PhD THESIS

The interrelation between Anti-Müllerian hormone and ovarian pathology in canine species

SUMMARY OF THE DOCTORAL THESIS

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INTRODUCTION

In domestic animals, ovarian cysts have a high prevalence and are responsible for the onset of hyperestrogenism. Ovarian cysts can originate from different ovarian structures, and their development, frequency, and size vary depending on the species. These formations are classified histologically, with the most common type of ovarian cyst being the follicular cyst (Knauf şi colab., 2014).

In canines, ovarian cysts are of particular clinical importance, as they are a significant source of hyperestrogenism in bitches. This condition can lead to prolonged estrus, anovulatory heat, shortened interestrus, cystic endometrial hyperplasia, pyometra, mammary tumors, and vaginal tumors, and in chronic forms, it may even cause skin pathologies and hematogenous bone marrow suppression (Johnston şi colab., 2001) (Arlt şi colab., 2011). Follicular cysts are endocrinologically active, secreting estradiol and progesterone, which has led to research into the hormonal profile of bitches with ovarian cysts (Chuffa şi colab., 2016). These conditions have a significant impact on the general health and reproductive function of affected dogs, particularly those in breeding programs, highlighting the relevance of this subject. The impact of hypothyroidism on reproduction is clinically significant, especially in purebred dogs, where the aim is to improve health and phenotypic traits. In bitches, there is limited knowledge about the effects of thyroid hormone deficiency on reproductive performance (Panciera, D.L., 2012).

An important marker used in human medicine for infertility is the anti-Müllerian hormone. Produced by the granulosa cells of the ovary, this hormone is initially involved in the development of ovarian follicles (Broekmans, 2008). Serum levels of AMH closely correlate with the number of antral follicles, reflecting the size of the primordial follicle reserve. Evaluating ovarian reserve is important in in vitro fertilization, with anti-Müllerian hormone also being used to assess fertility potential. Measuring this hormone aids in diagnosing and monitoring cases of ovarian granulosa cell tumors and, more recently, in evaluating polycystic ovary syndrome, with serum AMH levels being elevated in polycystic ovary syndrome, serving as a useful marker for assessing the extent of this condition (Broekmans, 2008; Jenny Visser şi colab., 2006; Fleming, 2005).

In veterinary medicine, the implications of the anti-Müllerian hormone have been studied to a limited extent in the canine species, with research attempting to outline AMH values in various ovarian pathologies, using testing kits designed for human use or for laboratory animals. Since testing kits for canine species are newly available on the market, there are significant differences among the studies conducted, highlighting the need to clarify issues and align them with ovarian pathologies in canines, as well as the scope of research in canine endocrinology.

THESIS STRUCTURE

The doctoral thesis titled "The Interrelation Between Anti-Müllerian Hormone and Ovarian Pathology in Female Canines" contains 97 pages, 20 figures, and 19 tables. The doctoral candidate adhered to the methodology for writing doctoral theses. The thesis is divided into two parts as follows:

The first part of the thesis includes 27 pages and is structured into three subsections.

The second part comprises 70 pages and is structured into three chapters, which present the purpose, objectives, materials and methods, research results regarding AMH, hypothyroidism, hormonal profiles in female canines diagnosed with ovarian cysts, general discussions, general conclusions, and finally, aspects of originality and innovative contributions of the research.

THE AIM AND OBJECTIVES OF THE THESIS

This study explores in detail the pathology of ovarian cysts in dogs, considering the lack of knowledge regarding the etiology, pathophysiological mechanisms, and the effectiveness of current treatments. Hypothyroidism was investigated as a possible etiological factor, analyzing the hormonal profile of female canines to clarify the relationship between estrogen, progesterone, and testosterone in this condition. Additionally, I studied the use of anti-Müllerian hormone (AMH) as a marker in the diagnosis of ovarian cysts in dogs, highlighting its potential in veterinary medicine.

The research was conducted with the following objectives:

1. Serum dosing of anti-Müllerian hormone in bitches using the double sandwich ELISA method to highlight differences in cases of ovarian cystic pathology.
2. Conducting serum measurements of thyroid hormones T4 and fT4 using the ELFA method to establish the prevalence of hypothyroidism in cases of ovarian cystic pathology in female dogs and correlating these with serum AMH values.
3. Serum testing of steroid sex hormones Testosterone II, Progesterone, and Estradiol II using the ELFA method to create a hormonal profile, determine their dynamics, and define the relationship between sex hormones and AMH in female dogs with ovarian cystic pathology.

MATERIALS AND METHODS

The research conducted in Chapter 2 was based on a total of 131 female cases, divided into a control group and a group of females diagnosed with ovarian cysts. In Chapter 3, there were 48 females diagnosed with cysts, divided into a group with normal

thyroid hormone levels (euthyroid individuals) and a group with hypothyroidism. In Chapter 4, a total of 96 females were divided into a control group and a group of females diagnosed with ovarian cysts.

For diagnosing female canines with ovarian cystic pathology, the Esaote My Lab X5 ultrasound machine with a 5-10 MHz microconvex probe was used at the Small Animal Reproduction Clinic of the Faculty of Veterinary Medicine at UASVM Cluj-Napoca and the private clinic for small animal reproduction, Quantas Repro Vet SRL in Cluj-Napoca.

The measurement of AMH was performed using the ELISA method, with the ELISA Canine Anti-Müllerian Hormone (AMH) kit (Shanghai Coon Koon Biotech Co., Ltd, Shanghai, China), at the Institute of Life Sciences USAMV Cluj-Napoca, in the Microbiology Discipline laboratory, following the manufacturer's instructions. For quantitative hormonal determinations of AMH, blood serum was used.

Hormonal measurements were performed using the Biomerieux MiniVidas hormone analyzer. This is an automatic analyzer that uses immunodetection techniques. This automatic quantitative immunoenzymatic test for determining the levels of free thyroxine (fT4), protein-bound thyroxine (T4), Estradiol II, Progesterone, and Testosterone in serum uses the ELFA (Enzyme Linked Fluorescent Assay) detection technique.

Statistical analysis was carried out using two programs: IBM® SPSS Statistics version 29.0 (Armonk, NY, USA) and MedCalc® Statistical Software version 22.032 (MedCalc Software Ltd, Ostend, Belgium).

RESULTS AND CONCLUSIONS

Chapter 5, titled "Serum Values of Anti-Müllerian Hormone in Bitches Diagnosed with Ovarian Cysts," aims to evaluate and compare serum levels of anti-Müllerian hormone (AMH) between healthy bitches and those diagnosed with ovarian cystic pathology.

The results for the statistical analysis of AMH levels in the control group (females without ovarian cysts) indicated a mean of 10.0842 ng/ml (95% CI for the mean: 9.7953-10.3731, SD: 1.0686). For the study group, the AMH results indicated a mean of 10.6266 ng/ml (95% CI for the mean: 10.3704-10.8828, SD: 1.1212). Based on the application of the t-test for independent samples, a statistically significant difference ($p = 0.006$) was observed between the AMH levels in the control group and those in the study group. Thus, the mean concentrations of anti-Müllerian hormone were higher in the study group of bitches diagnosed with polycystic ovarian pathology compared to the control group, which consisted solely of clinically healthy females without ovarian cystic pathology.

Results from another statistical analysis indicated that there is no significant correlation between age and AMH levels in either the control or experimental groups.

Another noteworthy aspect is that there are no statistically significant differences between the breed categories included in the study group.

To better outline the values between the study group of bitches with ovarian cystic pathology, the reference interval for 95% of AMH values was calculated by excluding extreme low and high values. For the giant breed category, the 90% confidence interval for the lower limit was 6.05 – 8.32 ng/ml, and the upper limit was 11.96 - 14.23 ng/ml. For large breeds, the 90% lower confidence interval was 8.07 - 9.42 ng/ml, with the upper limit being 12.26 – 13.35 ng/ml. For medium breeds, the lower limit was 8.08 – 9.30 ng/ml, and the upper limit was 12.13 – 13.35 ng/ml. For small breeds, the 90% interval was 8.42 – 9.63 ng/ml.

The highest mean and standard deviation in the control group based on size categories were recorded in medium-sized bitches with a mean of 10.43 ng/ml (SD: 1.07 ng/ml, RI: 8.33–12.54 ng/ml), followed by large breeds with a mean of 10.03 ng/ml (SD: 1.47 ng/ml, RI: 7.15–12.92 ng/ml), and giant breeds with a mean of 9.99 ng/ml (SD: 0.91 ng/ml, RI: 8.20–11.79 ng/ml). Lastly, the small breed had an average AMH value of 9.78 ng/ml (SD: 0.72 ng/ml, RI: 8.36–11.20 ng/ml).

The 95% reference interval for the control group was as follows: for giant breeds, the lower limit was 7.55 – 8.84 ng/ml and the upper limit was 11.14 – 12.43 ng/ml; for large breeds, the lower limit was 5.90 – 8.40 ng/ml and the upper limit was 11.67 – 14.16 ng/ml; for medium breeds, the lower limit was 7.52 – 9.14 ng/ml and the upper limit was 11.73 – 13.35 ng/ml; and for small breeds, the lower limit was 7.72 – 9.00 ng/ml and the upper limit was 10.56 – 11.85 ng/ml.

To study whether there are statistically significant differences between the four breed categories, a one-way ANOVA was used. The results indicated that F_stat = 1.29 and p = 0.29 (> 0.05), therefore, there are no statistically significant differences between the breed categories included in the study group.

Using Spearman's rank correlation coefficient, no significant difference was observed in the serum concentrations of estrogens (median: 10.70 ng/ml, 95% CI for the median: 9.86–12.30) compared to those of AMH (median: 10.17 ng/ml, 95% CI for the median: 9.56–10.63) in the control group (p = 0.1947) with a correlation of rho = -0.190. In the study group, similar to the control group, the differences between the two hormones, AMH (median: 10.81 ng/ml, 95% CI for the median: 10.16–11.18 ng/ml) and estrogens (median: 21.83 ng/ml, 95% CI for the median: 14.65–25.37 ng/ml), were not statistically significant (p = 0.8368), rho = 0.0305.

Comparing the correlation coefficients between the two groups (healthy bitches and bitches with cystic formations) using Fisher's z-test showed no significant correlation (p = 0.2905).

Based on the conducted research, the following conclusions are supported: serum AMH levels were higher in females with ovarian cysts compared to healthy females. According to linear regression analysis, age and AMH values do not show significant correlations. In healthy bitches from different breed categories, a positive correlation was observed exclusively in the giant and large breed groups regarding their AMH levels.

The relative values of anti-Müllerian hormone (AMH) can vary significantly depending on the method and ELISA kit used, whether it is a kit intended for human, murine, or canine use. Due to the small differences in serum AMH values between the two groups, although statistically significant, they cannot be used for diagnostic purposes.

Chapter 6, titled "The prevalence of canine hypothyroidism in females with ovarian cysts and its influence on AMH values" aims to establish the degree of involvement of hypothyroidism in the development and progression of polycystic ovarian pathology in dogs. The research seeks to determine if there is a significant correlation between hypothyroidism and the prevalence and evolution of ovarian cysts in dogs.

The prevalence of hypothyroidism among female dogs diagnosed with ovarian cysts and who underwent thyroid hormone testing (n=48) was 12% (n=6). In 15% (n=7) of the individuals, thyroid hormone levels were at the lower limit with T4/fT4 values of: 1.37 µg/dl/0.86 ng/ml, 1.31 µg/dl/0.91 ng/ml, 1.26 µg/dl/1.10 ng/ml, 1.23 µg/dl/1.02 ng/ml, 1.48 µg/dl/0.87 ng/ml, 1.31 µg/dl/0.87 ng/ml, and 1.18 µg/dl/0.91 ng/ml. A percentage of 73% (n=35) of the cases were diagnosed with euthyroidism. Considering that thyroid marker values should be interpreted in a clinical context, I classified the cases with minimal values as hypothyroidism. Therefore, 27% (n=13) of cases were diagnosed with hypothyroidism, and 73% (n=35) of cases were diagnosed with euthyroidism.

The T4 values for female dogs diagnosed with ovarian cysts were compared between the euthyroid group (n = 35) and the hypothyroid group (n = 13). In the euthyroid group, T4 values ranged from 1.12 to 4.46 µg/dl (mean: 2.4154 µg/dl, SD: 0.7459). In the hypothyroid group, T4 values ranged from 0.57 to 1.55 µg/dl (mean: 1.1613 µg/dl, SD: 0.2952 µg/dl). Since the normality of T4 values in both groups was accepted, an independent samples t-test was applied. The results indicated a statistically significant difference ($p = 0.001$) in T4 values between the euthyroid and hypothyroid groups.

The independent samples t-test revealed no statistically significant difference ($p=0.531$) in AMH concentrations between females with euthyroidism and those diagnosed with hypothyroidism.

Based on the obtained data, the following conclusions can be drawn: Considering the less studied reproductive effects of hypothyroidism in canine species, greater attention should be given to this disorder in the pathological evaluation of infertility in females of this species. One-third of the female dogs with ovarian cysts in the research group were also diagnosed with hypothyroidism, indicating a significant correlation between the two conditions and highlighting a notable prevalence of hypothyroidism. Another noteworthy finding in this research is that medium and giant breeds are more predisposed to hypothyroidism; therefore, there is a high likelihood that hypothyroidism is an important cause of infertility in these breeds. Cases presenting clinical signs such as infertility, skin lesions, a tendency to obesity, etc., along with minimal T4 and fT4 values, should be considered cases of hypothyroidism.

Chapter 7, titled "The assement of sexual hormones profile in bitches with ovarian cystic pathology and the establishment of the relationship with AMH values" aims to establish the hormonal profile of patients diagnosed with polycystic ovaries in canine species to create a detailed hormonal profile for these patients. This effort seeks to enhance the understanding of polycystic ovarian pathology, thereby improving the clinical management of this condition.

In terms of incidence, mammary tumors and pyometra ranked first with an incidence of 19% (n=16), followed by cystic endometrial hyperplasia at 10% (n=8), and mortality and embryonic resorption at 9% (n=7). A lower incidence was observed in cases of abortion (4%), vaginitis, and obesity (2%). The lowest prevalence, at 1%, was seen in comorbidities such as uterine serous cysts, vaginal leukorrhea, and one case of diabetes.

The most frequently encountered clinical signs in bitches with ovarian cystic pathology were infertility at 18%, prolonged heat cycles at 9%, and short interestrus at 3%.

Following quantitative measurements of Testosterone II in females diagnosed with ovarian cystic formations, testosterone levels were found to be low, below 0.05 ng/ml, except for one case—a 9-year-old female German Shepherd with polycystic ovarian degeneration, where a serum testosterone level of 0.09 ng/ml was recorded. Due to the low values, testosterone levels were not included in the statistics.

Based on serum progesterone concentrations, the females included in the study were initially classified according to their estrous cycle stage, specifically diestrus and proestrus. In the control group, 22 bitches recorded a value lower than 3 ng/ml, corresponding to the anestrus period, while 26 females had serum progesterone levels ranging from 4.07 ng/ml to 80 ng/ml, out of a total of 48 females.

Samples from bitches diagnosed with ovarian cystic pathology were collected strictly during the diestrus and anestrus periods, thus the major differences in progesterone values can be attributed to the different stages of the estrous cycle. In 26 cases, a value lower than 3 ng/ml was recorded, typical for the anestrus period, while in 22 females, serum progesterone levels ranged from 7.30 ng/ml to 80 ng/ml, characteristic of the diestrus period.

When comparing progesterone values between the control group (median: 7 ng/ml) and the study group (median: 2.96 ng/ml), there was no statistically significant difference ($p = 0.6735$). Regarding age and breed size, no statistically significant differences in concentrations were observed.

In the control group, Spearman's rank coefficient analysis showed no significant difference between serum progesterone levels (mean: 20.91 ng/ml, SD: 23.30 ng/ml) and AMH levels (mean: 10.11 ng/ml, SD: 1.13 ng/ml), with a correlation of rho = 0.273 and a P-value of 0.0607. Similarly, in the group with cystic ovarian pathology, there was no significant difference between AMH levels (mean: 10.61 ng/ml, SD: 1.21 ng/ml) and progesterone levels (mean: 18.33 ng/ml, SD: 24.12 ng/ml), with a P-value of 0.9933 and rho = 0.00125.

The females in the study group had significantly higher estradiol levels ($p < 0.0001$), with a mean of 21.83 pg/ml (95% CI: 14.65-25.37), compared to the control group, which had a mean of 10.70 pg/ml (95% CI: 9.86-12.30).

To compare the estradiol values in the anestrous phase between the control group and the study group, it was observed that there was a statistically significant difference. Females in the study group had a significantly higher estradiol value ($p = 0.0105$) in the anestrous phase, with a median of 21.19 pg/ml (95% CI: 13.44-26.09), compared to the control group, which had a median value of 12.95 pg/ml (95% CI: 10.46-15.72).

The results of the comparison of estradiol values in the diestrus phase between the control group and the study group showed a statistically significant difference. Females in the study group had a significantly higher estradiol value ($p = 0.0105$) in the diestrus phase, with a median of 23.17 pg/ml (95% CI: 11.75-28.77), compared to the control group, which had a median level of 9.63 pg/ml (95% CI: 9.02-10.92).

The comparative analysis of estradiol values between females in the control group in diestrus and anestrus revealed that there was a statistically significant difference. Females in the anestrous phase had a significantly higher estradiol value ($p = 0.0105$), with a median of 12.95 pg/ml (95% CI: 10.46-15.72), compared to females in the diestrus phase, who had a median level of 9.63 pg/ml (95% CI: 9.02-10.92).

The results of the comparative analysis of estradiol values between females in the study group in diestrus and anestrus reflected that there was no statistically significant difference ($p = 0.8607$). Females in the anestrous phase had a median estradiol value of 21.19 pg/ml (95% CI: 13.44-26.09), compared to females in the diestrus phase, who had a median level of 23.17 pg/ml (95% CI: 11.74-28.77).

Based on the analyzed data, the following conclusions can be drawn: the incidence of ovarian cysts in bitches increases with age and after going through multiple heat cycles; females diagnosed with ovarian cysts exhibited significantly elevated estrogen levels, both in the diestrus and the anestrous phases; the most common concurrent conditions associated with cystic ovarian pathology were mammary tumors and pyometra, with an incidence of 19%, while infertility was observed in 18% of the cases studied; the presence of ovarian cysts does not significantly alter serum progesterone levels in either the diestrus or anestrous phases; in females with ovarian cysts, serum AMH levels remain elevated during both the diestrus and anestrus phases. In contrast, in healthy females, AMH levels decrease proportionally with progesterone levels from the diestrus to the anestrus phase; progesterone dominates the estrous cycle in this species and is a reliable marker for identifying the estrous period in female dogs, even when cystic formations are present. Although there is an increase in serum levels of both AMH and estrogens in bitches with ovarian cysts, no relationship was described between their serum levels.

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