Investigation of serum anti-Müllerian hormone levels at follicular phase and interestrus period in queens

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ABSTRACT

The objectives of this study were: *i)* to report overall mean AMH serum concentrations in queens, ii) to determine relationship between serum AMH concentration with estradiol level, *iii*) to investigate the relationship between serum AMH with follicular phase and interestrus phase of the estrous cycle, *iv*) to discuss the usability of AMH as a biomarker to diagnosis of ovarian remnant syndrome in breeding season in queens. In the study, 64 healthy queens referred to clinics for routine ovariohysterectomy were used. After anamnesis, vaginal cytology, macroscopic examination of the uterus and ovaries and estradiol measurement, queens were divided into two groups: follicular phase (n: 45) and interestrus (n: 19). Estradiol and AMH levels in serum samples were determined by ELISA. In the evaluation of serum AMH concentrations, a statistical difference was determined between the follicular phase and interestrus ($P<0.05$). A negative correlation was found between age and estradiol in both follicular phase ($r = -0.171$) and interestrus ($r = -0.385$) groups. A positive correlation was found between age and AMH in the interestrus ($P<0.01$, $r = 0.696$). Serum AMH levels in the interestrus were found to be significantly higher than follicular phase ($P<0.05$). It was thought that this increase in serum AMH levels during the interestrus period, which is the stage where oocytes were selected for ovulation, may be an indicator of the role of AMH in oocyte selection in queens, as in many other mammalian species.

Introduction

Clasically, the queen is described as a seasonally polyestrous, induced ovulator (11). However, some authors have reported cases of spontaneous ovulation in groups of queens housed together and in wild felids, some species may show alternatively induced or spontaneous ovulations (2, 7). Various studies are documented greatest frequency of cycling activity in queens in the Northern Hemisphere in January and February, with gradual frequency decline until September and October (3, 11).

Even in the absence of mating and ovulation, queens show successive proestrus, estrus, and interestrus under the influence of daylight. Proestrus and estrus, form the estrogen-dominated follicular phase of the estrous cycle (3, 7, 16, 25). Estrogen levels in cats are found to be above 20 pg/mL during the follicular phase (proestrus and estrus), and below 20 pg/mL during anoestrus and interestrus (5, 25). In particular, an estradiol level above 20 pg/mL is diagnostic for ovarian follicle activity (5, 22). The effect of estradiol on vaginal epithelium of the queen is to cause an increased number of epithelial cell layers and to cause vaginal cornification, resulting in change in the morphologic apperance of exfoliated epithelial cells (11).

Anti-Müllerian hormone (AMH) belongs to the Transforming Growth Factor-ß (TGF-ß) family and produces in females only in ovaries by the granulosa cells of growing follicles, it is known as a marker of ovarian follicular reserve and ovarian aging, tumor marker or tumor inhibitör (4, 6). The active form of AMH is made up of two identical monomer subunits connected by disulphide links and is synthesized as a prohormone. For

the molecule to function, both locations are critical (14). Measurement of AMH levels plays an important role for the diagnosis the presence or absence of the ovaries in bitch and queen (18, 19, 20) and also for diagnosis the ovarian pathologies (9) and ovarian remnant syndrome (1, 19, 20, 26). Although some studies have been carried to the determination of serum AMH levels in queens in recent years (1, 18, 20, 24) more studies are needed to understand if AMH is also a biochemical marker of follicular development in queens. It is also important to assessif AMH is a reliable diagnostic biomarker in ovarian pathologies or ORS cases, to avoid unnecessary exploratory laparotomy.

The aims of this study are: *i)* to report overall mean AMH serum concentrations in queens, ii) to determine relationship between serum AMH and estradiol levels, *iii*) to investigate the relationship between serum AMH with follicular phase and interestrus phase of the estrous cycle, *iv*) to discuss the usability of AMH as a biomarker to diagnosis of ovarian remnant syndrome in breeding season in queens.

Materials and Methods

Animals and Sample Collections: The queens were clinically healthy and were referred for routine ovariohysterectomy (OHE) by their owners with the request of spaying for population control at the Kırıkkale University Faculty of Veterinary Medicine Clinics. In mating season, queens who showed typical oestrus signs at least once according to the anamnesis before the surgery were included in the study. None of the queens had symptoms of systemic or gynecological disorders according to clinical and ultrasonographic examinations. Furthermore, all of the queens were unpaired, unmated, unstimulated and individually housed. Age, body weight, previous diseases and last estrus dates of queens were recorded as anamnesis information.

Ovariohysterectomy was performed on the animals under general anesthesia from the lateral side as stated previously (8); blood and vaginal smear samples were taken before the surgery. Vaginal smears were obtained from the anterior vaginal wall with a sterile cotton swab, stained with Diff-Quick (Bes-Quick) staining method and evaluated under a light microscope (Olympos CHKZ-F-GS). All epithelial cells were examined and classified as described previously (23). Blood samples were taken from *v. saphena medialis magna* into tubes without anticoagulant with 21 G needle and were centrifuged at 4000 rpm for 10 minutes (ElektromagCentrifuge M4808P). The serum samples stored in eppendorf tubes at -20°C until analysis.

The study was approved by the animal ethics committee, University of Kırıkkale, Türkiye (Approval Number: 2019/29).

Macroscopic Examination of the Uterus and Ovaries: Uterus and ovaries obtained from all animals were examined macroscopically after surgery. The ovaries were examinated for the presence of fresh ovulation scars and were dissected longitudinally to investigate the presence of the corpora lutea. After macroscopic examination, the queen who had pathological condition, early pregnancy signs and corpus luteum or fresh ovulation scar in their ovaries were excluded. Finally, 64 queens aged between 8 months and 5 years were used in this study.

Hormonal Analysis: AMH and estradiol levels in serum samples were determined by ELISA method using DRG Instruments Elisa Mat 2000 device. Anti-Müllerian Hormone level was determined using human specific ELISA kit (Beckman Coulter®, AMH Gen II, USA) and estradiol level was determined using ELISA kit (DRG Estradiol EIA-2693). We used a second-generation human-based AMH kit produced by Beckman Coulter Immunotech, that works based on a two-site immunoassay that targets the epitopes by utilizing two different monoclonal antibodies selectively in the mature area (14). We previously used the same kit for assessing AMH levels in queens (18). Aside from our research team, two independent researchers (1, 11) also chose to determine the AMH status of queens using human-based AMH kit. These studies revealed that both the sensitivity and specificity of the assay are higher than 90%. The linearity of the test was assessed by spiking two different samples, with the high-concentration AMH standard, followed by serial dilution for validation purposes.

Grouping: Based on the anamnesis, vaginal cytology results, macroscopic examination of the ovaries and uterus expelled after surgery, and estradiol results, the operated queens (n:64) were divided into 2 groups as follicular phase and interestrus. Queens in estrus according to the anamnesis and vaginal cytology, with follicular development in their ovaries and with estradiol results above 20 pg/mL were included in “Follicular Phase Group” (n:45). Beside, queens those whose estrus ended at least 1 week ago according to the anamnesis, without corpus luteum in their ovaries, and also with estradiol results below 20 pg/mL were included in “Interestrus Group” (n:19).

Statistical Analysis: All obtained variables were analyzed with Shapiro Wilk for normality and Levene test for homogenity of variance, before starting the significance tests. Statistical determination of the difference in parameters between the groups was evaluated with Student's T Test, and the correlation measurements of the parameters within the group were analyzed with the Pearson correlation test. The minimum margin of error was determined as 5% in all statistical analyses. Data was analyzed using the GLM for Repeated Measures procedure of SPSS 14.01 (SPSS Inc., Chicago, IL, USA).

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Table 1. Mean age, body weight, AMH and estradiol results of the queens (mean ± standard error).

	n	Age (month)	Body weight (kg)	AMH (ng/mL)	Estradiol (pg/mL)
Follicular Phase	45	21.31±2.01	3.15±0.12	5.92±0.57[a]	115.05±36.78[c]
Interestrus	19	18±2.44	3.05±0.10	9.44±2.01[b]	16.58±1.10[d]

[a,b] Different letters in the same column are statistically significant (P<0.05).
[c,d] Different letters in the same column are statistically significant (P<0.01).

Results

Average age, weight, AMH and estradiol results of the follicular phase and interestrus groups were given in Table 1. Overall mean AMH levels of all queens was founded 7.28±0.74 ng/mL. When the age and weight of the queens were compared, no difference were determined between the groups (P>0.05). In the evaluation of serum AMH concentrations, a statistical difference was determined between the follicular phase (5.92±0.57 ng/mL) and interestrus groups (9.44±2.01 pg/mL) (P<0.05). Although it was not statistically significant (P>0.05), there was a negative correlation between age and estradiol in both follicular phase (r = -0.171) and interestrus (r = -0.385) groups. A positive correlation was determined between age and AMH in the interestrus group (P<0.01, r = 0.696).

Discussion and Conclusion

Adult queens are seasonal polyestric animals, and their sexual activity begins in December, naturally continues until September or October (23). However, artificial light also affects ovarian activity in domestic cats. In queens exposed to artificial light for at least 10 hours, the sexual cycle does not end in September and can continue throughout the year (10). Estrous behavior in queens is associated with estrogen synthesis released from the follicles. Follicles that are smaller than 1 mm at the start of proestrus reach 1.5 mm in diameter at the beginning of estrus. Follicles with a diameter of 2-3 mm develop in the ovaries during estrus, and the plasma estradiol level, which is below 12-15 pg/mL during anoestrus and interestrus, rises above 20 pg/mL when follicular activity starts (22, 23). In this study, the mean estradiol value of the follicular phase group was 115.05±36.78 pg/mL, and the interestrus group was 16.58±1.10 pg/mL. In cow, during follicular waves, the development of a dominant follicle was paralleled by an increase in circulating estradiol level, as expected, confirming that estradiol is an endocrine marker of terminal follicular development (16, 21). Similar with cows, we suggest that estradiol levels could be a potantial endocrine marker to determine the terminal follicular development in queens.

In this study, serum AMH levels were higher in interestrous (9.44 ng/mL) than follicular phase (5.92 ng/mL). In bitches, changes in AMH concentration throughout the oestrous cycle have been identified with many studies (17, 28) but in queens AMH studies have focused more on detecting the presence of ovaries and changes in AMH concentration over the entire oestrous cycle have not yet been clearly determined. In a study on serum AMH concentration and ovarian follicle population in queens (15), similarly with cows (21), anestrous bitches (13) and womens (4,6), serum AMH levels in queens were founded highly correlated with small antral follicle count and also higher in anestrus than follicular phase. In females, AMH has an inhibitory effect on follicular development. It plays a role in selection at the beginning of the recruitment period and reducing the sensitivity of antral follicles to FSH (Follicle Stimulating Hormone). Thus, by preventing excessive follicular recruitment and follicular development, it has a role in determining the physiological limits in follicle development. In the absence of AMH, the recruitment rate may increase, leading to rapid depletion of the follicular pool (27). Similarly to the study by Lapuente et al. (15), low AMH level in follicular phase obtained in this study could be due to the termination of the follicles development. Considering this new study, it was thought that the statistical difference between follicular phase and interestrus in this study may be due to developing follicles and especially the selection of dominant follicles in queens. At this moment, it is very difficult to explain and in order to better understand this, more detailed studies are needed on this hypothesis.

There were some AMH variations have been found, although cyclical changes may be different in different species. The FSH stimulus result in an increase in serum estradiol but did not affect serum AMH concentrations in queens (1). In our study, we found a negative correlation between AMH and estradiol concentrations. Thus, increasing in follicular growth towards the end of interestrus in queens and the high AMH concentration in this period suggested that oocyte selection was completed with the transition to oestrus. Furthermore, since exact physiological follicular size limits could not be determined, oocytes was thought to be selected before estradiol exceeded the 20 pg/mL limits. Further studies are

required to demonstrate the cyclical changes in estradiol and AMH concentrations, serial samples from the same individuals are necessary.

Higher AMH levels were found in intact cats (7.28 ng/mL) than intact bitches (0.32 ng/mL) in our previous study (19). Although AMH is inversely correlated with age in prepubertal queens; along with folliculogenesis, AMH increases with age in sexually active queens in breeding season. Granulosa cells in primary, secondary and early antral follicles are producing AMH, but the main source of circulating AMH is early antral follicles (11). In the study conducted by Korkmaz et al. (13), they reported that with increasing age in bitches, the number of primordial and primary follicles, and especially the granulosa cell numbers in the secondary and preantral follicles are decreased in parallel with AMH hormone concentration.

In studies conducted in queens to date, the average of circulating AMH level has been detected in the range of 1.3 to 19 ng/mL (1, 11, 15, 18). In this study, similar to others, overal mean AMH was 7.28±0.74 ng/mL of all queens. However mean AMH levels in queens with follicular phase was lower than this level (5.92±0.57 ng/mL). In cases of feline (11) and canine (11, 19, 26) ovarian remnant syndrome, AMH concentrations were intermediate to those of spayed and intact queens and bitches. But, queens are reffered to veterinary clinics with the suspicion of ovarian remnant syndrome, mostly because they show signs of estrus and according to this study and Lapuente et al. (15), AMH levels were lower in follicular phase than interestrus or anestrus. Therefore, determining the reference limits of phsyolocigal AMH levels in queens, especially in follicular phase or others, is much more valuable for definitive diagnosis in feline ORS cases with unknown gyneacologic history.

In conclusion, in queens, changes of serum AMH and estradiol concentrations between follicular phase and interestrous period shows that AMH can play a role in determining the physiological limits in follicle development and also selection of dominant follicles as in other mammalian species. AMH gives a critical information regarding the presence of functional ovaries during the mating season or inactive period. Finally, it is extremly important to develop practical, economic, easy and also standart measurement methods developed for use by veterinary clinicians. Further studies are needed to confirm to role of AMH on reproductive physiology in queens.

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Author Contributions

IPY and GS conceived and planned the experiments. IPY, GS, IMP and TBE carried out the experiments. IPY, GS, IMP and MP planned and carried out the simulations. IPY, GS and TBE contributed to sample preparation. IPY, IMP and MP contributed to the interpretation of the results. IPY, IMP and MP took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Ethical Statement

The study was approved by the Animal Ethics Committee, University of Kırıkkale, Türkiye (Approval Number: 2019/29).

Conflict of Interest

The authors declare no conflict of interest.

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