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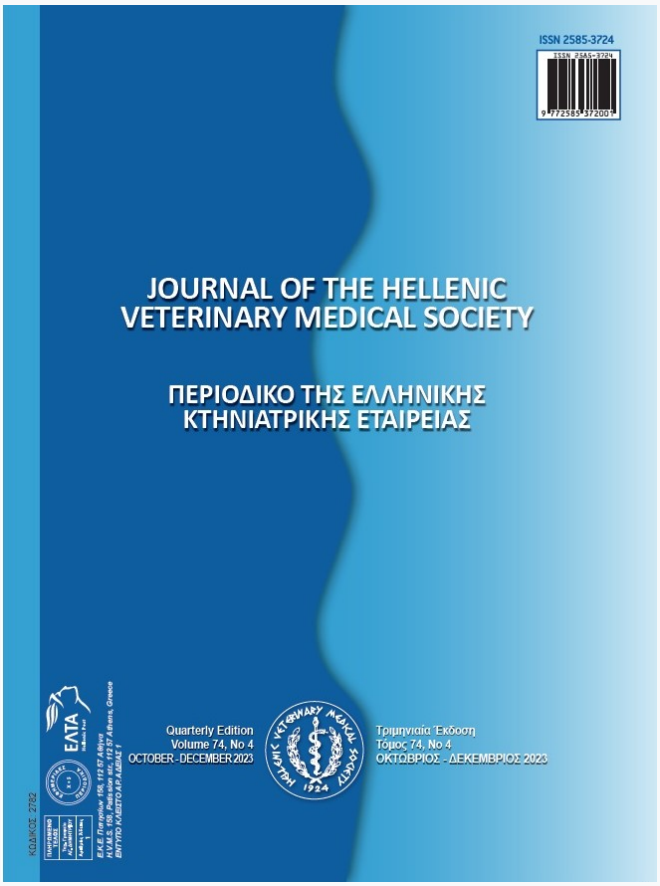

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Research article
Ερευνητικό άρθρο

Anti-Müllerian Hormone Expression in the Ovarian Follicles and Factors Related to Serum Anti-Müllerian Hormone Concentrations in the Domestic Queens

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ABSTRACT: Anti-Müllerian Hormone (AMH) is a glycoprotein with 140 kDa molecular weight secreted by the granulosa cells, playing a crucial role in folliculogenesis. The present study aimed to reveal (1) the relationship between age, body weight, estrouscycle, and ovariohysterectomy with the serum AMH concentrations and (2) expression of the AMH in the ovarian follicles. Serum AMH concentrations were determined by a commercial ELISA. Ovarian expression of the AMH in the granulosa cells was detected by immunohistochemistry. Twenty femalecats were allocated to three groups according to the period of the sexual cycle, age, and body weight. The phaseof their estrouscycle was determined according to serum estradiol 17β and progesterone levels. Blood samples were collected to detect serum AMH concentration on Day 0- preoperative, Day 3, and Day 10-postoperative. There was a decrease in AMH concentration on Day 3 compared to the preoperative level (Day 0) ($P<0.001$).No statistical difference in AMH concentration wasfound between Day 3 and 10 postoperative ($P>0.05$). Furthermore, the period of the sexual cycle, age, and body weight did not affect AMH levels ($P>0.05$). In immunohistochemical analyses,a mild AMH expression was first observed in the granulosa cells of the primary follicles, and moderate AMH expression was observed in the granulosa cells of the secondary follicles. Strong AMH expression was observed in the cumulus cells and mild AMH expression in the mural granulosa cells of the antral follicles. In conclusion, ovariohysterectomy caused a definite decline in serum AMH concentration, and AMH expression was found in all the ovarian follicles except for primordial follicles in domestic cats.

***Key Words*:** cat; ovary; folliculogenesis; antimüllerian hormone; immunohistochemistry

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INTRODUCTION

Female cats are seasonal polyestrous species with-induced ovulation (Bristol-Gould and Woodruff, 2006). Sexual activity depends on the day length. It starts with an increase in daylight, which causes a decrease in melatonin concentration, consequently promoting the Gonadotropin-Releasing Hormone (GnRH) secretion from the hypothalamus. GnRH provokes further Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH) secretion and promotes folliculogenesis (Schäfer-Somi, 2017). Folliculogenesis is controlled by various factors, including gonadotrophins and local intraovarian peptides (Bristol-Gould and Woodruff, 2006). Several factors, such as insulin-like growth factors (IGFs), epidermal growth factors (EGFs), and transforming growth factor- ß (TGF-ß) family members [inhibin, activin, growth differentiating factors (GDFs), bone morphogenetic proteins(BMPs)] are essential for folliculogenesis in domestic cats. These factors regulate oocyte development and granulosa cell proliferation, transformation, and steroidogenesis in ovarian follicle development (Kitiyanant et al., 2003; Phillips and Woodruff, 2004; Fujihara et al., 2014; Yıldırım et al., 2014; Kehoe et al., 2021; Perego et al., 2021).

Anti-Müllerian Hormone (AMH), also known as Müllerian inhibiting substance, is a glycoprotein with a molecular weight of 140 kDa belonging to the transforming growth factor-β family (Cate et al., 1986; Baarends et al., 1995). Its best-known role in the male fetus was described by Jost (1953) as inhibiting the development of the Müllerian ducts consequently allowing the male genital tract development. In females, AMH is only secreted by granulosa cells of the ovarian follicles and has a significant role in folliculogenesis (Bezard et al., 1987). The critical actions of the AMH are suppression of the primordial follicle activation mediated by stem cell factor (SCF) and fibroblast growth factor-7 (FGF-7) and suppression of the FSH-dependent follicle development, consequently preventing premature depletion of ovarian follicle reserve (Durlinger et al., 2002; Nilsson et al., 2007; Lebbe and Woodruff, 2013; Hu et al., 2014).

During the last decade, many studies have investigated the role of AMH in feline reproduction. These studies have focused on the diagnostic usefulness of AMH in various reproductive diseases, such as ovarian remnant syndrome, granulosa cell tumour, and pyometra (Heaps et al., 2017;Ottosson, 2017; Flock et al., 2022a;Gozer et al., 2023),and the detection of gonadectomy status (Gozer et al., 2023). Moreover, the effect of age and sexual cycle on serum AMH concentration has been identified (Flock et al., 2022b; Lapuente et al., 2023).However, knowledge of the role of AMH in feline folliculogenesis is scarce (Snoeck et al., 2017; Gültiken et al., 2022). The present study was carried out to reveal or reevaluate (1) the relationship between age, body weight, estrouscycle, and ovariohysterectomy with the serum AMH concentration and (2) the expression of the AMH in the ovarian follicles of the cat.

MATERIALS AND METHODS

The animal experiments required for the study were approved by the Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee with the decision number 2020/04-23 on 24.06.2020.

Animals

Twenty healthy female cats that have developed at least one estrus and without any gynecological diseases were included in the present study. The age of the cats varied between 5-48 months. The average body weight of the cats was 3.07± 0.5 kg. The cats were classified according to a) the phaseof the estrouscycle: estrus (n=8), diestrus (n=4), and anestrus (n=8), b) age: <1-year-old (n=8); > 1-year-old (n=12) as previously suggested by Pir Yagci et al. (2016) and c) body weight: 2-3 kg (n=10), > 3 kg (n=10).

Blood samples

Blood samples were collected from *v.cephalica*inserum tubes without anticoagulant to determine serum AMH, estradiol 17**β** (E_2), and progesterone (P_4) on Day 0 (preoperative) and Day 3 and 10 (postoperative). The blood samples were centrifuged at 3000 rpm for 5 minutes, and the sera were obtained and stored at -20 °C until analysis (Pir Yağcı et al., 2016).

Surgery

The cats were anesthetized by intramuscular administration of xylazine hydrochloride (1 mg/kg, Basilazin 2%, Bavet, Turkey) and ketamine hydrochloride (10 mg/kg, Ketasol 10%, Interhas, Turkey) ten minutes later. For prophylaxis, cefazolin sodium (20 mg/kg, Cefazol, 250 mg, Mustafa Nevzat, Turkey) was administered intramuscularly before the operation.Mid-line ovariohysterectomy was performed with the 3-clamp method (Santos et al., 2022). Meloxicam (0.2 mg/kg, Maksikam, Bavet, Turkey) was administered subcutaneously as an analgesic. Ovariohysterectomy operations were performed be-

tween 8 October 2020 and 1 March 2022.

Ovary Samples

Ovaries were kept in 10% formaldehyde solution after the ovariohysterectomy until immunohistochemical and histopathological examination (Alkan-Karakaş et al., 2019).

Detection of the estrouscycle

The concentrations of E_2 and P_4 were measured to determine the phaseof the estrous cycle. Queens with E_2 values less than 20 pg/ml were classified as anestrus, P_4 values greater than 1.5 ng/ml were classified as diestrus, and E_2 values above 20 pg/mlwere classified as estrus (Hamouzova et al., 2017).

Anti-Müllerian Hormone (AMH) measurement

A commercial enzyme-linked immunosorbent assay (Beckman Coulter ® AMH Gen II, USA) was used for the serum AMH measurement, following the manufacturer's instructions. Samples, standards, anti-AMH biotin conjugate, and streptavidin enzyme conjugate were added to the wells coated with primary antibodies, one step at a time, and incubated as suggested by the test procedure.After each incubation, the wells were washed with a washing solution five times.After the last washing step, chromogen solution was added and incubated. At the end of the incubation, stop solution was added into wells. Wells were read with an ELISA reader (ERBAManheim, Lisascan) at 450 nanometers.

Estradiol 17β and progesterone hormone measurements

Serum Estradiol 17**β** (Siemens, product no 10491445, Germany)and progesterone (Siemens, product no 01586287, Germany)levels were measured bydirect chemiluminescent method with fully automatic immunoassay autoanalyzer using commercial reagents (Siemens, Advia Centaur XP, Germany). The measurement ranges of the E_2 and P_4 kits were 11.80-3000 pg/ml and 0.21-60 ng/ml, respectively.

Histopathological examination

Formoldehyde-fixed tissue samples were dehydrated by successive immersion in 70, 80, 96, and 100% alcohol. Tissues were cleared with xylene before embedding in paraffin. Sections were cut at 4μm, deparaffinized in xylol, and stained with hematoxylin and eosin (H & E) after successive passage through 100, 96, 80,70% alcohol, and water.

Immunohistochemical examination

To demonstrate AMH expression and localization in tissues following immunostaining, the Avidin-Biotin-Peroxidase Complex (ABC) technique was performed according to the standard procedure prescribed in the commercial kit (SensiTek HRP, ScyTek Laboratories, Logan, UT®). Anti-AMH antibody (ab103233/1:50dilution/overnight/+4 °C) was used as the primary antibody. Hydrogen peroxide (3%in methanol)was used to block endogenous peroxidase activity.Endogenous avidin/biotin blocking kit (ab64212) for endogenous biotin blocking, and proteinase K as antigen retrieval (Abcam, ab64220®) were also used. Phosphate Buffered Saline (PBS) was applied to the tissues as a negative control. 3,3'-diaminobenzidine tetrahydrochloride (DAB, ScyTek Laboratories, Logan, UT®) was used as chromogen. The samples were counterstained with hematoxylin. Follicles were classified into primordial, secondary, antral, and atretic follicles as previously described (Bristol-Gould and Woodruff, 2006; Saint-Dizier et al., 2007). Immunohistochemistry results were classified by the same specialist as 0 (no reaction), 1+ (mild), 2+ (moderate), and 3+ (strong).

Statistical Analyses

Considering the primary hypotheses of the planned study, to determine the required minimum sample size before the study, it was calculated that it would be appropriate to be a minimum of 18 cats in the study, using the criteria of type 1 error probability (α) = 0.05, power (1-ß) = 0.80 and the effect size (f) = 0.6. Before starting the significance tests, the variables were analyzed with the Shapiro-Wilk test for normality and the Levene test for homogeneity of variances. In the study, the analysis of variance of repeated measurements method was used to evaluate the serum AMH concentrations in terms of time (day 0 preoperative and day 3 and 10 postoperative). As a post hoc test, simple effects analysis with Bonferroni correction was performed. The two-way mixed ANOVA method was used to evaluate AMH levels in terms of body weight (2-3 kg and >3 kg) and age (<12 months and ≥12 months) groups, time (0 day preoperative and 3 and 10 day postoperative) and their interaction. Simple effects analysis with Bonferroni correction was used as a post hoc test. The Kruskal-Wallis test was used to evaluate the AMH,E_2, and P_4concentrations in terms of the period of the sexual cycle (estrus, diestrus, and anestrus). The Mann-Whitney U test with Bonferroni correction was preferred as the post hoc

Table 1. Effect of the ovariectomy on the serum AMH concentration (ng/ml, mean ± SEM).

AMH (n=20)	Day 0	Day 3	Day 10	P
	4.35 ± 0.18[a]	0.73 ± 0.19[b]	0.32 ± 0.19[b]	<0.001

[a,b]: Different letters show statistical differences (p<0.05).

Table 2. Effect of the phase of the estrouscycle on serum AMH concentration and E_2 and P_4 levels (mean ± SEM)

Hormone	EstrousCycle Phase			P
	Diestrus (n=4)	Estrus (n=8)	Anestrus (n=8)	
AMH (ng/ml)	4.74 ± 0.82	4.15 ± 0.67	4.35 ± 0.5	0.96
E_2 (pg/ml)	26.66±5.32 [a]	26.23 ±1.75[a]	13.59 ± 1.09[b]	0.001
P_4 (ng/ml)	9.81 ±3.08 [a]	0.51±0.07[b]	0.40±0.06 [b]	0.007

[a,b]: Different letters show statistical differences (p<0.05).

Table 3. Serum AMH concentration in different body weights and age groups (ng/ml, mean ± SEM)

		AMH (ng/ml)	P
Body weight	2-3 kg (n=10)	4.78 ± 0.42	0.079
	> 3 kg (n=10)	3.70 ± 0.61	
Age	<12 months (n=8)	4.60 ± 0.85	0.534
	>12 months (n=12)	4.14 ± 0.49	

test. All statistical analyzes were performed with Stata 12/MP4 statistical package program. P<0.05 was considered the statistically significant criterion.

RESULTS

Serum AMH concentration

In the present study, there was a significant decrease in the serum AMH concentration on the 3rd and 10th postoperative days compared to the preoperative concentration (day 0) (P<0.001). No significant differences were found between the postoperative 3rd and 10th days (Table 1). There was no significant relationship between serum AMH concentration and the phaseof the estrouscycle (Table 2), the age, or the body weight (Table 3).

Immunohistochemical results

The present study detected AMH immunoreaction in all follicular stages except primordial follicles. In primary follicles, mild (+1) AMH immunoreaction was observed in the granulosa cells (Fig 1). In secondary follicles, moderate (+2) AMH immunoreactions were detected in the granulosa cells. However, no immunoreaction was observed in the thecal cells of the secondary follicles (Fig 2). In antral follicles, strong (+3) AMH immunoreaction was observed in

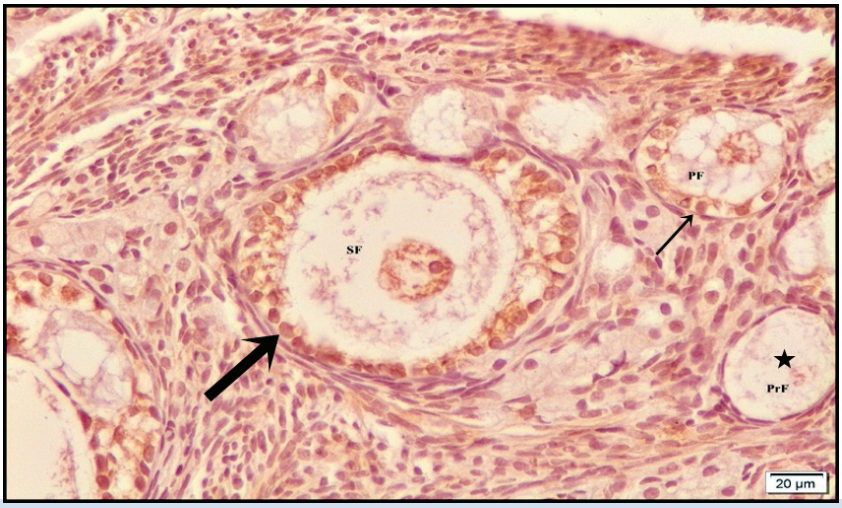

Fig. 1. AMH expression in the primordial, primary, and secondary follicle. Primordial follicle (PrF) (star), Primary Follicle (PF)(thin arrow), Secondary Follicle (SF)(thick arrow),40x, IHC.

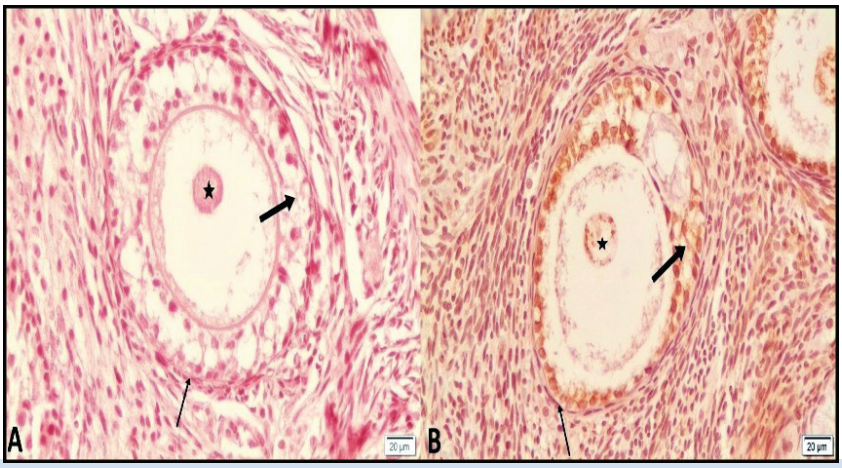

Fig. 2. AMH expression in the secondary follicle. A: Histopathological image of the secondary follicle; granulosa cell (thick arrow), thecal cell (thin arrow); oocyte (star) 40x, HE. B: AMH expression in the secondary follicle; granulosa cell (thick arrow), thecal cell (thin arrow); oocyte (star) 40x, IHC.

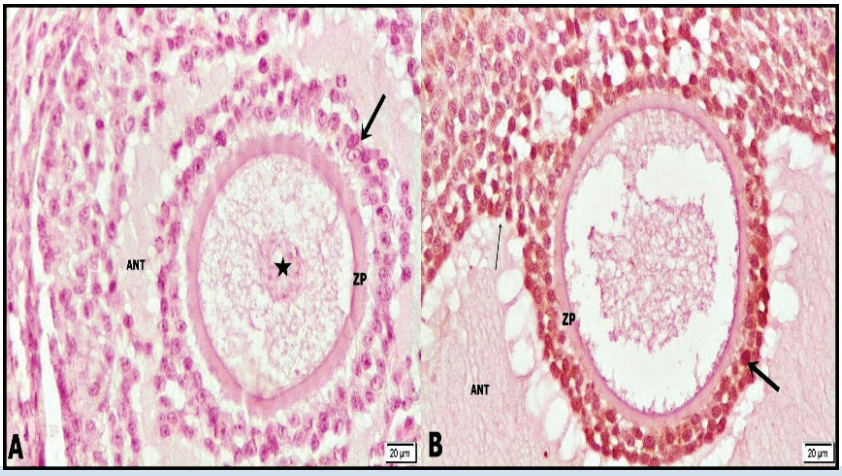

Fig. 3. Cumulus oophorus. A: Histopathological image of the cumulus oophorus; antrum (ANT), zona pellusida (ZP), oocyte (star) 40x, HE. B: AMH expression in cumulus oophorus; antrum (ANT), zona pellusida (ZP), in cumulus oophorus (arrow), oocyte (star) 40x, IHC.

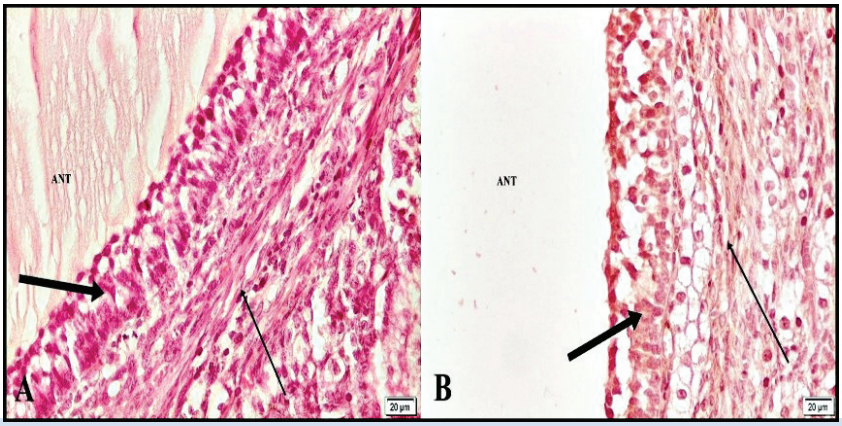

Fig. 4. AMH expression in mural granulosa and theca cell. A: Histopathological image of mural granulosa and thecal cell; antrum (ANT), granulosa cells (thick arrow), theca cell (thin arrow) 40x, HE. B: AMH expression in mural granulosa and theca cell; antrum (ANT), granulosa cells (thick arrow), theca cell (thin arrow) 40x, IHC.

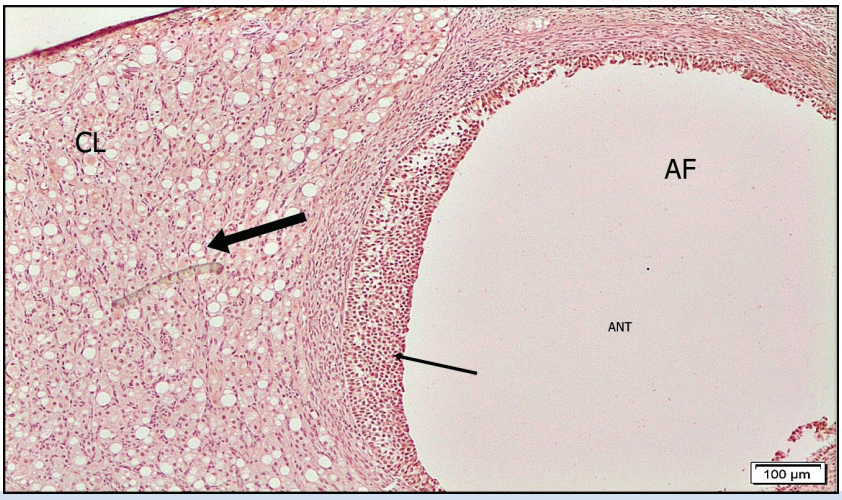

Fig. 5. NegativeAMH expression in corpus luteum (thick arrow); AMH expression in the antral follicle (thin arrow),antrum (ANT), the antral follicle (AF), corpus luteum (CL), 10x, IHC.

the cumulus oophorus cells (Fig 3), whereas mild (+1) AMH immunoreaction was detected in mural granulosa cells (Fig 4). No AMH immunoreaction was detected in the corpus luteum (Fig 5). In immunohistochemical examinations, the best-qualified pictureswere chosen to show the AMH immunoreaction in the ovarian follicles. There were no statistical analyses performed on the ovarian expression of the AMH according to estrous cycle phase. The current study only evaluated AMH expressions of ovarian follicles via immunohistochemical methods.

DISCUSSION

Anti-Müllerian Hormone (AMH) is a transforming growth factor-ß family member and is only secreted by the granulosa cells in the ovarian follicles (Durlinger et al., 2002). Ovariohysterectomy causes a rapid decline in serum AMH concentration (Pir Yağcı et al., 2016). Therefore, determining the presence of ovarian tissue through serum AMH measurement is a common practice in feline reproduction. Serum AMH concentration in the spayed cats varied between 0.01-0.5 ng/ml (Place et al., 2011; Axnér et al., 2015; Heaps et al., 2017; Flock et al., 2022b; Gültiken et al., 2022; Gozer et al., 2023). In this study, decreasing concentrations of AMH were observed following the spaying, and postoperative Day 10 AMH concentrations in line with the previous studies (Table 1).

It has been reported that serum AMH concentration declines as the female ages (Snoeck et al., 2017). Age-related decline in the follicular reserve led to decreased serum AMH concentration (Lahoz et al., 2014; Snoeck et al., 2017; Ball et al., 2019; Ferré-Dolcet et al., 2022; Lapuente et al., 2023). In our study, numerically higher serum AMH concentrations were observed in cats that were younger than oneyear than in cats older than 1year.This may be attributed to the small number of animals used in the study. Serum AMH concentration of cats older than 1year align with the ones reported by Snoeck et al. (2017). In our study, AMH concentrations in cats younger than 1year were lowercompared to those measured in other studies (Snoeck et al., 2017; Ferré-Dolcet et al., 2022; Lapuente et al., 2023). This could be the result of differences in the immunoassay, age classification, and individual differences (Flock et al., 2022b; Lapuente et al., 2023).

In cats, a definite pattern of how AMH courses in the estrus cycle hasnot been established yet (Lapuente et al., 2023). It has been reported that serum AMH concentration is high during anestrus and interestrus but low during estrus (Flock et al., 2022b); however, there is no difference between proestrus and estrus periods (Gültiken et al., 2022). Lapuente et al. (2023) stated that higher AMH concentration wasfound in the anestrus phase than in the follicular phase. Gültiken et al. (2022) reported that higher AMH concentration was observed in the pregnant cat. The current study found no statistical differences in the serum AMH concentration in different phasesof the estrous cycle (Table 2). AMH concentration in different phasesof the estrous cycle of cats is similar to that found in women (Hehenkamp et al., 2006), mares (Almeida et al., 2011), and goats (Monniaux et al., 2011).

In the current study, although not significant, numerically higher serum AMH concentration was-

found in the diestrus. This was unexpected since there was no immunoreaction of AMH in the corpus luteum (Fig.5).Flock et al., (2022b) stated that the median AMH concentration was 6.38 ng/ml (4.50-10.75) and 6.47 ng/ml (5.60-9.80) in metestrus and pregnant cats, respectively. Gültiken et al., (2022) also observed that mean serum AMH concentrationwas 2.95±1.21 and 2.83±1.33 ng/ml in 32-40 and 41-46 days of pregnancy in domestic cats. In the current study, mean AMH concentration was4.74 ± 0.82 ng/ml.Our results might be attributed to the small sample size and the individual differences, as previously reported (Flock et al., 2022b). A larger sample size may be more efficient to determine serum AMH concentration in domestic cat. However, it does not seem possible to predict how it can affect the results.It was also reported that median AMH concentration can be different among and within individual cats and high variation can be observed in a single cat throughout the estrus cycle (Flock et al., 2022b).Furthermore, a recent study by Gültiken et al. (2022) stated that the expression of AMH wasobserved in the feline corpus luteum and higher AMH concentration was found during pregnancy. Further investigations are needed to elucidate the relationship between AMH and corpus luteum in cat.

Previous studies have reported contradictory results regarding the relationship between the body weight and serum AMH concentration. Moy et al. (2015) stated that a negative relationship exists between obesity and serum AMH concentration. However, other studies stated no relationship between body weight and serum AMH concentration (Skałba et al., 2011; Sahmay et al., 2012). The current study found numerically higher serum AMH concentration in cats with a body weight of 2-3 kg compared to those with a body weight of >3 kg (p=0.079). However, these results should be cautiously interpreted since they might come from younger cats weighing 2-3 kg than from cats weighing>3 kg. More detailed studies are needed to investigate the effect of body weight on AMH concentration in cats.

AMH expressions in the ovarian follicles were studied in different species, such as sheep, human, cow, porcine, and dog. Generally, AMH expression had development-dependent characteristics in the ovarian follicles (Bezard et al., 1987; Weenen et al., 2004; Almeida et al., 2018; Teh et al., 2018; Alkan-Karakaş et al., 2019). In our study, AMH expression was first observed in the granulosa cells of the primary follicles. As the follicle size increased, stronger AMH expression was encountered in the granulosa cells of the ovarian follicles in the cat (Fig 1). These results may indicate that AMH expression in the ovarian follicles havestage-dependent characteristics in the domestic cat.

In the current study, strong (3+) AMH expression was encountered in the cumulus oophorus cells (COCs), whereas mild (1+) AMH expression was detected in mural granulosa cells (Fig 3). Stronger AMH expression in the cumulus cells might result from the effect of oocyte-originated growth factors on the COCs(Convissar et al., 2017; Zhao et al., 2018). Monniaux et al. (2012) stated that oocyte-originated growth factors, such as bone morphogenetic factors (BMPs) and growth differentiating factors (GDF-9), affect the expression of AMH. In various species, it has beenreported that BMP-4, BMP-6, BMP-15, and GDF-9 increase AMH mRNA expression (McNatty et al., 2005; Rico et al., 2011; Convissar et al., 2017; Zhao et al., 2018). Previous studies have illustrated that cumulus cells havegreater AMH expression than mural granulosa cells (Munsterberg and Lovell-Badge, 1991; Salmon et al., 2004; Grøndahl et al., 2011). Salmon et al. (2004) indicated higher AMH expression at the oocytes collected from the preovulatory follicles co-cultured with the cumulus granulosa cells.

It has been reported that serum AMH concentration iscorrelated with the antral follicles count (Kevenaar et al., 2006; Ireland et al., 2008; Anadol et al., 2020; Böhmer et al., 2022) and the relationship between serum AMH concentration and follicles count have beenstudied in many species (Monniaux et al., 2011; Rico et al., 2009). Lapuente et al. (2023) reported that serum AMH concentration is correlated with the small antral follicles in domestic cat. In the current study, follicle counting and correlation between follicle count and AMH concentration were not performed. However, immunohistochemical results showed that AMH expression was first observed in the primary follicles (Table 5). Therefore, it can be inferred that circulating AMH may originate from the primary, secondary, and antral follicles in domestic cat. Further studies are needed to reveal the contribution of the preantral follicles to the serum AMH concentration.

CONCLUSION

In brief, AMH can detect the ovariectomy status in domestic cat. Estrouscycle, age, and body weight

did not affect the serum AMH concentration. Primary, secondary, and antral follicles contribute to the serum AMH level. Strong AMH expressions were observed in the cumulus oophorus in the antral follicles. Anti-Müllerian Hormone expression showed development-dependent characteristics.

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CONFLICT OF INTEREST

None declared by the authors

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