulter, Inc.). The sensitivity of the ELISA kit was 0.08 ng/mL. The intra- and inter-assay precision of the kit were ≤ 5.4% and ≤ 5.6%, respectively. The analyses were performed according to the manufacturers' instructions.

Statistical analysis. IBM SPSS Statistics (version 10.00; IBM Corp., Armonk, NY, USA) was used for statistical analysis. Mean concentrations of circulating hormones during estrus and diestrus were compared by an independent sample Student's t test. $P < 0.05$ was considered statistically significant.

Results and discussion

All mares were in estrus on day 0, with a mean preovulatory follicle diameter of 41.4 ± 3.9 mm, teasing score of 5/5 and uterine edema of UE4, and they were in diestrus on day 7, with an ultrasonographically well-identified and developed corpus luteum. Circulating estradiol and progesterone concentrations were measured to verify the stage of the cycle. Estradiol and progesterone concentrations were significantly different ($P < 0.001$) between estrus and diestrus (Tab. 1). The higher serum estradiol concentrations measured on day 0 were compatible with reports that emphasize an increase in estradiol concentrations accompanying preovulatory follicle development (1, 16). Circulating progesterone concentrations on day 0 and day 7 were also in agreement with concentrations according to luteal activity (27).

Tab. 1. Serum estradiol, progesterone, and AMH concentrations on day 0 and day 7 (mean ± SE)

Hormones	Day 0	Day 7	P value
Estradiol (pg/mL)	38.93 ± 4.69	9.61 ± 0.80	< 0.001
Progesterone (ng/mL)	0.84 ± 0.02	29.49 ±2.65	< 0.001
AMH (ng/mL)	1.26 ± 0.20	0.77 ± 0.12	0.042

Women, cows, and mares, as members of monovular species, have been considered as comparative research models for each other because of their similar follicle selection characteristics (12). AMH levels have been postulated to show no consistent fluctuations throughout the estrous cycle by many researchers who studied women, cows, and mares (2, 14, 26, 28). In the present study, however, serum AMH concentrations on day 0 were significantly higher than on day 7 ($P = 0.042$). According to the authors' knowledge, this is the first report to suggest that the sexual cycle stage affects AMH concentration in mares. The reference interval for AMH levels was defined as 0.32-5.2 ng/mL in a study which examined the association between AMH levels, age of the mare, and fertility (13). The mean AMH concentrations obtained from the present study are within the reference interval of the above-mentioned study, but that reference interval does not refer to any stage of the estrous cycle. Almeida et al. (2) reported the mean serum AMH concentration as 0.96 ± 0.003 ng/mL, without any significant effect of the cycle stage in normal cyclic mares. On the contrary, several studies reported higher AMH concentrations in the follicular phase in cows and women (7, 23, 29). In a study conducted on cows, AMH concentrations decreased rapidly after estrus, which was followed by a slow increase until the next estrus (23). In women, it was postulated that luteinization leads to a negative effect on AMH production, which is followed by a decrease in AMH concentrations. In addition, diminished activity in granulosa cells has been suggested to play a role in decreased AMH fluctuations in advanced age (29). Not only central, but also local ovarian mechanisms are believed to play a role during folliculogenesis (8). AMH is also involved in this process as an ovary-originated hormone (4, 19, 22). Therefore, AMH concentrations may possibly fluctuate depending on the developmental stage of follicles and age. However, further investigations with larger sample size are needed.

Kusy et al. (20) ephasize the importance of comprehensive genital examination by all available methods, from inspection through uterine histology, in diagnosing and treating conditions that may lead to infertility. Since AMH has been well accepted as a fertility parameter, AMH measurement may become a routine procedure in equine reproduction. For instance, antral follicle count (AFC) serves as a marker for ovarian reserve (18). A positive correlation between AMH and AFC has been found in young (25), middle-aged and older mares (6). It was noted that AMH and AFC were highly repeatebale in mares (6). Ball et al. (5) discovered that mares in the lowest AMH quartile had a lower pregnancy outcome compared to mares in the mid-50% or upper quartile,

and attributed their findings to the relationship between AMH levels and fertility. The findings of the present study suggest that the sexual cycle stage is worth considering while evaluating fertility by AMH measurements.

AMH is also a very important biomarker for granulosa cell tumors (GCT), which is the most common ovarian neoplasia in mares. Serum AMH concentrations were found to be more sensitive than serum inhibin and/or testosterone concentrations for GCT detection (3). AMH immunostaining confirms high AMH expression from neoplastic granulosa cells (4, 9). The suppression of gonadotropin secretion starts to resolve after removal of the affected ovary, and consequently the contralateral ovary regains its normal function (17). It has been suggested that the depot form of gonadotropin-releasing hormone analog be used to hasten the return to normal cyclicity (21). During this process, AMH measurement may help to monitor the healing period of GCTs by observing its decline to physiological levels. An interesting study performed on mares with delayed uterine clearance revealed that their AMH concentrations were lower than those in healthy mares. It was suggested that low AMH concentrations were associated with luteal dysfunction, which was a potential contributor to ovary-lined mechanism (11). These reports suggest a potential use of AMH measurement in physiological circumstances as well as in pathological conditions.

In conclusion, mares have lower AMH concentrations in diestrus than in estrus. Although there are various studies with contrary findings in other species, according to the authors' knowledge, this is the first report to suggest that the sexual cycle stage affects AMH concentrations in mares. Further investigations are needed to reveal factors affecting the AMH production and secretion pattern throughout the sexual cycle which leads to discrepancy between studies in monovular species, including mares.

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