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RESEARCH ARTICLE

Serum Anti-Müllerian Hormone Levels during Estrus and Diestrus Phases of the Estrous Cycle and its Possible Effect on Fertility in Cross-bred Hamdani Sheep

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ABSTRACT

This study aimed to assess the variations in serum Anti-müllerian hormone (AMH) levels during the estrus and diestrus phases of the estrous cycle and its effect on fertility in cross-bred Hamdani ewes. In the study, 21 non-prolific cross-bred Hamdani ewes (aged 2-3 years with BCS 2.75-3.25) were subjected to estrous synchronization using intra-vaginal sponges. Following synchronization, ewes underwent natural mating and pregnancy examination was carried out on the 45th day after mating through trans-rectal ultrasonography. Jugular blood samples were collected on day of estrus and 8 days later and analyzed for serum AMH and progesterone concentrations. Results showed that serum AMH levels were 100-480 and 95-520 pg/ml during estrus and diestrus phases, respectively. There was no change in serum AMH levels due to age, BCS and during estrus and diestrus phases of the estrous cycle ($P>0.05$). Estrus and diestrus serum AMH levels were significantly higher in pregnant ewes compared to non-pregnant ewes ($P<0.05$). Receiver-operating characteristic (ROC) analysis revealed that serum AMH cut-off value of >270 pg/mL was feasible to predict fertility in cross-bred Hamdani ewes. In conclusion, serum AMH can be used as a marker of fertility in non-prolific cross-bred Hamdani ewes.

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INTRODUCTION

Sheep generally have a limited number of ovarian germ cells, which further decrease significantly with aging similar to other mammals. Oocytes are primarily located in antral and preantral follicles, forming the so-called ovarian reserve (McNatty *et al.*, 1995). Genetic material and maternal nutrition during pregnancy contribute to the inherent variability in the number of ovarian follicles in sheep (Kotsampasi *et al.*, 2009; Grazul-Bilska *et al.*, 2009). Previous findings also suggest a potential link between the number of growing follicle populations in the ovaries of cattle and sheep and their fertility. Consequently, the number of follicles in the ovaries of ruminant species seems to greatly influence their reproductive performance (Lahoz *et al.*, 2012; Xu *et al.*, 2022).

Ovarian functions are regulated by the locally expressed hormones and growth factors in different cell types during the estrous cycle. These factors are associated with angiogenesis (Turgut and Ağaoğlu, 2023; Ağaoğlu *et al.*, 2023), somatic cell proliferation, differentiation and maturation of oocytes in the ovary (Webb and Campbell, 2007). Anti-müllerian hormone (AMH) is a highly conserved dimeric 140 kDa glycoprotein composed of 70 kDa monomers (Pepinsky *et al.*, 1988). This hormone is a member of the transforming growth factor β (TGF-β) superfamily and can signal by its type 1 and type 2 receptors (Monniaux *et al.*, 2012). It was first identified in mice as a factor that controls sex differentiation in males (Rey *et al.*, 2003). On the other hand, this hormone is also expressed by granulosa cells of growing follicles in females (Monniaux *et al.*, 2012; Alward and Bohlen, 2020).

Anti-müllerian hormone has emerged as a valuable endocrine marker for the follicular reserve in the ovary. There are numerous factors that affect serum AMH levels, such as genetic material, puberty onset, parity and nutritional status of the animal (Umer *et al.*, 2019; Alward and Bohlen, 2020). Previous findings in cattle and sheep indicate that AMH can be used as a marker to assess superovulation response and select donors in embryo transfer (Aziz *et al.*, 2017; Pinto *et al.*, 2018). Moreover, serum AMH levels are related to fertility and the number of lambs born in sheep (Lahoz *et al.*, 2012; Acharya *et al.*,

2020). The concentrations of AMH are highly variable in prepubertal periods and detectable in lambs at two months of age (Lahoz *et al.*, 2012). Serum AMH level ranges between 0 and 500 pg/ml in lambs and there is a strong relationship between prepubertal AMH level and adult fertility. Therefore, it is suggested that prepubertal AMH levels can be used as a predictor of fertility in ewe lambs at a young age (Lahoz *et al.*, 2012). On the other hand, limited data exist on serum AMH levels during the estrous cycle in sheep (Waheeb, 2017). In this study, it was aimed to assess the variations in AMH levels during the estrus and diestrus phases of the estrous cycle and its effect on fertility in cross-bred Hamdani ewes.

MATERIALS AND METHODS

Animals: The study was conducted in Siirt University Small and Large Ruminant Application and Research Center during September-October 2023. Location of the research center is 37°58'05"N 41°50'14"E, Siirt, Türkiye. All experimental procedures were carried out during autumn season, which is normal breeding season for sheep. A total of 21 cross-bred Hamdani ewes, aged 2-3 years with initial body condition scores (BCS) of 2.75-3.25 were selected for the study. Ewes were weighed and subjected to nutritional flushing two weeks before and three weeks after mating, according to the National Research Council (NRC) recommendations. All ewes were clinically healthy and did not have any reproductive problem. The animals were housed in a shelter with sufficient ventilation facilities. Fresh water was supplied *ad libitum* for all animals during the experiment. The animals were fed twice a day, in the morning and in the evening.

Estrous synchronization and mating: The synchronization of estrous in all female sheep was achieved by using intravaginal sponges (containing 60 mg medroxyprogesterone acetate, Esponjavet®, HIPRA, Türkiye). The sponge was left in the vagina for 6 days. On the day of sponge removal, intramuscular injections of 500 IU eCG (Oviser®, HIPRA, Türkiye) and a $PGF2_{\alpha}$ analog (50 µg cloprostenol, Gestavet®, HIPRA, Türkiye) were administered. All ewes were closely monitored for estrus signs three times daily, each lasting for at least 40 minutes, over a three-day period, starting 24 hours after sponge removal. Estrus was observed in all ewes and ewes underwent natural breeding with two fertile rams. The mated ewes were recorded using their ear tags and placed in a different compartment.

Collection of blood samples: Blood samples were collected from jugular vein of ewes during the estrus and diestrus phases of the estrous cycle using 5 mL serum tubes. For this purpose, blood samples were collected on the day of estrus and 8 days after estrus (diestrus). After collection, blood samples were centrifuged at 3000 rpm for 15 minutes, serum was separated and stored at -20ºC for AMH and progesterone assay.

Pregnancy diagnosis: Pregnancy diagnosis was performed on 45th day after mating through trans-rectal ultrasonography, using a B-mode real time ultrasound scanner (HS-102V, Japan) fitted with a 7.5 MHz frequency linear-array probe. Feed and water intake were restricted for 12 hours before ultrasound examination. Pregnancy examination was performed for at least two minutes for each ewe and pregnancies were recorded.

Hormone assay: Serum samples were analyzed to determine the concentrations of AMH and progesterone, using the electrochemiluminescence immunoassay (ECLIA) method, as described earlier (Koca *et al.*, 2023; Marchetti *et al.*, 2023; Bartha *et al.*, 2023). The measurements of AMH and progesterone were conducted through the Elecsys® AMH (Human) and Elecsys® progesterone automated assay on the Cobas 601 platform (Roche, Germany), respectively. Prior to the main trial, the samples underwent method validation following the manufacturer's guidelines. Calibration and standard curves were scrutinized based on precise assigned values. The assay demonstrated analytical sensitivities of 10.0 pg/mL and 0.05 ng/mL for AMH and progesterone, respectively.

Statistical analysis: All statistical analyses were performed using Minitab® (version 21.4.1) and MedCalc (version 22.016). For the sample size analysis, the power of the test was determined to be 80%, and the type-1 error of 5% (G*Power statistics program, ver.3.1.9.4). Due to normal distribution of the data, parametric tests were used. The effects of age and BCS on serum AMH and progesterone levels were evaluated by one-way ANOVA. A paired t-test was used to compare estrus and diestrus phase serum AMH and progesterone levels. An independent sample t-test was performed to compare AMH levels between pregnant and non-pregnant ewes. Binary logistic regression analysis was used to evaluate the effect of AMH, BCS, and age on fertility. Receiver Operating Characteristic (ROC) analysis using estrus serum AMH was performed to detect the serum AMH cut-off value related to fertility. Scoring scale and interpretation of ROC curves was based on the area under curve (AUC) values as follows: $AUC \leq 0.5$=Poor, $0.5 < AUC \leq 0.6$=Accurate, $0.6 < AUC \leq 0.7$=Very accurate, $0.7 < AUC \leq 0.9$=Highly accurate and $0.9 < AUC \leq 1.00$=Excellent. The statistical significance level was defined as $p<0.05$.

RESULTS

In the present study, serum AMH levels were 100-480 and 95-520 pg/ml and serum progesterone levels were 0.10-0.50 and 1.80-3.70 ng/mL during estrus and diestrus, respectively (Table 1). Age and BCS did not affect serum AMH and progesterone levels during estrus and diestrus phases of the estrous cycle (Table 2). Paired t-test showed that there was no difference in serum AMH level between estrus and diestrus phases of the estrous cycle. On the other hand, serum progesterone levels were significantly higher ($P<0.01$) during diestrus than estrus phase, which confirms the luteal formation during the diestrus phase (Table 3).

Pregnancy detection revealed that nine ewes out of 21 were pregnant. As shown in Table 4, estrus and diestrus serum AMH levels were significantly higher in pregnant ewes compared to non-pregnant ewes ($p<0.05$). Logistic regression model revealed that serum AMH was

Table 1: Descriptive statistics showing serum AMH (pg/mL) and progesterone (ng/mL) during estrus and diestrus phases.

Hormone	Phase	Mean±SE	Minimum	Median	Maximum
AMH	Estrus	247.6±23	100	260	480
	Diestrus	235.7±21.9	95	200	520
Progesterone	Estrus	0.29±0.02	0.10	0.30	0.50
	Diestrus	3.10±0.10	1.80	3.20	3.70

Table 2: The effect of age and BCS on serum AMH (pg/mL) and progesterone (ng/mL) levels during estrus and diestrus phases (Mean±SE).

		AMH		Progesterone	
Age (Years)	n	**Estrus**	**Diestrus**	**Estrus**	**Diestrus**
2.0	7	244.3±27.4	226.9±18.6	0.23±0.05	3.02±0.17
2.5	4	277.5±73.6	235.0±60.6	0.42±0.04	3.37±0.13
3.0	10	238.0±36.8	242.1±39.8	0.23±0.03	3.07±0.16
	P	>0.05	>0.05	>0.05	>0.05
BCS	n	**Estrus**	**Diestrus**	**Estrus**	**Diestrus**
2.75	6	283.3± 42.3	269.7± 53.1	0.21 ± 0.03	3.11 ± 0.16
3.00	8	238.8± 25.6	230.1± 27.1	0.35 ± 0.03	3.01 ± 0.22
3.25	7	227.1± 53.7	212.± 39.5	0.29 ± 0.06	3.21 ± 0.10
	P	>0.05	>0.05	>0.05	>0.05

Table 3: Serum AMH (pg/mL) and progesterone (ng/mL) levels in estrus and diestrus phases of estrous cycle (Mean±SE).

	n	Estrus	Diestrus
AMH	21	247.6±23[a]	235.7±21.9[a]
Progesterone	21	0.29±0.02[b]	3.10±0.10[a]

Values with different letters within a row differ significantly from each other (P<0.01).

Table 4: Estrus and diestrus serum AMH levels (pg/mL) in pregnant and non-pregnant ewes. (Mean ± SE).

	Pregnant (n=9)	Non-pregnant (n=12)
Estrus	320±35[a]	193.3±20[b]
Diestrus	305±37[a]	183.8±14[b]

Values with different letters within a row differ significantly from each other (P<0.05).

Table 5: Binary logistic regression analysis results showing the effect of serum AMH, age, and BCS on fertility.

Variables	SE	Z-value	P-value	Odd ratio
Serum AMH	0.009	2.25	0.02*	1.02
Age	1.40	1.10	0.27[NS]	4.73
BCS	3.28	0.30	0.76 [NS]	2.64

*P<0.05; [NS] Non-significant; SE= Standard error.

Table 6: Classification table for logistic regression model

	Predicted		
Observed	Non-pregnant	Pregnant	% Correct
Non-pregnant	11	1	91.67
Pregnant	2	7	77.78

Table 7: ROC analysis results for estrus serum AMH

Sensitivity (%)	Specificity (%)	Youden Index	Area Curve	Under P
77.78	91.67	69.45	0.88	0.0001***

***P<0.001 indicates that area under the ROC curve does have an ability of predicting fertility in cross-bred Hamdani ewes.

significantly related to fertility (p<0.05). However, there was no relationship between fertility and age or fertility and BCS (Table 5). Table 6 represents classification table for logistic regression model. Because there was non-significant difference in serum AMH levels between estrus and diestrus phases, estrus serum AMH level was preferred for ROC analysis. ROC analysis revealed that >270 pg/mL AMH level could be used for the evaluation of fertility in cross-bred Hamdani ewes (Fig. 1). The sensitivity and specificity of the ROC analysis for AMH cut-off value were 77.78 and 91.67% respectively (Table 7). In this study, AUC was 0.88, which reflects highly accurate results.

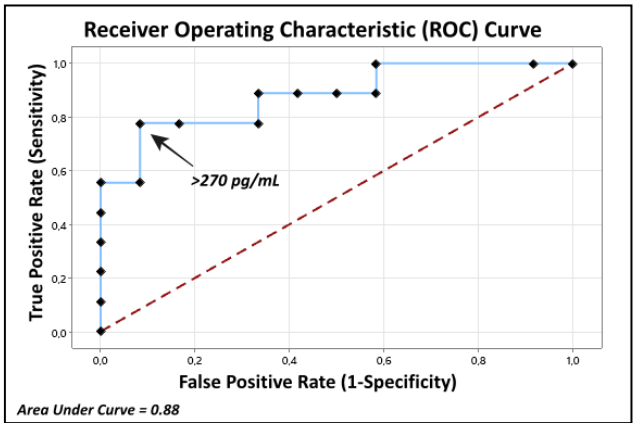

Fig. 1: Receiver-operating characteristic (ROC) curve for predicting fertility in cross-bred Hamdani ewes. The arrow indicates the cut-off point maximizing the Youden index.

DISCUSSION

Anti-müllerian hormone is accepted as an important biomarker for ovarian reserve in livestock species. In cattle, serum AMH levels show significant variations during the prepubertal period while it remains stable during the estrous cycle of the post-pubertal period in cattle (Mossa *et al.*, 2017; Umer *et al.*, 2019; Koca *et al.*, 2023). Studies also suggest that AMH shows a strong correlation with the number of healthy preantral and antral follicles, and cows with high serum AMH exhibit better superovulation response compared to cows with low AMH levels (Mossa and Ireland, 2019).

There is limited data on serum AMH levels during the estrous cycle in sheep (Waheeb, 2017). This study revealed that there was no change in serum AMH level during estrus and diestrus phases of the estrous cycle in cross-bred Hamdani sheep. This indicates that serum AMH level does not change during estrus and diestrus phases of the estrous cycle which is similar to cattle. However, its levels showed a wide variation among ewes and ranged between 100-480 and 95-520 pg/ml during estrus and diestrus phases, respectively. Waheeb (2017) reported significant positive correlation between serum AMH level and the number of pre-ovulatory follicles in Barki sheep. Furthermore, ewes with reduced numbers of antral follicles had lower serum AMH levels (Torres-Rovira *et al.*, 2014). Therefore, variation in serum AMH levels between ewes in this study may be related to their ovarian reserves. According to Acharya *et al.* (2020), serum AMH levels did not change in Katahdin ewes at 1-7 years of age. In the present study, age of ewes (2-3 years) did not affect serum AMH levels. Similarly, BCS did not affect serum AMH levels during estrus and diestrus phases of the estrous cycle in cross-bred Hamdani ewes. These results suggest that age (2-3 years) and BCS (2.75-3.25) do not affect ovarian reserve in adult ewes.

Extremely low concentrations of AMH are linked to infertility, especially in cases of premenopause and premature ovarian failure (Visser *et al.*, 2012). Similar to women, Cushman *et al.* (2023) showed that infertile ewes with fewer antral and primordial follicles also had lower serum AMH levels. Therefore, a relationship between AMH and pregnancy in sheep can be expected. In this

study, nine of 21 ewes were pregnant and their serum AMH levels were significantly higher compared to non-pregnant ewes during estrus and diestrus phases of the estrous cycle. This supports the findings of other studies which reveal that serum AMH levels may affect fertility in sheep. The AMH may affect fertility by regulating ovarian and uterine functions. Ferdousy *et al*. (2020) reported that AMH type 2 receptors were localized in both oviduct and uterus of cows. A previous study has also indicated that AMH exhibits a substantial degree of heritability (0.36) in cattle (Nawaz *et al.*, 2018). Because of low heritability of most of the reproductive traits in farm animals, favorable heritability of AMH may be beneficial to improve production in sheep breeding. Therefore, using AMH as a marker could be feasible to accelerate the genetic improvement of reproductive traits in sheep breeding.

According to different earlier studies, serum AMH could be used for predicting fertility and superovulation response in mammals including livestock species. In women, a cut-off value of 1.83 ng/mL for AMH was reported to predict response to ovarian stimulation (Riggs *et al*., 2008). In Japanese Black cattle, it was reported that >342 pg/mL of serum AMH may be used to predict superovulation response (Hirayama *et al*., 2017). On the other hand, AMH cut-off value for superovulation response was reported as 632.4 pg/mL in Gir (*Bos indicus*) breed of cattle (Feres *et al.*, 2021). However, data on cut-off serum AMH in sheep is limited. In a previous study, Lahoz *et al*. (2012) reported that a cut-off value of >97 pg/mL of serum AMH in young lambs could be used for predicting their fertility at first mating. In this study, it was found that >270 pg/mL of serum AMH could be used as a reliable marker for the evaluation of fertility in adult cross-bred Hamdani ewes.

Conclusion: In summary, this study is the first report showing serum AMH levels in cross-bred Hamdani ewes during estrus and diestrus phases of estrous cycle. Moreover, this study revealed that serum AMH levels do not change with age (2-3 years), BCS (2.75-3.25) and during estrus and diestrus phases of estrous cycle in cross-bred Hamdani ewes in their breeding season. However, its levels showed wide individual variations among animals. In addition, estrus or diestrus serum AMH could be used as a marker to predict fertility in non-prolific cross-bred Hamdani ewes. However, comprehensive studies may be carried out regarding the effect of AMH on fertility in sheep.

Ethical statement: This study was approved by Siirt University Animal Experiments Local Ethics Committee (Approval No: 2023/07-50).

Conflict of interest: There is no conflict of interest.

Authors' contributions: AOT conceived the idea, arranged necessary funding and software, carried out experimental work and prepared original draft of the manuscript. DK also conceived the idea, helped in experimental work, data analysis and preparation of original draft. Both authors reviewed and approved final version of the manuscript.

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