

Association of Anti-Müllerian Hormone concentrations between the pregnancy rates and pregnancy continuity of cows in different age groups

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Abstract: The potential relationships between Anti-Müllerian Hormone (AMH) concentrations and fertility were investigated by examining pregnancy rates and early pregnancy loss in different age groups of cows. Holstein heifers (17.35 ± 1.35 months, $n = 20$), young cows (3.25 ± 1.02 years, $n = 20$), and old cows (6.7 ± 0.80 years, $n = 20$) were synchronised and time fixed inseminated. A single blood sample per animal was taken during oestrus just before artificial insemination (AI), (Day 0) for the AMH analysis. The highest plasma AMH concentrations were determined in the heifer group (149.01 ± 12.62 pg/ml, $P < 0.001$) in the study. The AMH concentrations and conception rates decreased with age. The Day-0-AMH concentrations were higher in the pregnant animals at Day 20 after AI than in the non-pregnant animals ($P < 0.05$) in each group. The mean Day-0-AMH concentrations in the pregnant animals at Day 60 after AI was higher ($P < 0.001$) than the AMH concentrations in those with a detected pregnancy loss between D₂₀ and D₆₀, and also in those with a pregnancy loss and not being pregnant by Day 20 considered together. When each group was assessed on its own, based on the pregnancy results of Day 60, the Day-0-AMH concentrations were found to be significantly higher in the pregnant heifers than in the non-pregnant heifers and heifers with a pregnancy loss ($P < 0.05$). In conclusion, the AMH analysis might provide valuable information on the reproductive efficiency of the animals in a herd.

Keywords: bovine livestock management; herd management

Anti-Müllerian hormone (AMH) is a homodimeric glycoprotein structured hormone which is secreted from sustentacular (Sertoli) cells. It is vital in foetal sex discrimination by providing the regression of Müllerian ducts (Rota et al. 2002; La Marca and Volpe 2006; Ball et al. 2008). AMH is

also secreted from granulosa cells of preantral and small antral follicles in postnatal females (La Marca and Volpe 2006; Ball et al. 2008). AMH is actively involved in the follicle development by reducing the sensitivity of the growing follicles to the follicle stimulating hormone, thereby limiting the num-

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ber of actively growing follicles (Ball et al. 2008; Pfeiffer et al. 2014). AMH has a wide array of clinical applications such as the determination of the ovarian reserve, the monitoring of the menopausal transition, the diagnosis of a polycystic ovary syndrome and granulosa cell tumours, the prediction of a poor ovarian response, and the prognosis of *in vitro* fertilisation studies in human medicine (La Marca and Volpe 2006).

Ovarian dynamics have been regarded as a potential index of fertility and have also gained interest in the bovine species. AMH, which is secreted from the developing follicles, is an endocrine indicator of small antral follicles susceptible to gonadotrophins. Consequently, AMH is potentially related to the ovarian reserve (Pfeiffer et al. 2014).

When studies on the ovarian reserve and ovarian response to superovulation procedures are reviewed, it is seen that the reference ranges were still unclear for plasma AMH concentrations in cattle (Rico et al. 2009; Pfeiffer et al. 2014; Vernunft et al. 2015). Additionally, the results of various studies show that the AMH concentrations in cows are similar without any significant fluctuations throughout the oestrous cycle. Therefore, AMH measurements are considered as an independent marker of the follicle reserve in cows (Baruselli et al. 2015; Vernunft et al. 2015).

The aims of the present study were to determine the plasma AMH concentrations in dairy cows of different age groups who had undergone an oestrus synchronisation protocol, and also to investigate the relationship of AMH concentrations between the pregnancy rates and continuity. The objectives of the study were to contribute to the elucidation of the AMH reference interval in heifers, young cows, and old cows; to investigate the determining effect of the AMH levels on reproductive efficiency in cattle; and to spread the use of this hormone analysis in forming herds with high rates of reproductive efficiency, hence contributing to animal husbandry economics and leading to new investigations.

It was expected, as a hypothesis, that higher AMH levels correspond to higher pregnancy rates; additionally, low AMH levels represent a similar manner with the age-dependent decrease of the reproductive efficiency, thus, the AMH measurements were expected to give valuable information for a herd's management.

MATERIAL AND METHODS

Animals and experimental design. The study was approved by the Istanbul University Local Committee on Animal Research Ethics (permit No. 2015/95).

Holstein heifers and cows were selected according to their general health and gynaecological and udder conditions. The animals were kept at the Education, Training and Research Farm of Faculty of Veterinary Medicine, Istanbul University – Cerrahpasa. Among them, 60 healthy cows formed the experimental groups. They were kept in a 10 m² barn space per animal. The cows were machine milked twice per day in milking parlours. The mean milk yields were 22.9 ± 1.74 l/day and 27.85 ± 1.84 l/day, respectively, for the young and old cows. The animals received a complete diet prepared according to their nutritional requirements by the Department of Animal Breeding.

The animals were divided into three groups: heifers (18.35 ± 1.35 months old, $n = 20$, cycling regularly), young cows (3.25 ± 1.02 years old, $n = 20$, cycling regularly and delivered before), and old cows (6.70 ± 0.80 years old, $n = 20$, cycling regularly, delivered before). The cycles of the cows were monitored by pedometers and a computer-aided recording system in the study.

Oestrus synchronisation. All groups were subjected to the Co-synch oestrus synchronisation protocol after the determination of the completion of the uterine involution ultrasonographically following the 50th postpartum day. According to this protocol, a gonadotropin releasing hormone (GnRH) injection (0.01 mg/cow intramuscularly (*i.m.*), Lecibreed 10-ml flacon, Vetas, Turkey) and a prostaglandin F_{2 α} (PGF_{2 α}) injection (500 μ g/cow, *i.m.*, Senkrocin 10-ml flacon, Vetas) were administered to the animals on Day 0 and 7 of the protocol, respectively. Then, the animals received the second GnRH injection (0.01 mg/cow, *i.m.*, Lecibreed 10-ml flakon, Vetas) after 56 h from the PGF_{2 α} injection; a fixed-time artificial insemination was carried out at the same time.

Plasma AMH measurements. Single blood samples were collected from the coccygeal vein into tubes containing EDTA when the animals were in the oestrus just before the artificial insemination (Day 0). The oestrus was detected using the clinical techniques mentioned by Roelofs et al. (2010). The collected blood samples were centrifuged at 3000 g

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for 15 min to obtain the plasma. The discarded plasma samples were stored at -20°C until analysis. The plasma AMH concentrations were measured by a private laboratory (Bilim Laboratory, Bilim Sag. ve Lab. Hiz.Tic.Ltd.Sti., Istanbul). An AMH Gen II enzyme-linked immunosorbent assay (ELISA) commercial kit (AMH Gen II ELISA, Beckman Coulter, Inc., California, USA) with 80 pg/ml sensitivity, with an intra-assay and inter-assay coefficient of variations of 1.41% – 3.30% and 3.04% – 5.76%, respectively, was used for the analysis as described by Kumar et al. (2010).

Pregnancy diagnosis and monitoring. The pregnancies were assessed by identifying the asymmetry in the uterine horns and the presence of an embryonic vesicle with a transrectal ultrasonographic examination (real-time B-mode ultrasonography device with a 6.5-MHz linear probe, BCF Easy-Scan, BCF Technology Ltd., UK) on the 20th day (Day 20) after insemination. The animals were noted as pregnant and non-pregnant according to this first pregnancy examination. Then, the animals were checked again for the pregnancy continuity by rectal ultrasonography on the 60th day (Day 60) after insemination, and the pregnancy losses were recorded.

Statistical analysis. The Statistical Package for the Social Sciences (SPSS) 13.0 packet analysis program was used for the statistical analysis. A one-way analysis of the variance test comparison was used for the AMH concentrations in the heifer, young cow, and old cow groups. Tukey's test was used to determine the significance between the groups. The Mann-Whitney U test was used to assess the pregnancy findings. The significance level was accepted as $P < 0.05$.

RESULTS

According to the single blood sample collected in oestrus for the AMH measurement, the 20th and 60th day of pregnancy findings were evaluated.

The highest plasma AMH concentrations were detected in the heifer group, followed by the young cow and old cow groups, respectively. The difference in the mean AMH concentrations between the heifer group and other groups was statistically significant ($P < 0.001$). The experimental groups of the plasma AMH concentrations are shown in Table 1.

Table 1. The plasma AMH concentrations in the heifer, young, and old cow groups on Day 0

Groups	<i>n</i>	Mean age \pm S.E.M.	Mean AMH concentrations \pm S.E.M. (pg/ml)
Heifer	20	18.35 \pm 1.35 months	149.01 \pm 12.62 ^a
Young cow	20	3.25 \pm 1.02 years	110.84 \pm 12.61 ^b
Old cow	20	6.70 \pm 0.80 years	79.56 \pm 9.29 ^b

^{a,b}The difference between the mean values with the different letters in the same line is significant, $P < 0.001$

S.E.M. = standard error of the mean (concentration)

In the first pregnancy examination performed on Day 20, the numbers of pregnant animals were 17, 16, and 14 for the heifer, young cow, and old cow groups, respectively. The numbers of pregnant animals on Day 60 were detected as 14 for the heifer group; 12 for the young cow group; and 8 for the old cow group.

In the first pregnancy test performed on Day 20 after the artificial insemination, the AMH concentrations were higher in the pregnant animals than in the non-pregnant animals ($P < 0.05$) in each group. Additionally, the AMH concentrations were higher in all the pregnant animals than in the non-pregnant animals ($P < 0.001$). The plasma AMH concentrations for the pregnant and non-pregnant animals in the first examination are shown in Table 2.

No significant difference ($P > 0.05$) was detected in the Day 0 plasma AMH concentrations for the animals that were pregnant or with a pregnancy loss at Day 60 within all the groups, however, the AMH concentrations were significantly higher in all the pregnant animals than in the animals with a pregnancy loss ($P < 0.001$) in the last examination performed on Day 60 of the pregnancy. The plasma AMH concentrations of the pregnant animals and the animals with a pregnancy loss on Day 60 are shown in Table 3.

When the plasma AMH concentrations in the pregnant animals, the non-pregnant animals, and the animals with a pregnancy loss were assessed on Day 60, a statistically significant difference was detected in the heifer group ($P < 0.05$). However, the difference in the other groups was not significant. Significant differences were found when all the animals were evaluated together ($P < 0.001$). The findings are shown in Table 4.

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Table 2. The plasma AMH concentrations from the day of insemination for the animals becoming pregnant and non-pregnant in the examination on the 20th day after insemination

Groups	Pregnant		Nonpregnant		P
	n	mean ± S.E.M. (pg/ml)	n	mean ± S.E.M. (pg/ml)	
Heifer	17	159.27 ± 13.33	3	90.90 ± 6.47	*
Young cow	16	123.30 ± 13.86	4	60.98 ± 12.73	*
Old cow	14	94.01 ± 10.75	6	45.83 ± 8.19	*
Total	47	127.59 ± 8.29	13	60.89 ± 7.28	***

S.E.M. = standard error of the mean (concentration)

*P < 0.05; ***P < 0.001

Table 3. The plasma AMH concentrations from the day of insemination of the pregnant animals and the animals with a pregnancy loss detected on the 60th day after insemination (lost between D₂₀ and D₆₀)

Groups	Pregnant		With pregnancy loss		P
	n	mean ± S.E.M. (pg/ml)	n	mean ± S.E.M. (pg/ml)	
Heifer	14	168.19 ± 14.90	3	117.63 ± 16.28	n.s.
Young Cow	12	132.02 ± 17.88	4	97.15 ± 4.59	n.s.
Old Cow	8	94.48 ± 15.62	6	93.40 ± 15.65	n.s.
Total	34	138.08 ± 10.54	13	100.15 ± 8.17	***

n.s. = not significant (P > 0.05); S.E.M. = standard error of the mean (concentration)

***P < 0.001

Table 4. The plasma AMH concentrations from the day of insemination of the pregnant animals and the non-pregnant animals in the examination on the 60th day after insemination

Groups	Pregnant		Nonpregnant D60		P
	n	mean ± S.E.M. (pg/ml)	n	mean ± S.E.M. (pg/ml)	
Heifer	14	168.19 ± 14.90	6	104.27 ± 9.86	*
Young cow	12	132.02 ± 17.88	8	79.06 ± 9.27	n.s.
Old cow	8	94.48 ± 15.62	12	69.62 ± 11.06	n.s.
Total	34	138.08 ± 10.54	26	80.52 ± 6.65	**

n.s. = not significant (P > 0.05); S.E.M. = standard error of the mean (concentration)

*P < 0.05; **P < 0.01

DISCUSSION

Ovarian dynamics, which are considered to be an indicator of fertility, have also gained attention in cattle. Recent studies have tried to obtain information about the quantity and quality of the ovarian reserve by evaluating the number of morphologically healthy follicles in the ovary and its relationship to fertility (Pfeiffer et al. 2014). Although the AMH measurements are used in the paediatric

endocrinology of prepubertal males, more sensitive ELISA kits have been developed for the use in the field of gynaecology and assisted reproductive techniques subsequent to the recognised success of the AMH concentration as a marker of an ovarian reserve in women (Arouche et al. 2015). It is much easier to determine the ovarian reserve and potential fertility by evaluating the AMH concentrations in a blood sample rather than performing an ultrasonographic examination (Pfeiffer et al.

2014). Recently, kits containing bovine-specific monoclonal antibodies have been developed, and finally, an AMH Gen II ELISA kit has been developed for use in the veterinary field (Arouche et al. 2015). The present study aimed to investigate the relationship of fertility to the AMH measurements in different age groups of cows who had undergone an oestrus synchronisation, which is considerably feasible compared with the ultrasonographic antral follicle count (AFC) in veterinary practice.

Previous studies demonstrated that plasma AMH concentrations in cattle slightly decreased 1 week after ovulation, which was not sufficient to make a significant difference (Vernunft et al. 2015). These concentrations displayed a minimal change throughout the oestrous cycle unless a super-stimulation protocol was performed (Baruselli et al. 2015). Superovulation procedures simultaneously increased the number of antral follicles and AMH concentrations; however, the AMH concentrations returned to the previous values after a resting period (Rico et al. 2009). Similar AMH concentrations were measured during natural and synchronised oestrus cycles, and oestrus synchronisation procedures were found to be ineffective on the AMH concentrations (Pfeiffer et al. 2014). The AMH measurement has been recently characterised as an independent marker of ovarian activity in cattle due to its relatively stable concentrations throughout the oestrus cycle (Baruselli et al. 2015; Vernunft et al. 2015). In a study reported by Tanaka et al. (2018), the oestrus cycle did not affect the AFC, even when the blood samples were collected at different times. Furthermore, the AFC and AMH measurements were found to be associated with fertility. A synchronisation protocol was used in the present study to provide sexual uniformity during the evaluation of the relationship between the pregnancy rates and the continuity with the AMH concentrations of the cows with the different age groups. However, the feasibility of the AMH measurement at any time during the sexual cycle exhibits a great advantage from a practical point of view and makes it valuable especially in the health of the herd and reproductive management.

Jimenez-Krassel et al. (2015) found a shorter reproductive herd life, a reduced survival rate of the first calf, and lower pregnancy rates with low AMH concentrations in Holstein heifers. They concluded that a single AMH measurement is a predictive

tool for the longevity of the future herd. In the present study, the mean AMH concentrations of the entire pregnant animals were found to be significantly higher than in the non-pregnant animals ($P < 0.001$). Additionally, the AMH concentrations in the pregnant heifers was higher than the non-pregnant heifers ($P < 0.05$). Similar data was obtained for the young and old cow groups, exhibiting the relationship between the AMH measurement and the pregnancy rates ($P < 0.05$). These results were inconsistent with the findings of the study in which heifers with low AMH concentrations were reported to have poor fertility (Jimenez-Krassel et al. 2015). Although our results, with a limited animal number, reflect the outcome that the higher AMH concentrations corresponded to higher fertility, further studies are needed with expanded populations.

The highest plasma AMH concentrations were determined in the heifer group (149.01 ± 12.62 pg/ml, $P < 0.001$) in the present study. Additionally, the AMH concentrations and pregnancy rates decreased with the age. Although the subgroups with high and low AMH concentrations per group could not be established due to the sample size in the present study, the highest concentrations found in the youngest group and the lowest concentrations found in the oldest group supported the association of the AMH concentrations with the ovarian reserve, which decreased with the age. In agreement with the present study, Baruselli et al. (2015) reported a higher plasma AMH concentration in younger cows.

Recent studies report that cows with low AMH concentrations exhibit lower pregnancy rates in their first service and have a higher pregnancy loss incidence between 30 and 65 days of the pregnancy (Riberio et al. 2014). Interestingly, the pregnancy rate at the first service was detected to be higher in animals with intermediate AFC rather than in animals with low and high AFC after 36 days post-insemination in another study (Mossa et al. 2012). The higher Day-0-AMH levels of animals with a pregnancy loss rather than the animals which were not pregnant and with a pregnancy loss – according to the last pregnancy examination on Day 60 – may reflect the presumptive role of AMH on the reproductive efficiency in the presented study. However, Mossa et al. (2012) did not report any finding about the pregnancy loss in their study. As the AFC and AMH measurements serve the same intent, the dif-

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ferent pregnancy control schedules performed may lead to the discrepancy between these studies.

When each group was assessed on its own on Day 60, the AMH concentrations were significantly higher in the pregnant heifers than in the non-pregnant heifers and the heifers with a pregnancy loss ($P < 0.05$). Despite the lack of statistical significance among the other age groups, the AMH concentrations were found to be relatively higher in the pregnant cows than in the cows with a pregnancy loss or in the non-pregnant cows and the cows with a pregnancy loss considered together. However, in a study in which the predictive effect of the plasma AMH measurement on an ovum pick-up (OPU) and the embryo production outcomes were investigated in Holstein-Friesian heifers, the AMH measurement was found to be insufficient (Vernunft et al. 2015). Although the AMH levels were found to be positively correlated with the number of follicles aspirated per OPU session, the recovery oocytes per OPU, and in the vitro produced embryos per OPU; they concluded that it was difficult to predict the success of the in vivo produced embryos based on the AMH measurements alone. Moreover, it was recommended to use the AMH levels for classifying the heifers into groups as very good and very poor oocyte donors (Vernunft et al. 2015). As many factors may affect the embryonic development, and these results cannot be attributed to the AMH measurements only, the AMH levels may be a good tool in fertility evaluation in the light of the present study's results. The AMH concentrations in the pregnant animals were found to be higher, and a decrease in the AMH concentrations, pregnancy rates, and continuity was detected with the increasing age for all the groups. This study indicated that heifers with a mean AMH concentration of 170 pg/ml might have a high reproductive yield. Furthermore, forming herds with animals having AMH concentrations higher than 140 pg/ml and evaluating the animals with AMH concentrations lower than 130 pg/ml for withdrawal from the herd were highly recommended.

The following conclusions were obtained from the present study: (1) when considering the correlation between the ovarian reserve and age, the AMH levels might decrease with the increasing age; (2) only one AMH measurement may give information about the reproductive efficiencies even for nulliparous heifers; (3) the AMH measurements may help reduce economic losses, such as labour,

feed, and health expenditures, by providing valuable information about a herd's management via guidance through the possible pregnancy rates and pregnancy continuity of the animals; (4) this variable has the potential to be routinely used in the field and livestock management. The scientific data emerging from the present study might lead to new investigations on AMH, a valuable parameter for veterinary reproduction. Further studies are needed to evaluate the AMH measurement, especially for bovine livestock management.

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