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Concentrations of anti-Müllerian hormone in the domestic cat. Relation with spay or neuter status and serum estradiol

E. Axné^{a,*}, B. Ström Holst^b^a Division of Reproduction, Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Health, Swedish University of Agricultural Sciences, Uppsala, Sweden^b Division of Diagnostics and Large Animals, Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Health, Swedish University of Agricultural Sciences, Uppsala, Sweden

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ABSTRACT

Female cats with unknown history can be diagnosed as spayed or intact with a GnRH-stimulation test or an LH test independent of the stage in the estrous cycle. However, although most females are correctly diagnosed with the LH test, the sensitivity and specificity are not 100%. The GnRH-stimulation test, although reliable, requires an injection of buserelin 2 hours before the blood sample is collected. Granulosa cells are the only cell type that produces anti-Müllerian hormone (AMH) in females, whereas Sertoli cells produce AMH in males. Anti-Müllerian hormone has been linked to spay status in dogs and cats and to ovarian and testicular pathology and fertility in different species. Our aim was to evaluate serum AMH concentrations in spayed female cats and in intact female cats of known age and reproductive stage (inactive ovaries or luteal phase). In addition, our aim was to compare serum AMH concentrations in intact and neutered male cats. We analyzed serum AMH concentrations in 15 spayed and 16 intact females and in 15 intact and 12 neutered male cats. Serum AMH was below the lowest standard point (<0.14 ng/mL) in all spayed females and neutered males, ranged between 1.3 and 19.0 ng/mL in the intact females and between 4.8 and 81.3 ng/mL in intact males. Thus, the AMH test had 100% sensitivity and specificity to diagnose the presence or absence of ovaries and testes in this study. In addition, in contrast to serum estradiol, serum AMH was not affected by buserelin stimulation ($P = 0.459$). Serum AMH was not correlated with serum estradiol before ($r_s = -0.188$, $P = 0.519$) or after ($r_s = 0.335$, $P = 0.242$) buserelin stimulation in the intact females. Four 6-month-old intact cats (two females and two males) had the highest AMH concentrations which in the females might represent a prepubertal peak previously described in other species and in males is likely due to high concentrations before puberty. In conclusion, we found that the AMH Gen II ELISA is reliable for diagnosing spay and neuter status of cats and that the domestic cat might be an interesting model for studies on AMH dynamics.

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1. Introduction

Female cats with unknown history can be diagnosed as spayed or intact with a GnRH-stimulation test or an LH test

independent of the stage in the estrous cycle [1]. However, although most females are correctly diagnosed with the LH test, the sensitivity and specificity are not 100% [1,2]. The GnRH-stimulation test, although reliable, requires an injection of buserelin 2 hours before the blood sample is collected. Granulosa cells are the only cells that produce anti-Müllerian hormone (AMH) in females [3] why it is likely that the AMH test would have a high specificity and

* Corresponding author. Tel.: +46(0)18672181; fax: +46(0)18673545.
E-mail address: Eva.Axner@slu.se (E. Axné).

sensitivity for diagnosing the presence of ovaries. Recently, it was demonstrated that the serum concentration of AMH differs significantly between intact and spayed female cats, but it was stated that sample size of cats should be enlarged before a definitive cutoff is identified [4]. To our knowledge, there are no studies on AMH in the male cat. In males, AMH is produced by the Sertoli cell [5]. Anti-Müllerian hormone assays have also been used to evaluate fertility and gonadal pathology in humans and domestic animals [6–10]. In women, serum AMH concentrations decrease with age, independently of the menstrual cycle and the number of pregnancies [7]. Although cats do not experience menopause, evaluation of AMH might be useful for diagnosing reproductive aging and gonadal pathologies also in cats. Before the test can be used routinely for diagnostic purposes in the domestic cat, within and between individual variations have to be evaluated.

Our aims were to (1) evaluate the sensitivity and specificity of an AMH ELISA test to diagnose the spay and neuter status in cats, (2) evaluate if stimulation with the GnRH analog buserelin has an effect on serum AMH concentrations in female cats, and (3) evaluate if serum AMH concentrations were related to serum estradiol and progesterone concentrations in nonestrus female cats.

2. Materials and methods

2.1. Animals

Stored samples collected for a previous study from 16 intact and 15 females previously spayed according to their history and previous diagnostic tests were included [1]. From four intact females, there was only enough serum left in either the sample before or after the buserelin stimulation. Females with unknown history were included if laparotomy/spaying was performed immediately after the samples had been collected to confirm reproductive status. For the purpose of our previous study [1], females in the follicular phase were not included. One intact female was found to be approximately 3 weeks pregnant at ovariectomy. Ages ranged between 6 months and 11.5 years (median, 1.0 years) for the intact females and 2.2 and 4.8 years (median, 3.6 years) for the spayed. Previously collected serum from 15 intact male cats [11] and serum collected for this study from 12 neutered male cats were also included. The age of the intact males ranged between 6 months and 4 years (median, 9.6 months) and of the 12 neutered males between 2 and 14 years (median 8.9 years). For one of the neutered males, age was unknown.

The study was approved by the Ethical Committee for Experimentation with Animals in Uppsala, Sweden, and by the Swedish Animal Welfare agency for the use of private cats. Cat owners were informed about the study and signed an agreement to allow participation of their cats in the study.

2.2. Administration of the GnRH analog and collection of blood samples

Blood samples were collected from the cephalic vein. Two samples were collected from each female cat, before (time 0) and 2 hours after an injection with 0.4 µg/kg

buserelin (Receptal; Intervet, Danderyd, Sweden) intramuscularly. The serum was frozen and stored at -20°C . From male cats, only one serum sample was collected.

2.3. Evaluation of estradiol and progesterone

Concentration of estradiol-17 β was analyzed in one batch with a radioimmunoassay (Double antibody Estradiol; Siemens, LA, USA) modified and validated for cats. The intra-assay coefficients of variation were 16% at 4 pmol/L; 7% at 54 pmol/L; and 7% at 169 pmol/L. Concentrations of progesterone were analyzed with chemiluminescence (Immulite 2000; Siemens), validated for cats. The lowest standard point for estradiol-17 β was 2 pmol/L and for progesterone 0.6 nmol/L.

2.4. Evaluation of AMH

Stored samples were thawed and analyzed in one batch for AMH. Serum concentrations of AMH were analyzed using an enzyme-linked immunosorbent assay (AMH Gen II ELISA; Beckman Coulter), according to the manufacturer. Briefly, 20 µL of standards, controls, and samples were incubated in an anti-AMH antibody-coated microtitration plate. After incubation and washing, anti-AMH biotin conjugate was added to each well. After a second incubation and washing step, streptavidin-horseradish peroxidase was added. After a third incubation and washing step, the substrate, tetramethylbenzidine, was added and incubated briefly before adding an acidic stopping solution. The degree of enzymatic turnover of the substrate was determined by dual-wavelength absorbance measurement at 450 nm and 620 nm. The assay was validated for linearity of dilution and for imprecision.

Linearity of dilution was studied by diluting (1/2–1/16) one sample (19.2 ng/mL) with the sample diluent. Observed results were then plotted against the expected values and a regression fit performed. Expected values were calculated by dividing the concentration from the undiluted sample by the dilution factor used. Percentage recovery was calculated by dividing the observed values by the expected values and multiplying by 100.

Imprecision was calculated as intra-assay and interassay coefficient of variation (CV). Intra-assay CV was calculated from results of two samples (concentration, 7 and 19 ng/mL) analyzed 10 times in duplicate. Interassay CV was calculated after analyzing aliquots of two samples (1.5 and 10 ng/mL) five times in duplicate.

2.5. Statistics

Data are presented as individual values, medians, and ranges. Sensitivity was calculated as the proportion of intact cats that were correctly identified and specificity as the proportion of spayed females or neutered males that were correctly identified. All calculations and graphs were made with Minitab (Minitab Inc., State College, PA, USA). Comparisons of AMH values before and after buserelin stimulation in intact females are presented as range in the difference between the first and second samples, Wilcoxon test statistics, and as Spearman correlation of values. As

buserelin stimulation did not affect AMH concentrations, Spearman correlations were performed for mean AMH in each intact female and estradiol concentrations in blood samples collected before and after buserelin stimulation.

3. Results

Linearity under dilution was confirmed ($r^2 = 99.8\%$; Fig. 1), and recovery was 92 to 106%. Intra-assay CV was 2% at 7 ng/mL and 7% at 17 ng/mL. Interassay CV was 9% at 1.5 ng/mL and 12% at 10 ng/mL.

3.1. Females

All spayed females had AMH values below the lowest standard point (<0.14 ng/mL) in samples taken before and after the buserelin stimulation. Anti-Müllerian hormone concentrations in intact females ranged between 1.3 and 19 ng/mL (Fig. 2). At a cutoff value of 0.14 ng/mL, the test had thus 100% sensitivity and specificity.

Stimulation with the GnRH analog buserelin did not affect the AMH concentrations ($P = 1.0$; difference between first and second samples, 0 to 2.4 ng/mL; $n = 12$), and values before and after stimulation were highly correlated ($r_s = 0.945$, $P < 0.001$, Fig. 3).

There was no correlation between estradiol and AMH concentrations before ($r_s = -0.188$, $P = 0.519$) or after ($r_s = 0.335$, $P = 0.242$) buserelin stimulation or between mean AMH concentrations and progesterone concentrations ($r_s = -0.102$, $P = 0.706$; Fig. 4).

The two youngest females had the highest mean AMH concentrations (Fig. 5).

3.2. Males

All neutered males had AMH values below the lowest standard point of the test (<0.14 ng/mL). Anti-Müllerian hormone concentrations in intact males ranged between 4.8 and 81.3 ng/mL. The test had thus 100% sensitivity and specificity (Fig. 2). The two youngest males had the highest serum AMH concentrations (Fig. 6).

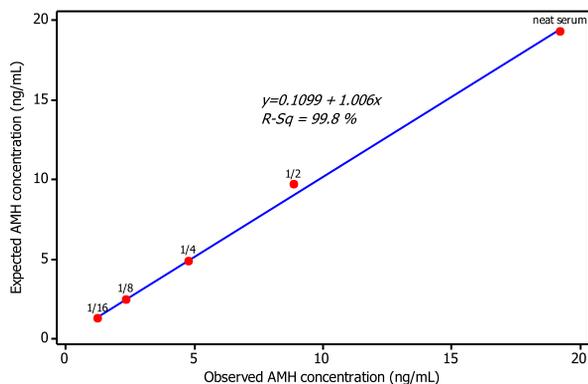


Fig. 1. Serial dilution of a feline serum sample illustrating adequate linearity with regression fit of measured concentrations of anti-Müllerian hormone (AMH). R-Sq indicates the coefficient of determination. (For interpretation of the references to color in this Figure, the reader is referred to the Web version of this article.)

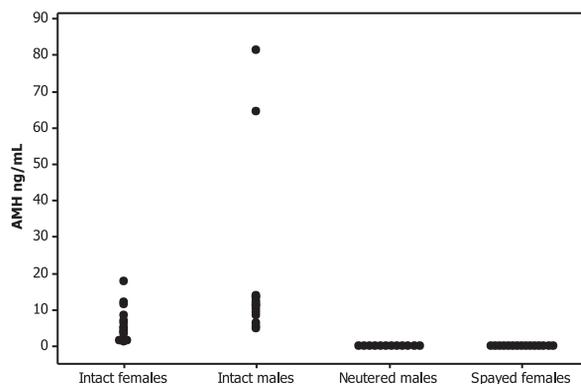


Fig. 2. Mean serum anti-Müllerian hormone (AMH) concentrations in spayed ($N = 15$) and intact ($N = 16$) female cats and in neutered ($N = 12$) and intact ($N = 15$) male cats. Hundred percent specificity and sensitivity at the detection limit of the test.

4. Discussion

We could confirm that analysis of the AMH had a 100% sensitivity and specificity to diagnose the presence or absence of ovaries and thus the spay status of female cats as also demonstrated by Place et al. [4] and in addition the neuter status of the males. The accuracy and precision of the AMH Gen II ELISA thus allows it to be used for feline serum samples with this indication.

All cats included in the present study had their spay status confirmed both by the inclusion criteria in our previous study and by the results of previous testing with estradiol concentrations after buserelin stimulation and LH test [1]. In addition, in contrast to the study by Place et al. [4], the stage of the reproductive cycle was known for all intact females. All females were either in the inactive or the luteal phase of the reproductive cycle as confirmed by ovarian inspection after spaying, progesterone concentrations, and vaginal cytology [1].

Buserelin stimulation resulted in an increase in estradiol concentrations in intact females [1] but did not affect the AMH concentrations in this study, neither did progesterone concentrations have an effect on the concentration of AMH.

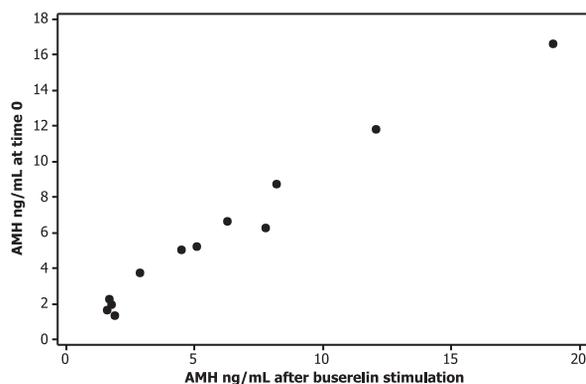


Fig. 3. Serum anti-Müllerian hormone (AMH) concentrations in intact female cats before and 2 hours after buserelin stimulation. $r_s = 0.945$, $P < 0.001$, $N = 12$.

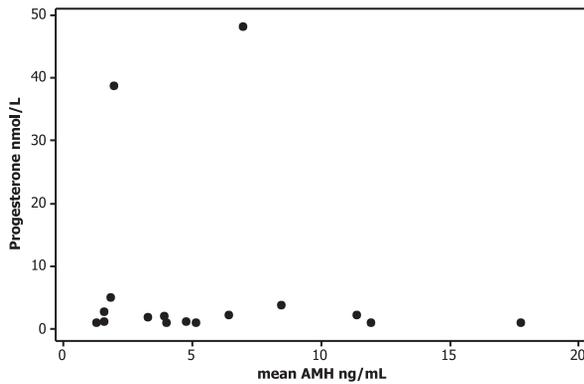


Fig. 4. Serum anti-Müllerian hormone (AMH) and progesterone concentrations in intact female cats. $r_s = -0.102$, $P = 0.706$, $N = 16$.

Thus, determining AMH concentration seems to be a reliable method for diagnosing the spay status in females both in the inactive and the luteal stages of the reproductive cycle. More samples from the luteal phase might, however, be required before strong conclusions can be made for females in the luteal phase. It is not possible to draw any conclusions about females in the follicular phase as females in this stage of the cycle were excluded from the study. In the follicular phase, it is, however, usually easy to diagnose the presence of ovaries with vaginal cytology [1] why blood samples are not required if a microscope is available. In addition, it is likely that AMH concentrations in the follicular or luteal stages of the cat's reproductive cycle would differ significantly from concentrations in spayed females as studies in other species have demonstrated that intraindividual differences are relatively small also during different stages of the reproductive cycle, because of a small numerical variation in the number of small antral growing follicles, that contribute the most to serum AMH concentrations [3,12].

As demonstrated in other species and in the domestic cat [3,4], interindividual variations in serum AMH were large in both the intact females and the intact males. In other species, low plasma AMH concentrations can be used as an indication of ovarian aging, whereas high AMH concentrations indicate that the size of the pool of preantral and small antral follicles is large [3]. In addition, some cyclical

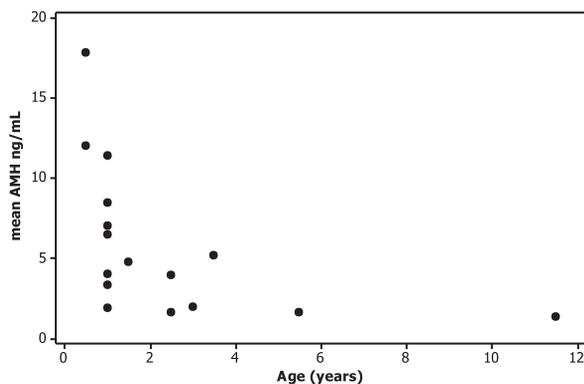


Fig. 5. Serum anti-Müllerian hormone (AMH) concentrations plotted against age in intact female cats. $N = 16$.

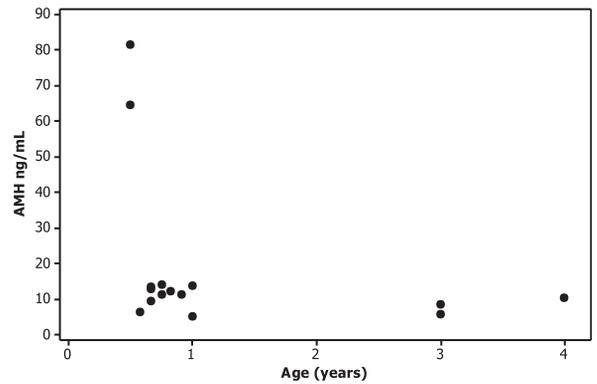


Fig. 6. Serum anti-Müllerian hormone (AMH) concentrations plotted against age in intact male cats. $N = 15$.

variations have been found, although cyclical changes may be different in different species [3]. The FSH stimulus caused by the busserelin injection that resulted in an increase in serum estradiol [1] did not affect serum AMH concentrations in this study. Rico et al. [13] suggested that AMH serum concentrations are independent of terminal follicular development. Serum estradiol is thus not expected to show a strong correlation with serum AMH concentrations, as estradiol is a marker of terminal follicular development [13]. To study possible cyclical changes in AMH concentrations in cat serum, serial samples from the same individuals would, however, be valuable. The distribution of ages was limited with only one intact female being older than 5.5 years and the oldest intact male being 4 years. In addition, the variability of serum AMH concentrations was high for the 1-year-old females. The two youngest females and the two youngest males had the highest AMH concentrations which in females might represent a prepubertal peak previously described in other species as for example the sheep (Lahoz et al. [14]). Further studies are warranted before any conclusions can be drawn about AMH as a marker for fertility or ovarian aging in the domestic cat. In male cats, the high concentrations for the two 6-month-old cats are probably a consequence of them not having reached puberty yet. In human males, a decrease in AMH is seen at puberty [15]. The domestic cat seems, however, to have comparably high concentrations of serum AMH and large interindividual variations [3,4] indicating that it might be an interesting model for further studies on AMH dynamics.

In conclusion, we could confirm that analysis of AMH was accurate for diagnosing the spay/neuter status of cats included in this study.

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