Uterine Androgen Receptor mRNA Expression in Metestrous and Anestrous Bitches being healthy or suffering from Pyometra

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Summary

The importance of androgens for the female reproductive system has been investigated for decades and a number of androgen sensitive processes has now been identified in female reproductive organs. For carnivore species no data were available so far about uterine androgen sensitivity and its regulation. The present study therefore aimed to investigate whether androgen receptors (AR) are present in the dog uterus, whether they are regulated throughout the ovarian cycle and whether pyometra affects their expression rate. Uterine tissue samples were collected from 28 bitches of different ages and various breeds. The samples were grouped according to the stage of estrous cycle (metestrus ME or anestrus AE) and the pathological status of the uterus (i. e. suffering from pyometra or not). Androgen receptor mRNA (AR mRNA) was quantified from 500 ng of total RNA isolated from the tissue samples using an internally standardized reverse transcription polymerase chain reaction (RT-PCR) described previously. The amount of total RNA extractable per g tissue was elevated during pyometra. The successful amplification of the expected 172 bp fragment from canine uterine RNA together with the confirmation of the identity of this fragment by sequence analysis, demonstrates that AR is expressed in this particular tissue. Comparing the expression rates in uteri from bitches during ME or AE being healthy (H) or suffering from pyometra (P), the only significant ($p<0.01$) difference was found between H and P uteri during ME with 3.5-fold lower expression rates in P. Although the same seems true for AE bitches, a significant difference could not be demonstrated due to the low number (n=2) of diseased animals in the AE group. There was no evident effect of the stage of ovarian cycle on uterine AR mRNA levels.

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Zusammenfassung

Die Bedeutung von Androgenen für das weibliche Reproduktionssystem wird seit Jahrzehnten untersucht und es wurde bereits eine Reihe androgensensitiver Prozesse im weiblichen Genitale entdeckt. Für Carnivoren lagen bislang keine Daten zur Androgensensitivität des Uterus und deren Regulation vor. Es wurde deshalb hier untersucht, ob Androgenrezeptor-mRNA (ARmRNA) im Uterus der Hündin nachweisbar ist, ob diese während des Ovarialzyklus reguliert wird und ob deren Expression durch das Vorliegen einer Pyometra verändert wird. Von 28 Hündinnen verschiedener Rassen und Altersstufen wurden Gewebeproben aus dem Uterus gewonnen und entsprechend dem Zyklusstand (Metöstrus ME und Anöstrus AE) und dem Gesundheitszustand (Pyometra oder gesund) eingeteilt. Die AR mRNA wurde in je 500 ng Gesamt-RNA, die aus den Geweben isoliert wurde, anhand einer intern standardisierten reversen Transkription mit anschließender Polymerasenkettenreaktion (RT-PCR) quantifiziert. Die Menge an extrahierbarer Gesamt-RNA pro g Gewebe war in Proben von Pyometra-kranken Tieren erhöht. Die erfolgreiche Amplifikation des erwarteten 172 bp AR-Fragments aus Hunde-Uterus zeigt zusammen mit der sequenzanalytischen Identitätsbestätigung, daß AR in diesem speziellen Gewebe exprimiert wird. Ein Vergleich der AR mRNA-Expressionsraten in Uteri von Hündinnen währed ME und AE, die gesund (H) waren oder Pyometra (P) hatten, zeigte 3.5 -fach niedrigere Werte bei den ME/P- als bei den ME/H-Tieren ($p < 0.05$). Für AE-Proben war zwar eine ähnliche Tendenz ($p = 0.07$) erkennbar; es wurde aber, vermutlich aufgrund der geringen Tierzahl in der AE/P Gruppe, die Signifikanzgrenze ($p < 0.05$) nicht erreicht. Ein Zusammenhang der AR mRNA Gehalte mit dem Zyklusstand wurde nicht beobachtet.

In veterinary practice, the use of androgens in females is limited to anabolic purposes in cachectic animals and, especially in bitches, to suppress or to postpone heat (O`CONELL, 1981). In contrast to Europe where gestagen application is commonly used for the latter indication, androgens are used more frequently in the United States and other countries. There testosterone implants or the anabolic-androgenic Mibolerone are used (ARBEITER, 1994). The mode of action of steroid hormone application in postponing estrus is based on the negative feedback of increased sex steroid concentrations on the pituitary release of gonadotropic hormones and thus on ovarian function. However, it remains open as to whether there is a functional role of endogenous androgens for female reproductive function. During the ovarian cycle of a non-pregnant bitch testosterone and androstenedione reach maximal circulating concentrations during late proestrus and beginning of metestrus; compared to estradiol, the basal and the peak concentrations of testosterone are about 10 to 20 fold higher (CONCANNON, 1986). A similar effectiveness of circulating estrogens and androgens in activating their respective receptors can be assumed since the affinity of the estrogen receptor (ER) is markedly higher than the one of androgen receptor (AR). An approximately 10-fold difference has for example been demonstrated for the dissociation constant of ER and AR in bovine skeletal muscle (K_d ER: 60 pM; MEYER and RAPP, 1985; K_d AR: 0.7 nM ; SAUERWEIN and MEYER, 1989). Based on these in principle considerations we aimed to elucidate the importance of endogenous androgens for the uterus by characterizing the androgen receptor mRNA expression in different stages of the ovarian cycle both in healthy as well as in pyometra affected bitches.

Material and Methods

Animals and tissue collection: Uterine tissue samples were collected from 28 bitches of different ages and various breeds. The samples were grouped according to the stage of the estrous cycle (metestrus or anestrus) and the pathophysiological state of the uterus (i. e. suffering from pyometra or not). Estrous cycle stage classification was based on the individual anamnesis (i. e. time interval since the last heat) and on vaginal inspection. Table 1 shows a detailed list of the different animals. Uterine tissue samples were dissected during hysterectomy, cut into small pieces (about 1 x 1 cm squares), aliquoted and immediately frozen in liquid nitrogen. Further storage was at –80 °C until RNA was extracted.

Tissue RNA extraction: After homogenization of the tissues according to CHIRGWIN et al. (1979), total cellular RNA was isolated using RNA-Clean™ (AGS, Heidelberg, Germany). Total RNA concentrations were determined by OD_{260} readings. The amount of RNA extracted per g of tissue was determined in 20 out of the 28 samples. The integrity of the RNA was assessed by inspection of the ethidium bromide-stained gels after agarose-formamide gel electrophoresis.

Quantification of AR mRNA: AR mRNA was measured by using an internally standardized reverse transcription polymerase chain reaction (RT-PCR) test system which has been described in detail earlier (MALUCELLI et al., 1996). In brief, a 172 bp fragment coding for the ligand binding domain of the AR protein was selected for amplification. The internal standard was obtained by deleting a 38 bp fragment from an amplified bovine AR sequence, which was then subcloned and transcribed into cRNA. Known dilutions of the competitor cRNA were spiked into a series of RT-PCR reaction tubes containing 500 ng of tissue RNA each. Following RT-PCR, the amplification products were separated by gel electrophoresis and quantified by densitometric analysis of ethidium bromide stain. Identical efficiencies of amplification rates were demonstrated for both templates. To obtain the concentration of AR mRNA initially present in the tissue RNA, the yields of the amplification products were compared by plotting their ratio against the $\log_{10}$ of the internal standard template (SIEBERT and LARRIK, 1992). The amount of competitor cRNA yielding equal molar amounts of PCR products was then calculated by extrapolating from the intersection of the curves, where the amounts of target and competitor are equal to the x-axis.

Statistical comparisons: data were analyzed by non-parametric analysis of variance (Kruskal-Wallis-Analysis); the significance of differences between groups was then assessed by the Mann-Whitney-Test.

Results

The successful amplification of a 172 bp fragment from dog uterine RNA together with the confirmation of the identity of this fragment by sequence analysis demonstrates that AR is expressed in this particular tissue. The obtained fragment which corresponds with the AR gene domain coding for the ligand binding region of the receptor protein, showed a 94 % homology to the human AR sequence. The protein sequence derived from this canine AR fragment is identical to the human one.

The amount of total RNA extractable per g tissue was 2.2 and 2.9 fold higher in samples from pyometra affected bitches during metestrus than in the samples from healthy anestrous or metestrous bitches, respectively ($p < 0.05$). Due to the low number of anestrous and pyo-

Table 1: **Breed, age, and ovarian gross appearance of the bitches allocated to the four different groups.**

Group allocated to Number *breed*	age (years)	gross appearance of the ovaries (size, functional structures)	remarks
Anestrous animals, healthy			
14 *Schnauzer (s)*	4	BS, residual C.L. present	s. f. s.
17 *Collie*	3	PS, no functional structures	s. f. s.
41 *Papillon*	1.5	LS, no functional structures	-
49 *Alaskan-Malamut*	2	PS - BS, no functional structures	-
50 *Yorkshire Terrier*	3	LS, no functional structures	-
61 *Poodle*	5	LS, no functional structures	s. f. s.
Anestrous animals, suffering from Pyometra			
11 *St. Bernhard*	8	BS, residual C.L. present	-
26 *Alsatian*	3.5	PS- BS, no functional structures	2.5 years gestagen treatment*
Metestrous animals, healthy			
1 *Alsatian (cb)*	2.5	BS, C.L. present	s. f. s.
3 *BMS*	5	BS, C.L. present	s. f. s
46 *Alsatian*	9	BS, C.L. present	-
52 *Alsatian*	7	BS, C.L. present	-
56 *Pekingese*	2.5	BS, C.L. present	-
71 *Alsatian*	1.5	BS, C.L. present	s. f. s.
Metestrous animals, suffering from Pyometra			
12 *Alsatian*	10	right ovary: BS, C.L. and follicle present, left ovary: BS, residual C.L.	-
13 *Poodle*	12	residual C.L. and follicle present	diabetes
15 *Dachshund (wh)*	14	BS, residual C.L. present	s.f.s
19 *Dachshund (wh)*	8	BS, C.L. present	-
22 *Boxer*	8	BS, C.L. present	-
29 *Schnauzer (g)*	12	BS, residual C.L. present	-
33 *Poodle*	11	PS - BS, C.L. present	-
35 *Alsatian*	3	CS, cysts	-
36 *Poodle*	12	BS, C.L. present	pregnant
39 *Chow Chow*	3	BS, C.L. present, cysts	-
42 *Dachshund (wh)*	7	PS, C.L. present	-
47 *Alsatian (cb)*	8	BS, C.L. present	-
57 *Alsatian (cb)*	10	BS, C.L. present	-
58 *Tyrolean Braque (cb)*	8	BS, C.L. present	estrogen therapy

Abbreviations used: *s* = standard size, *g* = giant, *cb* = cross breed, *wh* = wire-haired, *BMS* = Bavarian Mountain Slothound, C.L. = corpus luteum, BS = bean sized, LS = lens sized, PS = pea sized, CS = chestnut sized, s.f.s. = surgery for reasons of spaying.
* medroxyprogesterone acetate (Perlutex®) for estrus suppression

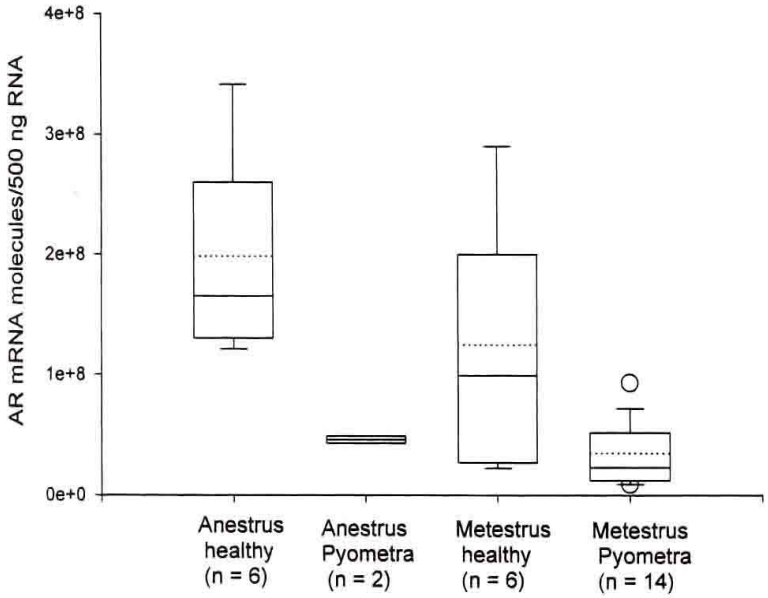

Fig. 1: Box plot representing the statistical characteristics of the AR mRNA concentrations measured in uterine tissue from anestrous or metestrous bitches being healthy or suffering from pyometra.
The boundary of the box closest to zero indicates the 25th percentile, the dotted line within the boxes marks the mean, the solid line is the median. Whiskers above and below the box indicate the 5th and 95th percentiles. Outlyers are depicted as open circles.

metra affected animals, this group could not be compared.

Figure 1 shows the AR mRNA expression rates in uteri from bitches during met- or anestrus being healthy or suffering from pyometra in a box plot format. The only significant ($p<0.05$) difference was found between healthy uteri and pyometra during metestrus. Although the same seems true for anestrous bitches, the difference ($p = 0.07$) did not reach the level of significance, probably due to the low number (n=2) of anestrous animals with pyometra. There was no significant effect of the stage of ovarian cycle on uterine AR mRNA levels in either the healthy ($p = 0.179$) or the affected ($p = 0.44$) animals. In the group of bitches suffering from pyometra in metestrus, the sample from animal #58 was classified to be an outlyer. AR mRNA expression rates showed no obvious relation with neither the size of the ovaries nor the presence of corpora lutea.

Discussion

The importance of androgens, being regarded as the classical male sex hormones, for the female reproductive system has been investigated for decades and a number of androgen sensitive processes has been identified in female reproductive organs. In another carnivore species, the spotted hyena, androgens are an essential part of female reproductive physiology (GLICKMAN et al., 1992). Testosterone as well as dihydrotestosterone (DHT) have been demonstrated to increase uterus weight in ovarectomized rats with similar effectiveness as estrogens (GONZALEZ-DIDDI et al., 1972). The uterotrophic effect of DHT supports the specifically androgenic mode of action since DHT cannot be converted to estrogens. Such a conversion has initially been assumed to explain the observed „estrogenic" effects of androgens in females (HARPER, 1967) but has additionally been refuted by GONZALEZ-DIDDI et al. (1972) who showed, that antiestrogen treatment does not inhibit the uterotrophic effects of androgens. Moreover, a divergent mode of action of estrogens, testosterone and DHT being differentially effective in endometrium and myometrium was observed (GONZALEZ-DIDDI et al., 1972).

With regard to the physiological importance of endogenous androgens for the female reproductive system and, especially for the uterus, different aspects are discussed:

- Modulatory effects on the expression of other sex steroid receptors, as indicated by investigations in hen oviduct uterus (KAWASHIMA et al., 1996), in which a stimulatory effect of DHT or testosterone injections on progesterone binding capacity was observed. Similarly, IWAI et al. (1995) have reported, that testosterone affects gestagen, androgen and estrogen receptor mRNA levels in cultured human endometrial stromal cells and concluded that the androgen may influence the responsiveness to progesterone of decidual change in these cells.
- Maintenance of decidual cell reaction as demonstrated in mice (ZHANG and CROY, 1996).
- Inhibition of apoptosis in neonatal and in adult mouse uterine epithelial cells induced to proliferate by estrogen (TERADA et al., 1990; JO et al., 1993).

Looking solely at the peripheral androgen concentrations during the canine ovarian cycle, a maximal effectiveness of androgens might be postulated for late proestrus/estrus and early metestrus. Provided that the uterine androgen sensitivity is constant throughout the ovarian cycle, this assumption seems true, however,

dynamical changes of steroid receptor concentrations have to be taken into consideration. Ovarian cycle dependent alterations in steroid hormone sensitivities as e. g. reported for gestagen receptors in bovine endometrium (MEYER et al., 1988), are related to the actual steroid hormone concentrations. There is a complex interplay of estrogens, gestagens and androgens with their respective receptors. For AR, estrogens seem to be the major regulators exerting a stimulatory effect on AR mRNA expression and AR synthesis (FUJIMOTO et al., 1994 and 1995). Coming back to the canine ovarian cycle, a maximum androgen sensitivity might thus be postulated for those phases in which endogenous estrogens are elevated, e. g. during proestrus and estrus. The present investigation compares metestrous and anestrous animals in which little differences of estrogen secretion are to be expected. Only at late anestrus estrogens are secreted in increasing amounts and might thus increase AR mRNA. Although the AR mRNA levels appeared tendencially higher in anestrous than in metestrous animals, the level of significance was not reached. From sample #58 there is a hint that estrogens do indeed stimulate AR mRNA in the dog uterus: this animal had undergone estrogen therapy and showed untypically high AR mRNA concentrations for the metestrous animals suffering from pyometra.

As indicated by the amounts of total RNA extractable per g of tissue, tissue RNA concentrations are elevated in the presence of pyometra. This might reflect generally increased transcription rates occurring during pyometra, being also characterized as cystic endometrial hyperplasia (BREITKOPF et al., 1997). When looking at the decreased AR mRNA per given amount of RNA in the affected animals, the general increase is not true for this specific mRNA. However, when extrapolating the AR mRNA concentrations to one g of tissue basis, the highest levels are still seen in samples from healthy animals. Considering the tissue reactions in pyometra, the observed reduction of androgen sensitivity might be explained by a shift within the relative importance of the various cell functions towards the defense mechanisms of inflammation. Thus hyperplasia during pyometra seems to occur independently from androgenic and possibly other endocrine control mechanisms.

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