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**RESEARCH ARTICLE**

# Investigation of Anti-Müllerian Hormone Presence in Bitch Urine and Comparison of Anti-Müllerian Hormone Levels in Blood Serum and Urine

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## ABSTRACT

The presented study aimed to investigate the presence of AMH in bitch urine and to determine whether there is a correlation between blood serum and urine AMH levels. Forty-two healthy crossbreed bitches brought to Kafkas University, Faculty of Veterinary Medicine, Animal Hospital, Department of Obstetrics and Gynecology with a request for ovariohysterectomy were included in the study. Blood samples were taken from the Vena cephalica antebrachii, and urine samples were collected using a urinary catheter of all bitches. After the blood and urine samples were centrifuged, AMH levels were determined using the ELISA method. The average AMH concentration was determined to be 4.56±0.53 ng/mL in urine and 7.75±1.19 ng/mL in blood serum. No significant correlation was found between blood and urine AMH levels (P>0.05). It was concluded that urine AMH levels were not related to blood serum AMH levels.

**Keywords:** Anti-Müllerian Hormone, Bitch, Blood serum, Urine

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## Köpeklerde İdrarda Anti-Müllerian Hormon Varlığının Araştırılması ve Kan Serumu ve İdrarda Anti-Müllerian Hormon Düzeylerinin Karşılaştırılması

## ÖZ

Sunulan çalışmada köpek idrarında AMH varlığının araştırılması ve kan serumu ile idrar AMH düzeyi arasında korelasyon olup olmadığının belirlenmesi amaçlanmıştır. Kafkas Üniversitesi, Veteriner Fakültesi Hayvan Hastanesi, Doğum ve Jinekoloji Kliniği'ne ovaryohisterektomi isteğiyle getirilen sağlıklı 42 melez ırk köpek çalışmaya dahil edildi. Tüm köpeklerin Vena cephalica antebrahii'den kan örnekleri alındı ve idrar kateteri kullanılarak idrar örnekleri toplandı. Kan ve idrar örnekleri santrifüj edildikten sonra AMH düzeyleri ELISA yöntemiyle belirlendi. Kan serumunda ortalama AMH düzeyi 7,75±1,19 ng/mL, idrarda ise 4,56±0,53 ng/mL bulundu. Kan ve idrar AMH düzeyi arasında anlamlı bir korelasyon saptanmadı (P>0,05). İdrar AMH düzeyinin kan serumundaki AMH düzeyi ile ilişkili olmadığı kanaatine varıldı.

**Anahtar kelimeler:** Anti-Müllerian hormon, İdrar, Kan serumu, Köpek.

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## INTRODUCTION

Anti-Müllerian hormone (AMH), which is produced exclusively in ovarian tissue (Yağcı et al. 2016) and has a glycoprotein structure, belongs to the transforming growth factor-β (TGF-β) family (Bedenk et al. 2020). It limits the number of actively growing follicles by reducing the sensitivity of follicles with growth potential to FSH. Thus, it prevents premature exhaustion of primordial follicles (Akbarinejad et al. 2020). Since the oocyte reserve is preserved (Holst 2017), the reproductive life of the dog is extended (Akbarinejad et al. 2020). AMH levels are used both to investigate the ovarian reserve (Hollinshead et al. 2017; Yağcı et al. 2016) and to determine the presence or absence of the ovary in adult bitches with no information about their medical history (Axner and Holst 2015; Alm and Holst 2018; Yagci et al. 2016). It is also a reliable diagnostic tool for diagnosing ovarian remnant syndrome (Yilmaz et al. 2015). AMH is investigated either in ovarian granulosa cells (Karakaş Alkan et al. 2019) or blood (serum or plasma) samples (Kaya et al. 2024; Themmen et al. 2016). Since these methods are invasive, minimally invasive or non-invasive methods are being investigated as alternatives (Cai et al. 2023; Hallberg et al. 2024; Kaya et al. 2024; Pankhurst et al. 2016).

The excretion mechanism of AMH is not fully known (Griesinger et al. 2012). It is reported that only AMH that undergoes proteolytic degradation can be excreted in the urine (Pankhurst et al. 2016). It is stated that neuraminidases are effective in the destruction of AMH. It is thought that after exposure to the effect of neuraminidase, it is removed from the circulation by endocytosis in liver cells and destruction in hepatic lysozymes (Griesinger et al. 2012). In recent years, many studies have investigated the excretion pathway of AMH. In one of the studies investigating whether it is excreted via seminal plasma, the presence of AMH in the seminal fluid could not be detected (Hallberg et al. 2024), while AMH was detected in another study (Muhammed et al. 2018). There are a few studies investigating its excretion via urine. In one study, the presence of AMH in human urine was detected, and its level was determined quantitatively (Cai et al. 2023). However, another study reported that AMH in the urine of women with polycystic ovary syndrome was much lower than blood AMH levels and that urine AMH levels could not diagnose the disease (Ipandi 2024). No AMH was detected in urine samples from mice injected with recombinant human AMH, leading researchers to believe that AMH would not be excreted by the kidneys in mice (Pankhurst et al. 2016). In a study investigating the presence of AMH in the urine of cats, it was reported that AMH was present in urine, but its level did not fully reflect blood AMH levels. In this study, blood AMH levels were higher than 1 ng/mL in all non-neutered cats, whereas urine AMH levels were <1 ng/mL in 7 cats (Kaya et al. 2024). It is unknown whether the urinary excretion of AMH is species to the specific. The presence of AMH in urine has not been investigated in bitches. Therefore, in the present study, we investigated the presence of AMH in bitch urine and the correlation between blood AMH and urine AMH levels.

## MATERIALS and METHODS

### Animal Material

Forty-two crossbreed bitches brought to Kafkas University, Faculty of Veterinary Medicine, Animal Hospital, Obstetrics and Gynecology Clinic with a request for ovariohysterectomy were included in the study. These bitches, weighing an average of 11-31 kg (21.67±0.97) and aged 1-3 years. The study protocol was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK/2021-090).

### Method of Study

Bitches that were found to be healthy as a result of clinical and ultrasonographic examinations were included in the study. Vaginal cytology was performed in these bitches to determine the period of the sexual cycle. Vaginal smear samples were taken from the dorsal wall of the vagina by gently rolling with the help of cotton swabs and rolling onto the slide. The samples were stained with Diff Quick (MGG Quick Stain, Chembio Laboratory Research, Türkiye). The period of the sexual cycle was determined according to the morphology of vaginal cell types in the microscopic examination (Olympus Cx23). Bitches in anoestrus and diestrus were included in the study. According to vaginal cytology results, 27 bitches were determined to be in anoestrus and 15 bitches were in diestrus. Bitches were classified as <15 kg, 15-24 kg, and ≥25 kg according to their body weight. When the bitches in the study were grouped according to body weight, it was determined that 7 bitches were small (9.5-14 kg), 24 bitches were medium (15-24 kg), and 11 bitches were large (25-41 kg).

### Obtaining Blood Serum and AMH Analysis

Blood samples were taken from all bitches (Vena cephalica antebrahii). Urine samples were collected via a urinary catheter (Coloplast, EasiCath, Denmark). Urine and blood samples were centrifuged at 3000 rpm for 20 minutes, and the serum was separated. The serum was transferred to Eppendorf tubes and stored at -20°C until analysis. Analysis of serum and urine AMH levels was performed by the ELISA method using a commercial canine-specific kit (BT LAB, Bioassay Technology Zhejiang, China) according to

the manufacturer's recommendations. The measurement range was 0.2-60 ng/mL. Intra-assay and inter-assay coefficients of variation were <8% and <10%, respectively.

**Statistical Analysis**

Data analysis was performed using the IBM SPSS 26 statistical package program. All data were given as mean±standard error. Normality tests were performed using the Shapiro-Wilk test. Groups were compared according to sexual cycle period using the Mann-Whitney U test. Blood AMH levels in groups according to body weight were compared using the Kruskal-Wallis test. A Spearman correlation test was used to investigate whether there was a relationship between urine and blood AMH levels. P value <0.05 was considered statistically significant.

## RESULTS

AMH levels in blood serum and urine were 7.75±1.19 ng/mL (1.29-40.55 ng/mL) and 4.56±0.53 ng/mL (1.20-20.30 ng/mL), respectively. There was no significant correlation between blood and urine AMH levels (P>0.05).

During the anoestrus period, the mean blood serum AMH level was determined as 8.91±1.83 ng/mL and in the urine as 4.56±0.78 ng/mL. During the diestrus period, the blood serum and urine AMH levels were determined as 5.87±0.83 ng/mL and 4.56±0.64 ng/mL, respectively. Blood AMH levels did not change statistically significantly according to the period of the sexual cycle.

Mean blood and urine AMH levels were determined as 7.53±2.18 ng/mL, 3.54±0.52 ng/mL in small breed, 5.38±0.61 ng/mL, 4.87±0.80 ng/mL in medium-sized, and 13.06±3.80 ng/mL, 3.54±0.52 ng/mL in large breed bitches, respectively. No statistically significant difference was found between the groups in blood AMH levels (P>0.05).

## DISCUSSION

The anti-Müllerian hormone is used to detect females who have undergone surgical sterilization, to the presence of ovarian remnant syndrome, to diagnose granulosa cell tumors, and to determine ovarian reserve (Kaya et al. 2021). AMH is found in the bloodstream in 2 forms (proAMH and AMHN,C) (Pankhurst et al. 2016). ProAMH is a proprotein that is incapable of binding to AMH receptor II. Proproteins are converted to AMHN,C, which can bind to the receptor by undergoing proteolytic degradation via converting enzymes (such as subtilisin/kexin) (Pankhurst and McLennan 2016). While the proAMH form is more abundant in the ovary (follicular fluid and granulosa cells), the non-covalent complex AMHN,C is most abundant in the bloodstream (Pankhurst et al. 2016). Commercially available methods for determining AMH levels cannot distinguish between these two forms (Pankhurst and McLennan 2016). In recent years, the presence, levels, and clearance times of AMH in body fluids have been revealed in various studies. In a study investigating the level of AMH in intraperitoneal fluid, it was found that there was a similar level of AMH with blood serum and that there was a significant positive correlation between the AMH levels in the two fluids (Kostrzewa et al. 2020). Human recombinant AMH was administered to rats intraperitoneally and intratesticularly to investigate the changes in AMH levels in blood serum and testicular fluid. With intratesticular administration, AMH levels were detected in both serum and testicular fluid (Sriraman et al. 2001). While AMH in seminal plasma could not be detected in a study (Hallberg et al. 2024), AMH has been detected in urine in humans and cats (Cai et al. 2023; Kaya et al. 2024). This shows that AMH is present in some body fluids and its level can be detected. However, it is not found in all body fluids.

It is reported that the time it takes for AMH to be cleared from the body varies depending on the animal species. In a study conducted on rats by Anadol et al. (2016), the change in AMH levels in rats whose ovaries were removed was investigated. There was a statistically significant decrease even one day after the operation. AMH level decreased significantly until day 5 (0.52±0.13 ng/mL) and below the non-detectable level (0.31 ng/mL) on day 10 after the surgery. In another study, the AMH level, which was 5.8±1.5 ng/mL before ovariohysterectomy in anestrous dogs, decreased to 3.1±1.1 ng/mL on the first day following the operation and to 1.9±1.5 ng/mL on the fifth day. It decreased below 1 ng/mL on the 10th postoperative day (Anadol et al. 2020). In rats given human recombinant AMH, it was observed that the half-life was 8-12 hours, and then it was rapidly cleared from the circulation. Since the rat's own AMH did not react in primate-based MIS ELISA measurements, the rat's own AMH was not detected in the measurements, and it was reported that it did not affect the results (Sriraman et al. 2001). All these results are based on the rate of AMH loss after gonad removal. The most appropriate explanation is thought to be that this situation is due to differences in clearance between species or differences between recombinant and native AMH. It is reported that AMH in circulation is cleared from the body by proteolytic degradation and excretion in the urine (Pankhurst et al. 2016). The presence of AMH in urine was first determined in humans by Cai et al. (2023) and in cats by Kaya et al. (2024). No previous study was found in bitch urine. In the presented study, the presence of AMH in bitch urine was determined and presented quantitatively. When all these results were evaluated, it was seen that AMH was excreted via urine in bitches, but there was no correlation between blood and urine AMH levels.

## CONCLUSION

Urinary AMH level in bitches was measured and assessed quantitatively for the first time. Urine AMH level is not consistent with blood AMH level.

**Conflict of interest:** The authors have no conflicts of interest to report.

**Authors' Contributions**: SK contributed to the project idea, design, and execution of the study. GK, MCD, and MAK contributed to the acquisition of data. SK evaluated vaginal cytology. İK analyzed the hormone levels. SK and CK analyzed the data. SK and CK drafted and wrote the manuscript. All authors have read and approved the finalized manuscript.

**Ethical approval:** The study protocol was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK/2021-090).

**Explanation:** We have presented as an oral at the Anadolu 11th International Conference on Applied Sciences (2022).

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