Polish Journal of Veterinary Sciences Vol. 23, No. 3 (2020), 391–397

DOI 10.24425/pjvs.2020.134683

Original article

Investigation of diagnostic use of serum anti-Müllerian hormone concentration in dioestrus and anoestrus bitches before and after ovariohysterectomy and the relationship with ovarian follicle numbers

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Abstract

The aims of the study were to (1) compare the serum concentration of anti-Müllerian hormone (AMH) with the number of follicles in ovaries and (2) determine the serum AMH concentration before and after ovariohysterectomy in dioestrus and anoestrus bitches. Sixteen bitches were divided into two groups: Group I (n=8) consisted of dioestrus and group II (n=8) anoestrus bitches. The blood samples for AMH assesment were taken before ovariohysterectomy (day 0) and on day 1, 5 and 10. Both in group I and II, serum AMH concentrations on day 1 and 5 were significantly different compared to day 0 ($p<0.05$). However, the concentrations at day 10 were under the minimum detectable concentration (1.0 ng/mL) and this finding revealed that ovaries are the only source of AMH synthesis. Follicle counts were not statistically different between the groups ($p>0.05$). Significantly positive correlation in serum AMH with secondary follicle numbers ($r=.942$, $p<0.01$), as well as negative correlation with antral follicle numbers ($r=-.765$, $p<0.05$) were determined in the group I. In the group II, positive correlations between serum AMH concentration and secondary follicle numbers ($r=.960$, $p<0.01$) and early antral follicles ($r=.726$, $p<0.05$) were noted. Assesment of AMH concentration seems to not only provide the diagnosis of the presence of ovaries but also correlate with the number of secondary follicles in young dioestrus and anoestrus bitches.

Key words: anti-Müllerian hormone, aneostrus, bitch, dieostrus, ovarian follicles

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Introduction

Unknown history of the gonadal status of female dogs might be an important issue particularly regarding adoption of shelter dogs. Since the dogs are not microchipped in most countries, determination of whether they are ovariectomized or not becomes difficult. The probability of the appearence of a surgical scar depends upon the techniques of ovariectomy or ovariohysterectomy, therefore explarotory laparotomy might be necessary to detect the presence or absence of gonads, especially for abandoned animals in shelters. Real time ultrasonographic examination might be used to determine the follicular growth in the bitch (Hase et al. 2000); although ovaries are easier to identify as follicular development progresses (Wallace et al. 1992). Evaluation of serum oestrogen or progesterone concentrations together with vaginal cytologic examination might be useful but only during proestrus and oestrus (Feldman and Nelson 2004). It is known that basal plasma luteinizing hormone (LH) concentration is higher in ovariectomized bitches than in oestrous bitches and GnRH administration leads to an increase in plasma oestradiol concentration of intact bitches (Buijtels et al. 2006, Anadol and Bastan 2007). However, the sensitivity of the measurement of LH is not reliable (Scebra and Griffin 2003, Rohlertz et al. 2012) and single assesment was not found to be safe for distinguishing between spayed and intact bitches (Lofstedt and Vonleeuwen 2002). As a promising alternative, anti-Müllerian hormone (AMH) has been investigated in different species. AMH is a member of the transforming growth factor-beta superfamily and is known for its role in male sex differentiation. It is produced by Sertoli cells of the male embryo in order to induce regression of Müllerian ducts (Munsterberg and Lovell-Badge 1991). Hence, testosterone stimulates differentiation of the Wolffian ducts. In females, Mullerian ducts differentiate into uterus, oviduct and upper third of the vagina in the absence of AMH (Lee and Donahoe 1993). In the domestic cat and dog, measurement of serum AMH was determined to be highly effective at distinguishing ovariohysterectomized from intact adults (Place et al. 2011) as well as for the diagnosis of canine ovarian remnant syndrome (Pir Yagci et al. 2016) and the prediction of litter size (Hollinshead et al. 2016).

Since AMH is secreted by small growing follicles in the ovary, its blood concentration exhibits the ovarian follicular pool (La Marca et al. 2013), and is therefore considered a powerful biomarker of ovarian reserve in the mouse (Durlinger et al. 1999) and human (Broer et al. 2011, Jamil et al. 2016). However, advanced and accurate prediction and control of the reproductive cycles of domestic dogs for assisted reproductive technologies are limited. Insight into the size of the ovarian reserve would greatly aid endangered canid conservation efforts and AMH might be a useful marker to predict the response to oestrus induction treatments similar to its utility in women. We therefore aimed in this study to determine the correlation between the blood serum concentration of AMH with the number of ovarian follicles and to evaluate the serum AMH concentration before and after ovariohysterectomy in dioestrus and anoestrus bitches.

Materials and Methods

Animals and collection of samples

Sixteen post-pubertal, intact mixed breed bitches of 1.5 to 2.5 years of age with a mean weight of 22.15±2.27 kg were assigned to this study that were presented to the clinic with a request for spaying by their owners. The age range was limited in this study since there is a significant effect of the age of the bitch on AMH concentrations (Hollinshead et al. 2016). All the bitches were subjected to routine physical, hematological and ultrasonographical examinations and only healthy ones were included in the study. The stages of oestrus cycle were determined by vaginoscopic and cytologic examinations (Johnston et al. 2001). Vaginal smears were obtained from the anterior vaginal wall with the use of a vaginoscope, stained using the Papanicolaou technique (Papanicolaou 1942) and evaluated with a light microscope (Leica Microsystems Inc., Illionis, USA). The bitches were then divided into groups: Group I (n=8) consisted of dioestrus and group II (n=8) anoestrus bitches. Ovariohysterectomy was performed under inhalation anaesthesia. Following the operation, both ovaries were fixed in 10% neutral formalin for follicle counting.

The blood samples for AMH assesment were taken immediately before ovariohysterectomy (day 0) and on day 1, 5 and 10 thereafter. Sera were removed after centrifugation at 1550 g for 10 min. and stored at -80°C until analyzed. All bitches were hospitalized until day 10, fed commercial dog food and given water *ad libitum.*

Ethical approval was obtained from the animal ethics committee, University of Ondokuz Mayis, Samsun, Turkiye (approval number 2015/35).

Serum AMH measurements

The serum AMH concentration was quantitatively assessed by an enzyme-linked immunosorbent assay using a commercially available canine-specific kit (MBS741626, MyBioSource, Inc. San Diego, CA

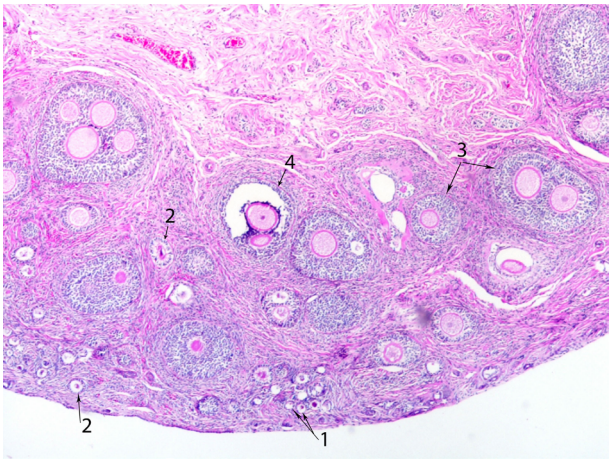

Fig. 1. Appearence of follicles in a bitch: primordial (1), primary (2), secondary (3) and antral follicle (4), Hematoxylin and eosin, x20.

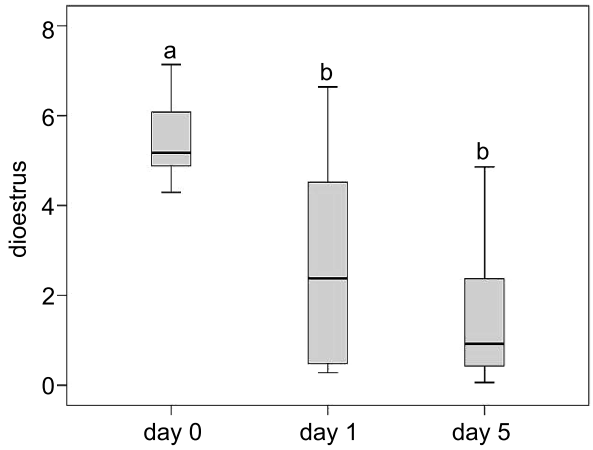

Fig. 2. Serum anti-Müllerian hormone (AMH) concentrations (ng/ml) in the dioestrus group ([a,b] Tamhane's T2 test, $p<0.05$).

92195-3308, USA) following the manufacturer's protocol. The assay was performed according to the manufacturer's instructions and assayed in duplicate. The detection range was 1.0-25.0 ng/mL. Intra-assay and inter-assay coefficients of variation were <5% and <10% respectively.

Counting of follicles

The ovaries were bisected longitudinally, embedded in paraffin wax, serially sectioned (50 slices on average per ovary) at 5 µm and stained with haematoxylin and eosin. Every tenth section was selected for observation under a light microscope (Nicon, Eclipse E600). The total count of five sections of each ovary was calculated. The mean number of follicles was then determined. Sections were analyzed by light microscopy at 240×magnification, and all follicles were counted. Two independent observers performed the histological evaluations (MY, EK). To minimize the possibility of counting follicles more than once, only follicles with a visible oocyte nucleolus were recorded (Li et al. 2013). The follicles were classified into five stages as follows: primordial, primary, secondary, early antral and antral follicles (Fig. 1). The morphological classification of growing follicles was done as described previously (Songsasen and Wildt 2007).

Statistical Analysis

Statistical analyses were performed with SPSS, version 22.0 for Windows. The results were given as the mean ± standard deviation. Normal distribution and homogeneity of variances were confirmed by Shapiro-Wilks and Levene's tests, respectively. Comparison of variables between the groups was carried out by Independent Samples T test and Tamhane's T2 Test. Pearson's correlation test was used to evaluate correlation among follicle numbers and serum AMH concentration. Values with $p<0.05$ were considered statistically significant.

Results

Clinical findings

Ultrasonographic examination of reproductive organs did not reveal any abnormalities. This finding of ovaries and uterus was confirmed macroscopically after ovariohysterectomy. Evaluation of vaginal smears displayed intermediate and parabasal cells and abundant neutrophils for dioestrus bitches and only basal and parabasal cells for anoestrus bitches (Johnston et al. 2001).

Serum AMH concentrations and numbers of follicles

Serum AMH concentrations in group I and group II were 5.5±0.9 ng/mL and 5.8±1.5 ng/mL on day 0, 2.9±2.3 ng/mL and 3.1±1.1 ng/mL on day 1 and 1.6±1.6 ng/mL and 1.9±1.5 ng/mL on day 5, respectively (Figs. 2 and 3). The difference between groups was statistically significant only at day 1 ($p<0.05$) (Fig. 4B), whereas there was no statistically significant difference at day 0 and 5 ($p>0.05$) (Figs. 4A and 4C). However, in both groups serum AMH concentrations on day 1 and 5 were significantly lower compared to day 0 ($p<0.05$) (Figs. 2 and 3). At day 10 AMH values were below the minimum detectable concentration of 1.0 ng/mL.

Counting of follicles showed that the numbers of primordial follicles were 216.3±153.1 and 195.6±156.5, primary follicles were 91.8±32.1 and 89.5±33.1, secondary follicles were 85.3±34.6 and 61.8±33.8, early antral follicles were 16.6±8.3 and 13.8±10.4, antral follicles were 5.1±4.8 and 6.4±3.3 in group I and group II, respectively. Follicle counts were not statistically diffe-

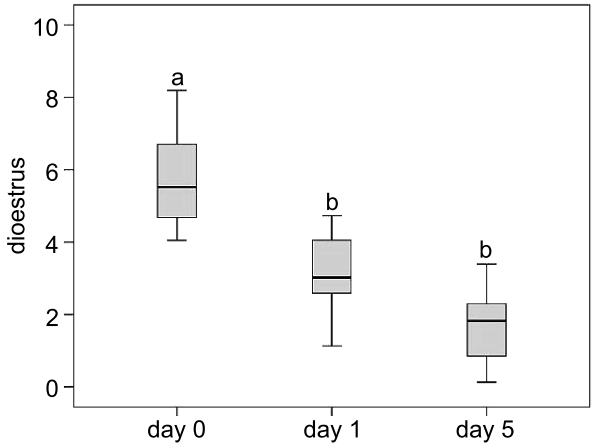

Fig. 3. Serum AMH concentrations in anestrus group ([a,b] Tamhane's T2 test, $p<0.05$).

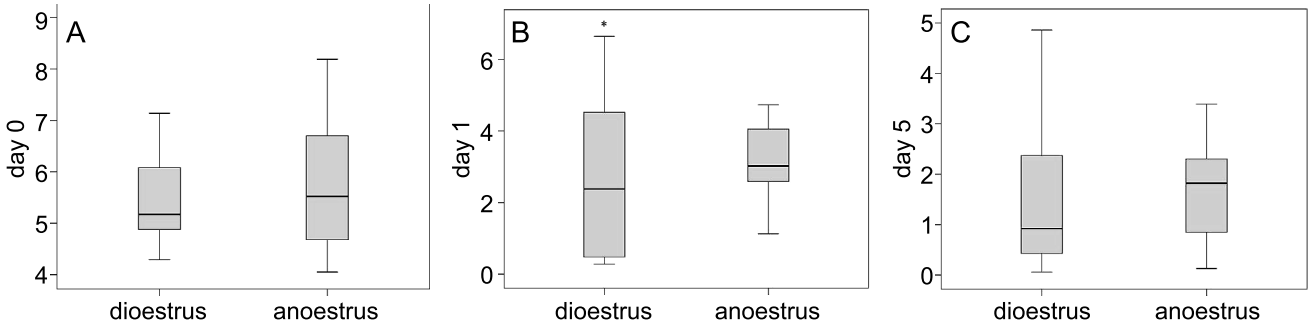

Fig. 4. Differences of the serum AMH concentrations between the groups on day 0 (A), day 1 (B) and day 5 (C) (*$p<0.05$, independent samples t-test). In each box, the central mark represents the median, the edges of the box represent the 25th and 75th percentiles, and the whiskers are the most extreme data points not considered to be outliers.

rent between the groups ($p>0.05$). Significantly positive correlation in serum AMH with secondary follicle numbers ($r=.942$, $p<0.01$), as well as negative correlation with antral follicle numbers ($r=-.765$, $p<0.05$) were determined in group I. In group II, positive correlations between serum AMH concentration and secondary follicle numbers ($r=.960$, $p<0.01$) and early antral follicles ($r=.726$, $p<0.05$) were noted.

Discussion

In the domestic cat, assessment of AMH was confirmed to have a hundred percent sensitivity and specificity to diagnose the presence or absence of ovaries (Axner and Strom 2015). Place et al. (2011) indicated that determination of serum AMH level can facilitate detection of the presence of ovaries in both adult dogs and cats. Similarly, Themmen et al. (2016) reported that AMH assay is a useful tool to diagnose gonadal status in bitches. Additionally, several studies showed that AMH level is higher in both intact bitches and bitches with ovarian remnant syndrome (ORS) than spayed ones. Therefore, measurement of serum AMH level provides diagnosis of ORS in bitches (Turna Yilmaz et al. 2015, Pir Yagci et al. 2016). In our study, serum AMH concentration on day 1 and 5 was significantly lower compared to day 0, however at day 10 it was under the detectable concentration showing that AMH level decreases gradually in the absence of dog ovaries. Accordingly, Pir Yagci (2016) measured the concentration less than or equal to 0.006 ng/mL at day 10 after OHE which was significantly lower than it was before OHE. Our previous study performed on rats also revealed that serum AMH concentrations gradually decreased from day 1 to 5 and were undetectable on day 10 after OHE, again proving that AMH concentration depends upon the presence of ovaries (Anadol et al. 2016). The need to determine gonadal status in bitches emerges in shelters or in the case of missing bitches. Compared with other methods such as detection of LH concentration or GnRH administration, measuring AMH concentration seems to be more reliable. On the other hand, AMH has been a subject of numerous studies focusing on infertility. In the cow, high plasma AMH concentrations were determined to be associated with highest follicular and ovulatory responses to treatment and it was concluded to be an useful indicative of the number of gonadotropin-responsive follicles (Rico et al. 2009). Another study revealed that concentrations of AMH were similar between synchronized

and natural oestrous cycle but differed between beef and dairy heifers (Pfeiffer et al. 2014). Similarly, Lahoz (2012) stated that AMH concentration might be a reliable marker for ovarian status of prepubertal ewe lambs to predict their fertility. The concentrations were also evaluated in different pathological conditions and detected to be reduced in hemorrhagic anovulatory follicles (Lahoz et al. 2012) and to be a useful marker for the diagnosis of granulosa cell tumours in mares (Ball et al. 2013) and in women (La Marca and Volpe 2007). The above-mentioned relationship between AMH and ovarian status brought to mind whether there is an association between serum AMH concentrations and the number of ovarian follicles in the bitch. Though there are studies concerning this association in the other species, to our best knowledge this is the first report in the bitch.

Kevenaar (2006) determined a strong correlation between AMH concentrations and numbers of growing follicles, particularly with the numbers of primordial follicles in mice. Furthermore, they suggested that the decrease in the concentration with increasing age might be caused by a decline in AMH expression per follicle. Similarly, several studies indicated a positive relationship between antral ovarian follicle population and plasma AMH concentration in cattle (Monniaux et al. 2012, Baldrighi et al. 2014, Batista et al. 2014). Claes (2016) determined a positive relationship between antral follicle count and AMH in the old mares and antral follicle count was significantly lower in older mares compared to middle-aged ones. However, there is no information on the correlation between AMH concentration and the number of ovarian follicles in the queen or bitch. Our results in the bitch revealed a significant negative correlation between serum AMH concentration and antral follicle numbers ($r=-.765$, $p<0.05$) in dioestrus; though a significant positive correlation was observed between serum AMH concentration and early antral follicles in anoestrus bitches ($r=.726$, $p<0.05$). On the other hand, there was a significantly positive correlation in serum AMH with secondary follicle numbers in both dioestrus and anoestrus bitches. However, there was no correlation with the number of primordial follicles as it was found in mice (Kevenaar et al. 2006).

Follicle stimulating hormone (FSH) is known to have a stimulatory effect on AMH expression (Josso et al. 2001) and AMH inhibits the initiation of primordial follicle growth in the mouse ovary, therefore exhaustion of the follicle pool (Durlinger et al. 2002). In other words, while FSH stimulates the growth of pre-antral and small antral follicles AMH inhibits that effect (Durlinger et al. 2001). Anti-Müllerian hormone is expressed in granulosa cells of growing follicles until they reach the stage of cyclic recruitment controlled by FSH (Rico et al. 2009). Similarly, AMH expression has been shown to be low in large antral follicles in human (Nielsen et al. 2010) and cow (Monniaux et al. 2008). Our results show that there is a significantly positive correlation in serum AMH with secondary follicle numbers and a negative correlation with antral follicle numbers in dioestrus bitches that agrees with the above-mentioned data.

According to the study performed by Hollinshead (2016) to establish the normal reference interval for AMH in oestrous bitches using a canine-specific AMH assay, the mean AMH concentration in bitches less than 4 years of age was 12.4±5.9 ng/mL, whereas it was 10.5±5.2 ng/mL in bitches older than 4 years. For each additional year, the concentration fell by 0.5 ng/mL. Our study revealed that serum AMH concentrations in dioestrus and anoestrous bitches between the ages of 1.5 and 2.5 years were 5.5±0.9 ng/mL and 5.8±1.5 ng/mL, respectively. Nagashima (2016) detected a rise in serum AMH during early proestrus and a decline back to the baseline values prior to the LH surge. Recently, Walter (2019) showed that there was a high inter-individual variation in AMH concentrations at every stage of the oestrous cycle between animals and the concentrations vary significantly during the cycle in the bitch. Karakas Alkan (2019) found significantly higher concentrations during proestrus and oestrus compared with other stages. They also stated that there was no statistical difference between the concentrations of dioestrus and anoestrous bitches. Similarly, our results did not reveal a statistical difference between the mean AMH concentrations of dioestrus and anoestrous bitches. In women, fluctuations of AMH concentrations in the menstrual cycle were shown to be minor, suggesting that AMH can be measured independently of the cycle phase (La Marca and Volpe 2007).

Many studies suggest that age-related decrease in the number of growing follicles may be correlated with a reduction in AMH concentration, as it reflects the ovarian pool in women (de Vet et al. 2002, van Rooij et al. 2002). Accordingly, Claes (2016) determined a strong correlation in old mares, a moderate correlation in middle-aged mares, and no significant correlation between antral follicle count and AMH in young mares. In the present study, both dioestrus and anoestrus bitches were between 1.5 and 2.5 years of age, and whether the correlation is affected by age and older bitches have positive correlation between the follicle count and AMH concentration needs to be shown by further studies. Hollinshead (2016) stated that the concentrations were lower in giant breeds than small, medium and large breeds, though there was no difference in concentrations among small, medium and

large breeds. In our study, both groups included mongrel dogs with a mean weight of 22.15±2.27 kg.

In conclusion, the fact that the concentrations were undetectable at day 10 after ovariohysterectomy revealed that ovaries are the only source of AMH synthesis and measuring circulating AMH concentrations is an effective way to detect the presence of ovaries. Moreover, there is a significantly positive correlation in serum AMH concentration with secondary follicle numbers in young dioestrus and anoestrus bitches.

Acknowledgements

An earlier version of this paper was presented at XXth EVSSAR Congress, University of Veterinary Sciences, Vienna, Austria, 29 June - 1 July, 2017.

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