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Research Article

# Differences in the Follicular Morphology of Young and Aged Bitches and Their Correlation with the Anti-Müllerian Hormone

Ömer KORKMAZ [1] Deniz KORKMAZ [2] İbrahim Mert POLAT [3] İlknur PİR YAĞCI [3]
Mert PEKCAN [4] Birten EMRE [1] Abuzer Kafar ZONTURLU [1]

[1] Harran Üniversitesi, Veteriner Fakültesi, Doğum ve Jinekoloji Anabilim Dalı, TR-63200 Eyyubiye, Şanlıurfa - TÜRKİYE
[2] Harran Üniversitesi, Veteriner Fakültesi, Histoloji-Embroyoloji Anabilim Dalı, TR-63200 Eyyubiye, Şanlıurfa - TÜRKİYE
[3] Kırıkkale Üniversitesi, Veteriner Fakültesi, Doğum ve Jinekoloji Anabilim Dalı, TR-71450 Yahşihan, Kırıkkale - TÜRKİYE
[4] Ankara Üniversitesi, Veteriner Fakültesi, Biyokimya Anabilim Dalı, TR-06110 Dışkapı, Ankara - TÜRKİYE

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## Abstract

This study aimed to investigate the structural, morphological and cellular differences of follicles at different stages of follicular development as well as to determine the correlation of the ovarian follicle population with serum Anti-Müllerian Hormone (AMH) levels in young and aged bithches. Sixteen bitches were divided into two groups according to their ages. Group A included young bitches aged 2 years (n=8) while Group B constituted of those aged 8 to 10 years (n=8). Diameters of the primordial, primary and preantral follicles were found to be significantly larger in Group B, in comparison to Group A. In Group A, the mean number of granulosa cells was 91.65±2.23 in the secondary follicles and 301.31±4.16 in the preantral follicles. In Group B, the same values were found to be 89.46±2.68 and 270.25±3.54, respectively. The mean serum AMH levels in Group A and Group B were 0.233±0.046 ng/mL and 0.099±0.008 ng/mL, respectively (P<0.05). In conclusion, the results indicated that the number of primordial and primary follicles as well as the numbers of granulosa cells in secondary and preantral follicles decreased with advanced age which resulted in lower serum AMH levels in aged bitches. The results suggested that the AMH, which is used as a fertility parameter in humans, could also be used for the same purpose in dogs.

***Keywords:*** *Bitch, Follicular morphology, Granulosa cell, Anti-Müllerian hormone*

# Genç ve Yaşlı Dişi Köpeklerde Foliküler Morfolojinin Farklılıkları ve Anti Müllerien Hormon ile İlişkisi

## Özet

Sunulan çalışmada genç ve yaşlı dişi köpeklerde, foliküler gelişimin farklı aşamalarında, foliküllerin yapısal morfolojik ve hücresel farklılıklarının ortaya konması, ovaryum follikül popülasyonunun Anti Müllerien Hormon (AMH) ile ilişkilerinin araştırılması ve bu hormonun genç ve yaşlı köpeklerdeki düzeylerinin belirlenmesi amaçlanmıştır. Çalışma, materyalini 8 adet, 2 yaşında, genç (grup A) ve 8 adet, 8-10 yaş aralığında yaşlı olmak üzere (grup B) toplam 16 dişi köpek oluşturmuştur. Primordial, primer ve preantral folikül çapları grup B de grup A'ya göre daha yüksek ölçülürken sekunder folikül çapları yönünden gruplar arası fark istatistiki olarak önemsiz bulunmuştur. Grup A'da Sekunder foliküllerdeki granuloza hücreleri ortalama 91.65±2.23 adet preantral foliküllerde ise 301.31±4.16 adet sayılmıştır. Grup B'de ise bu değerler sırasıyla 89.46±2.68 ve 270.25±3.54 adet olarak kaydedilmiştir. Çalışmada serum AMH sonuçlarına bakıldığında Grup A' da bu değer ortalama 0.233±0.046 ng/mL tespit edilmiştir. Grup B'de ise bu ortalamanın 0.099±0.008 ng/mL'ye düştüğü gözlemlendi (P<0.05). Sonuç olarak köpeklerde artan yaş ile primordial ve primer folikül sayılarının ve özellikle sekunder ve preantral foliküllerdeki granuloza hücre sayılarının azalması ve AMH hormonunun da buna paralellik göstermesi, insanlarda fertilite parametresi olarak kullanılan AMH hormonunun köpeklerde de kullanılabileceğini göstermektedir.

***Anahtar sözcükler:*** *Köpek, Foliküler morfoloji, Granuloza hücre, Anti Müllerien hormon*

## INTRODUCTION

Although bitches reach puberty at the age of 6-14 months, the optimal age range for mating is 2-6 years. The number of ovulatory follicles decrease with age, and the ovarian activity, which declines from 6 years onwards, nearly diminishes by the age of 10. In bitches older than 8 years, the oestrous cycle becomes irregular, fecundation

**İletişim (Correspondence)**
+90 414 3183859/3914
ahuramazda@harran.edu.tr

and fertility decrease, and the frequency of abortion increases. Therefore, it is suggested that dogs older than 7-8 years should not be used for breeding purposes [1].

Canine ovarian follicles are categorised into 5 classes including primordial, primary, secondary, early antral (pre-antral) and advanced antral (graafian) follicles on the basis of follicle morphology, follicle size, type and number of follicle cell layers, and the presence of follicular fluid [2]. Primordial follicles contain a small oocyte with a mean diameter of 25 µm, which lacks a zona pellucida (ZP) and is lined by a single layer of squamous granulosa cells [3]. By day 120, all primary follicles contain a small, pale oocyte with a distinct ZP, and have a single layer of cuboidal granulosa cells [4]. Secondary follicles are lined by two or more layers of granulosa cells [5]. Early antral follicles are observed with fluid-filled cavities among the granulosa cells [2]. In pre-pubertal bitches, the transition from a primordial follicle to an early antral follicle requires a time period of 70-150 days. Antral follicles are found in bitches at the age of 6 months, and shortly before proestrus [6,7]. Nonetheless, only a limited number of studies are available on folliculogenesis and the ovarian follicle population in dogs.

Granulosa cells, which provide the physical support and microenvironment required for oocyte development, are capable of active differentiation. Differentiation of the granulosa cells is regulated by several hormones and growth factors [8]. Granulosa cells bear specific receptors for several hormones and growth factors such as follicle stimulating hormone (FSH), luteinizing hormone (LH) [9] epidermal growth factor (EGF) [10], insulin-like growth factor (IGF) [11], and Anti-Müllerian hormone (AMH) [12].

The AMH known as the Müllerian inhibitory substance, is a homodimeric glycoprotein hormone belonging to the transforming growth factor-β superfamily [13] and is secreted by the sertoli cells in males [14,15] and by the granulosa cells of the secondary, preantral and small antral follicles in females. By inducing the regression of the Müllerian ducts, the AMH enables foetal sex determination and regulates the development of primordial follicles in females [8,16-18]. In male dogs, AMH is expressed from the time of sexual differentiation to puberty and is responsible for regression of the paramesonephric duct during sexual differentiation [19].

The AMH reduces the sensitivity of growing follicles to the FSH, and thereby, limits the number of actively growing follicles, and also acts in follicular development. In this way, the AMH contributes to the maintenance of oocyte reserves [17,20]. Detection of the AMH level has found common use in women, for determination of the ovarian reserve, which refers to the number of follicles that can be successfully recruited for a possible pregnancy, as well as for the monitoring of transition into menopause, the diagnosis of the polycystic ovary syndrome and granulosa cell tumours, the detection of low ovarian response, and prognosis in *in vitro* fertilization studies [16,20].

This study aimed to investigate the structural, morphological and cellular differences of follicles at different stages of follicular development as well as to determine the correlation of the ovarian follicle population with serum Anti-Müllerian Hormone (AMH) levels in young and aged bitches.

## MATERIAL and METHODS

Sixteen dogs, including eight 2-year-old young bitches, which were known to have undergone at least one proestrus bleeding and were referred to the clinic for routine ovario-hysterectomy (Group A), and eight 8 to 10-year-old aged bitches (Group B), constituted the study material. Before operation clinical examinations, a complete blood count, a regular vaginal cytological examination and gynaecological ultrasonography were performed, to ensure that healthy animals with no medical problem and gynaecological pathology were included in the study. This study was approved by the Dollvet Animal Experiments Local Ethics Committee (Aproval number: Dollvet A.Ş. HADYEK 2016/04).

Prior to surgery, blood samples were taken from each animal into dry tubes. After coagulation the blood samples were centrifuged at 3.000 rpm for 15 min, and the collected sera were stored at -20°C until being analysed. The serum AMH levels were measured with the enzyme-linked immunosorbent assay (ELISA) (DRG Instruments Elisa Mat 2000), using a commercial kit (Beckman Coulter, AMH Gen II, USA). All serum assays were performed in duplicate. The minimum detectable concentration of the assay was 0.375 ng/mL. The lower and upper limits of detection were 0.375 ng/mL and 150 ng/mL, respectively. The respective intra and inter assay coefficients of variations were <8% and <10%, respectively. Standard curve ranges were 0.07-22.5 ng/mL. The limit of detection value of the ELISA kit was <0.1 ng/mL.

The ovarian tissues obtained by routine ovariohysterectomy were fixed in 10% formaldehyde for 24 h and then washed. After being passed through a series of graded alcohol and xylol solutions, the tissue samples were embedded in paraffin. For each animal, four serial sections of 5 µm thickness cut from the paraffin blocks were stained with Crossman's modified triple staining technique [21]. The serial sections were examined under an Olympos Cover 018 model research microscope and measured using a Bs200Pro image analysis programme (BAB software).

In order to determine the number of follicles in different stages of the follicular development four sections pertaining to each animal were counted.

The follicles were categorised into five classes according to Songsasen et al.[2] on the basis of the characteristics described below.

*1- Primordial follicle:* No ZP surrounding the oocyte and the oocyte lined by a single layer of squamous granulosa cells.

*2- Primary follicle:* The oocyte surrounded by a ZP and lined by a single layer of cuboidal granulosa cells.

*3- Secondary follicle:* Oocyte lined by two or more layers of granulosa cells.

*4- Preantral follicle:* Oocytes surrounded by granulosa cells with small cavities (follicular antrum) among the granulosa cells or segmented cavities with two or more compartments.

*5- Antral follicle:* The formation of a single, large, continuous cavity (antrum).

Follicle diameters were determined as described by Griffin et al.[22], by averaging two measurements, at right angles, at the widest cross-section of the follicles.

The results obtained in the present study were analysed by the Statistical Package for Social Sciences (SPSS) Version 14.01 (Serial number: 9869264). The statistical significance of the differences observed between the groups for the values, which met the parametric assumptions of the test and were determined by measurement, including diameter (primordial, primary, secondary, preantral and antral follicle diameters) and theca folliculi wall length (in the secondary, preantral and antral follicles), was determined with Student's t-test, whereas the significance of the differences for follicle (primordial and primary follicles) and granulosa cell (of secondary and antral follicles) numbers, and quantitative features that did not meet the parametric assumptions of the test, was analysed by the Mann-Whitney U test. Furthermore, the significance of the correlation of the AMH with follicle number, follicle diameter, and granulosa cell number was determined with the Pearson correlation test.

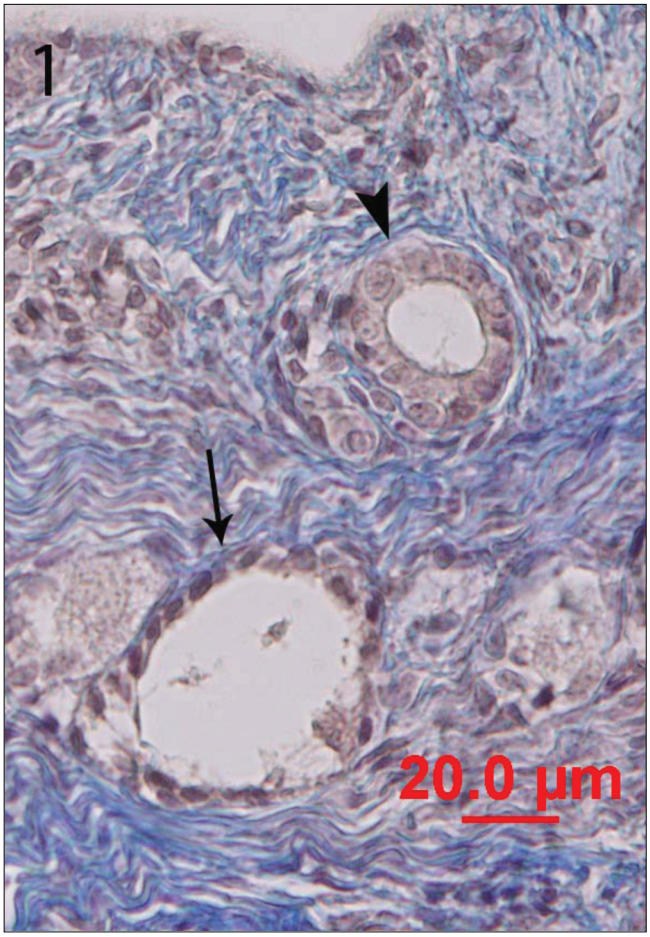



**Fig 1.** Primordial follicle *(arrow head)* and primary follicle *(arrow)*
**Şekil 1.** primordial folikül *(ok başı)* ve primer folikül *(ok)*

## RESULTS

The diameters of the primordial *(Fig. 1)*, primary *(Fig. 1)*, secondary *(Fig. 2)*, preantral *(Fig. 3)* and antral *(Fig. 4)* follicles and the theca folliculi wall lengths of the secondary, preantral and antral follicles observed in the ovarian sections of the group of young bitches (Group A) and the group of aged bitches (Group B) are presented in *Table 1*.

While the diameters of the primordial, primary and preantral follicles were found to be larger in Group B in comparison to Group A ($P<0.001$). The difference between the two groups for secondary follicle diameter was found to be statistically insignificant.

The numbers of the primordial and primary follicles and the granulosa cell numbers of the secondary and preantral follicles of both groups are presented in *Table 2*.

In the present study, the numbers of primordial and primary follicles were counted in an area of 1 $mm^2$ of the histological sections. The mean number of primordial follicles counted in this area was significantly higher in Group A than in Group B ($P<0.05$). Similarly, the mean number of primary follicles was higher in Group A, when compared to Group B, however, this was non-significant. In Group A, the mean number of granulosa cells averaged 91.65 in the secondary follicles and 301.31 in the preantral follicles. In Group B, both parameters were lower (89.46 vs 270.25). While the number of granulosa cells in the preantral follicles differed significantly between groups ($P<0.001$), this was not the case in the secondary follicles. The serum AMH concentrations measured in Groups A and B are presented in *Table 3*.

The assessment of the serum AMH concentrations demonstrated that the values averaged 0.166 ng/mL and ranged from 0.092 ng/mL to 0.478 ng/mL for both groups. In Group A, the average serum AMH level was significantly higher than in Group B ($P<0.05$).

The correlation of the serum AMH levels with the numbers and diameters of the primordial and primary follicles, and the diameters and the number of granulosa cells of the secondary and preantral follicles is presented in *Table 4*.

A significant correlation between serum AMH and the numbers of primordial ($r=0.611$) as well as primary follicles

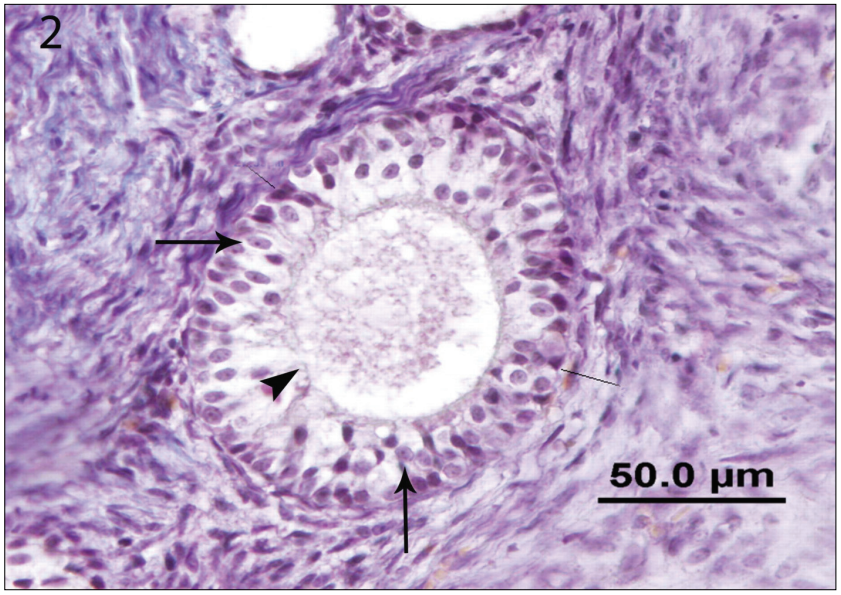
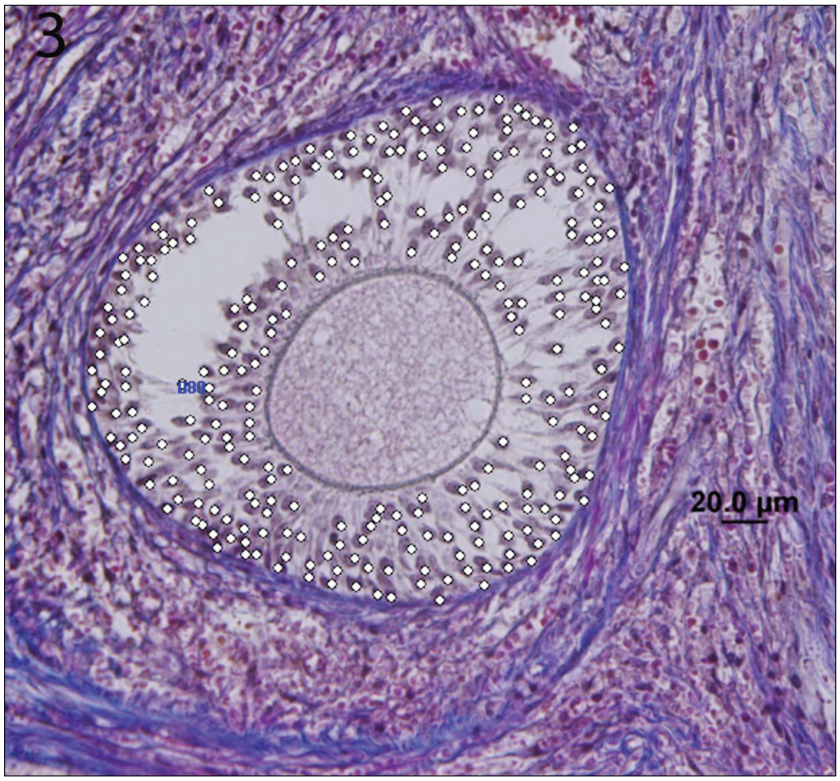



**Fig 2.** Secondary follicle. *Straight line:* teca follicle wall, *arrow head:* oocyte, *arrows:* granulosa cells

**Şekil 2.** Sekunder folikül. *İnce çizgi:* teka folikül duvarı, *ok başı:* oosit, *ok:* granuloza hücreler

**Fig 3.** Preantral follicle. *White circles:* granulosa cells

**Şekil 3.** Preantral folikül. *Beyaz noktalar:* granuloza hücreler




(r=0.572) was assessed (P<0.05); furthermore, between the serum AMH levels and the diameter of the primordial follicles (r=-0.593) (P<0.05). As the diameter of the primary, secondary and preantral follicles increased, the serum AMH levels decreased, however this correlation was non-significant (P>0.05).

## DISCUSSION

In mammals, female individuals are born with the pool of primordial follicles that develop throughout the reproductive life span of the female [8]. Clusters of primordial and primary follicles are referred to as 'egg nest', and are generally observed in mammalian species characterized by the ovulation of more than one oocyte at a time [4]. In the present study, it was observed that the numbers of the primordial and primary follicles were higher in the young bitches. This finding was in agreement with the report of Dolezel et al.[23] who, showed that the total number of follicles was lower in aged bitches, when compared to young bitches.

Studies of Griffin et al.[22] on ovarian follicle diameter in

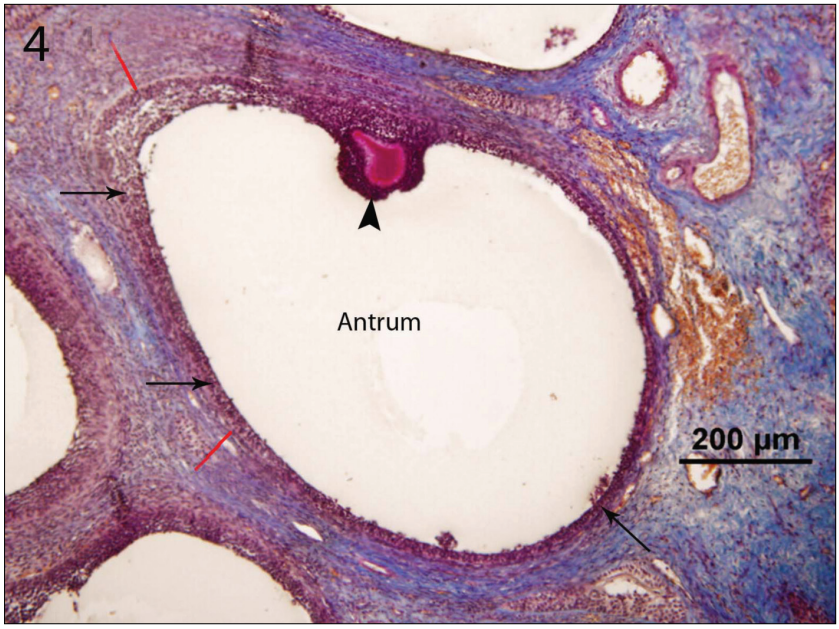



**Fig 4.** Antral follicle. *Straight line:* teca follicle wall, *arrow head:* corona radiata, *arrows:* granulosa cells

**Şekil 4.** Antral folikül. *İnce çizgi:* teka folikül duvarı, *ok başı:* korona radiyata, *ok:* granuloza hücreler

***Table 1.*** *Follicle diameters and theca folliculi wall lengths in Group A and Group B*
***Tablo 1.*** *Grup A ve Grup B deki folikül çapları ve teka duvarı uzunlukları*

| Diameter or Length | Group A (young) | | Group B (aged) | | t | P |
|---|---|---|---|---|---|---|
| | n | $\chi \pm_{\chi}$ (µm) | N | $\chi \pm_{\chi}$ (µm) | | |
| Primordial follicle diameter | 80 | 24.53±0.56 | 80 | 30.38±0.44 | 8.068 | *** |
| Primary follicle diameter | 80 | 59.73±0.62 | 80 | 67.29±0.49 | 9.570 | *** |
| Secondary follicle diameter | 45 | 137.87±2.64 | 48 | 143.71±2.36 | 1.654 | - |
| Wall length of secondary theca | 40 | 13.15±0.37 | 48 | 21.12±0.63 | 10.427 | *** |
| Preantral follicle diameter | 19 | 201.15±5.78 | 20 | 234.94±5.89 | 4.089 | *** |
| Wall length of preantral theca | 19 | 34.58±1.85 | 20 | 42.58±1.57 | 3.312 | ** |
| Antral follicle diameter | 13 | 367.14±22.51 | 6 | 508.88±40.87 | 3.295 | ** |
| Wall length of antral theca | 13 | 92.54±2.99 | 6 | 96.01±7.31 | 0.530 | - |

*-: P>0.05: non-significant, * P<0.05, ** P<0.01, *** P<0.001*

***Table 2.*** *Number of primordial and primary follicles and granulosa cell numbers of the secondary and preantral follicles in both groups*
***Tablo 2.*** *Her iki gruba ait köpeklerde primordial ve primer follikül sayıları ile sekunder ve preantral folliküllerdeki granuloza hücre sayıları*

| Number | Group A (young) | | | Group B (aged) | | | P |
|---|---|---|---|---|---|---|---|
| | n | $\chi \pm_{\chi}$ | Median | N | $\chi \pm_{\chi}$ | Median | |
| Primordial follicle number | 8 | 43.50±3.86 | 46.50 | 8 | 16.57±2.10 | 14.00 | * |
| Primary follicle number | 8 | 29.88±6.07 | 22.00 | 8 | 15.57±3.54 | 11.00 | - |
| Granulosa cell number of secondary follicles | 48 | 91.65±2.23 | 89.50 | 48 | 89.46±2.68 | 90.00 | - |
| Granulosa cell number of preantral follicles | 32 | 301.31±4.16 | 298.00 | 32 | 270.25±3.54 | 264.00 | *** |

*-: P>0.05: non-significant, * P<0.05, ** P<0.01, *** P<0.001*

mice, hamsters, pigs and humans have demonstrated that differences exist between these four mammalian species. The follicle diameters measured in the present study were found to be close to the follicle diameters measured by Griffin et al.[22] in hamsters. Furthermore, it was determined that the antral follicle diameter of the dog was smaller than that of human and pig, and larger than that of mouse. The differences observed between dogs and other animal species were attributed to the proportional relationship of the body mass index of a given species with its ovarian and follicle diameters. In the present study, it was observed that during the transition from the primordial follicle to the antral follicle, the follicle size displayed a nearly 15-fold increase in young animals, and a 17-fold increase in

***Table 3.*** *Blood serum AMH levels in both groups*
***Tablo 3.*** *Her iki gruba ait köpeklerde kan serum AMH değerleri*

| Group | n | $\bar{X}\pm_{\bar{X}}$ (ng/ml) | Z | P |
|---|---|---|---|---|
| Group A | 8 | 0.233±0.046 | -2.941 | 0.03 |
| Group B | 8 | 0.099±0.008 | | |

***Table 4.*** *Correlation of serum AMH levels with follicle number, follicle diameter, and granulosa cell number*
***Tablo 4.*** *Serum AMH düzeyi ile follikül sayısı, çapları ve granuloza hücre sayıları arasındaki korelasyon*

| Number or Diameter | Anti-Müllerian Hormone | |
|---|---|---|
| | R | P |
| Primordial follicle number | 0.611 | 0.016 |
| Primary follicle number | 0.572 | 0.026 |
| Primordial follicle diameter | -0.593 | 0.015 |
| Primary follicle diameter | -0.464 | 0.07 |
| Secondary follicle diameter | -0.228 | 0.397 |
| Granulosa cell number of secondary follicles | 0.193 | 0.473 |
| Preantral follicle diameter | -0.359 | 0.208 |
| Granulosa cell number of preantral follicles | 0.093 | 0.733 |

aged animals. The greatest increase in follicle diameter was observed during the transition to the antral follicle, similar to findings previously reported by Songsasen et al.[2].

Diameters of the primary and secondary follicles measured in the young dogs in the present study were found to be similar to those measured by Dolezel et al.[23]. Furthermore diameters of primary and secondary follicles measured in the young dogs in the present study were also similar to those reported by Songsasen et al.[2] while the antral follicle diameters determined in the present study were found to be larger. The follicle diameters measured in the aged dogs were larger than those in the young dogs and those reported in previous studies. The differences between the values observed in the present study and those reported by the researches above can be attributed to the differences in the age of the experimental animals used. The age of the animals included in the study conducted by Songsasen et al.[2] ranged from 5 months to 7 years which support this presumption. Hence, while the follicle diameters measured in the aged dogs were found to be larger than the diameters reported by Songsasen et al.[2], the follicle diameters measured in the young dogs, which were at an age close to that of the animals used by Songsasen et al.[2], were found to be similar to the measurements of these researchers.

In females although AMH is also secreted by the granulosa cells lining the secondary, preantral and small antral follicles [8] the major source of this hormone is the granulosa cells of early antral follicles [16,17]. In the present study, both the granulosa cells number and the diameter of the secondary follicles were similar to those reported by two study groups. An interesting finding was the granulosa cell number of the preantral follicles being smaller in the aged dogs (P<0.001).

It was considered that, the differences observed in the aged dogs for follicle diameter and granulosa cell number could be related to the ageing-related changes in the hormones regulating follicular development [24], the factors regulating follicular development (kit ligand, leukaemia inhibitory factor, bone morphogenic proteins 4 and 7, thrombocytic growth factor, basic fibroblast growth factor) [8,25] and the factors inhibiting follicular development (AMH and stromal cell factor-1) [26].

When compared to the values reported by Place et al.[27], the serum AMH levels measured in the young dogs in the present study were found to be lower (0.36 ng/mL) but were observed to be higher than the values measured in dogs with the ovarian remnant syndrome (0.195 ng/mL). This reflects that more clinical studies are required to develop the reference concentration ranges of serum AMH in bitches.

The lower serum AMH levels of the aged dogs in comparison to young dogs were probably attributed to the higher number of the granulosa cells of the preantral follicles in young animals, since these cells are directly involved in AMH synthesis. The negative correlation observed between serum AMH levels and age in dogs is in accordance with the decrease determined in the serum AMH levels of women in parallel with the decrease observed in gonad functions and ovarian reserves with advanced age [28-30]. In women, this is supposed to be caused by the aging process and changings towards menopause; even though in carnivores no menopause is described, the ovarian activity and fertility decrease in geriatric dogs.

Interestingly, even though the serum AMH levels increased with increased granulosa cell numbers of the secondary and preantral follicles, there was no correlation between serum AMH and follicle number, follicle diameter and granulosa cell number, which cannot be explained at present.

In conclusion, the decrease observed with advanced age in the number of primordial and primary follicles, and in particular, in the granulosa cell numbers of the secondary and preantral follicles, as well as the parallel changes determined in the serum AMH levels in bitches, makes us suggest that the AMH, which is used as a fertility parameter in humans, could be used for the same purpose in dogs. In this context, serum AMH measurement seems to be an interesting tool for the diagnosis and treatment of fertility problems in dogs, as well as for the selection of breeding animals.

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