Uterine androgen receptor mRNA expression in bitches of different physiological and pathological state

M. Pfaffl#, A. Brandstetter#, E. Möstl§, J. Handler*, K. Arbeiter*, H. Sauerwein#

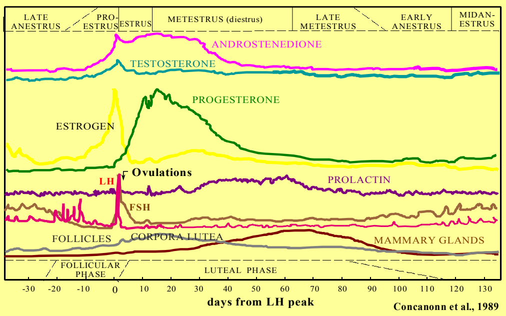
Institut für Physiologie, TU München, Freising-Weihenstephan, Germany#, Institut für Biochemie§ and Universitätsklinik für Geburtshilfe, Gynäkologie und Andrologie*, Veterinärmedizinische Universität Wien, Austria

Introduction

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 specifically androgen sensitive processes has now been identified in female reproductive organs.

During the ovarian cycle of a non-pregnant bitch, testosterone and androstenedione reach maximal circulating concentrations during late pro-estrus and beginning of metestrus; compared to estradiol, the basal and the peak concentrations of testosterone are about 10 - 20 fold higher (Fig. 1). A similar effectiveness of circulating estrogens and androgens in activating their respective receptors can be assumed since the affinity of the estrogen receptor (ER; $K_d \approx 60$ pM) is markedly higher than the one of the androgen receptor (AR; $K_d \approx 0.7$ nM; Sauerwein & Meyer, 1989). Based on these principial considerations we aimed to elucidate the importance of endogenous androgens for the uterus by characterizing ARmRNA expression in different stages of the dog ovarian cycle both in healthy as well as in pyometra affected bitches.

Fig. 1: The ovarian cycle of the dog


LATE ANESTRUS
PRO-ESTRUS
ESTRUS
METESTRUS (diestrus)
LATE METESTRUS
EARLY ANESTRUS
MIDAN-ESTRUS
ANDROSTENEDIONE
TESTOSTERONE
PROGESTERONE
ESTROGEN
Ovulations
LH
PROLACTIN
FSH
FOLLICLES
CORPORA LUTEA
MAMMARY GLANDS
FOLLICULAR PHASE
LUTEAL PHASE
-30 -20 -10 0 10 20 30 40 50 60 70 80 90 100 110 120 130
days from LH peak
Concannon et al., 1989

Materials and Methods

Animals and tissue collection: Uterine tissue samples were collected from 29 bitches of different ages and various breeds. The samples were grouped according to the stage of estrous cycle (metestrus or anestrus) and the pathophysiological state of the uterus (i.e. suffering from pyometra or not). Table 1 shows a detailed list of the animals. Uterine tissue samples were dissected during hysterectomy, aliquoted and immediately frozen in liquid nitrogen. Further storage was at -80ºC.

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) and quantified by OD_{260} readings. The amount of RNA extracted per g of tissue was determined in 20 out of the 29 samples. The integrity of the RNA was assessed by visual inspection of the ethidium bromide-stained gels after agarose-formamide gel electrophoresis.

Quantification of AR mRNA: AR mRNA was measured with an internally standardized **r**everse **t**ranscription **p**olymerase **c**hain 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 (Fig. 2). Known dilutions of the competitor cRNA were spiked into a series of cups 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 & 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.

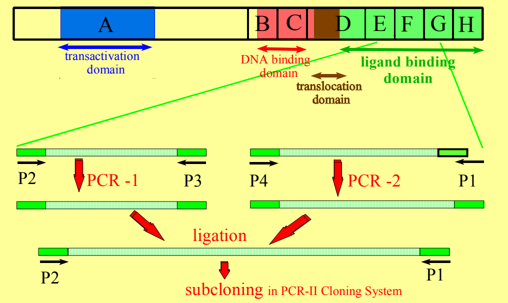
Statistical comparisons: data were analyzed by nonparametric analysis of variance (Kruskal-Wallis-Analysis); the significance of differences between groups was then assessed by the Mann-Whitney-Test.

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

Group allocated to Number	*breed*	age (a)	gross appearance of the ovaries (size, functional structures)	remarks
Anestric animals, healthy				
14	*Schnauzer (s)*	4	BS, residual C.L. present	s. f. s.
1	*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.
Anestric animals, suffering from Pyometra				
11	*St. Bernhard*	8	BS, C.L. present	-
26	*Alsatian*	3.5	PS- BS, no functional structures	2.5 years **Perlutex® treated**
Metestric 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.
Metestric animals, suffering from Pyometra				
12	*Alsatian*	10	right ovary: BS, C.L.+follicle present, left ovary: BS, residual C.L. present	-
13	*Poodle*	12	BS, residual C.L. and follicle present	Diabetes
15	*Dachshund (wh)*	14	BS, 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,	-
36	*Poodle*	12	BS, C.L. present	pregnant
39	*Chow Chow*	3	BS, C.L. present,	-
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, BS = bean sized, LS = lens sized, PS = pea sized, CS = chestnut sized, s.f.s. = surgery for reasons of spaying

Fig. 2: AR organisation and AR cRNA standard construction



Results

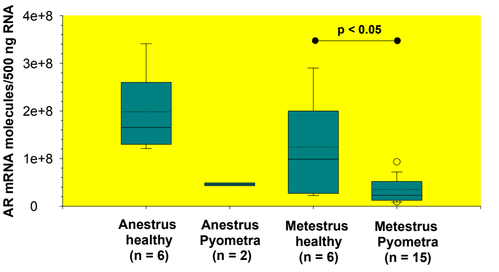
The successful amplification of a 172 bp fragment from dog uterine RNA together with the confirmation of the identity of this fragment by sequence analysis, demonstrates that AR is expressed in this particular tissue. The obtained fragment which corresponds to the AR gene domain coding for the ligand 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 recorded in 20 out of the 29 uterine samples; in samples from pyometra affected bitches during metestrus 2.2 and 2.9 fold higher RNA yields were obtained than in those samples from healthy anestric or metestric bitches, respectively ($p < 0.05$). Due to the low number of anestric and pyometra affected animals, this group could not be compared.

Fig. 3 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 and diseased uteri both during metestrus. Although the same seems true for anestric bitches, the difference ($p = 0.07$) did not reach the level of significance, probably due to the low number (n=2) of diseased animals in the anestrus group. 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 diseased ($p = 0.44$) animals. In the group of bitches suffering from pyometra during 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.

Fig. 3: AR mRNA concentrations measured in dog uteri

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. Outliers are depicted as open circles.



Discussion

The characterization of androgen sensitivity at the level of the mature protein, e.g. by radio receptor assay, is problematic since endogenous androgens induce a tight binding of the receptor in the nucleus from which a quantitative extraction by low ionic strength buffers is hardly possible. We therefore chose AR mRNA quantification in order to investigate as to whether AR expression rates are regulated during the dog ovarian cycle. Looking solely at the peripheral androgen concentrations during the dog ovarian cycle, a maximal effectiveness of androgens might be postulated for late pro-estrus/estrus and beginning 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. 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). For the dog ovarian cycle, a maximal androgen sensitivity might thus be postulated for those phases in which endogenous estrogens are elevated, e.g. during pro-estrus and estrus. The present investigation compares metestric and anestric animals in which little differences of estrogen secretion are to be expected. Only at late anestrus estrogens are increasingly secreted and might thus increase AR mRNA. Although the AR mRNA levels appeared in tendency to be higher in anestric than in metestric 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 had increased AR mRNA concentrations compared to the other metestric animals suffering from pyometra.

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 (KAWASHIMA et al., 1996; , IWAI et al., 1995).
- maintenance of decidual cell reaction as demonstrated in mice (ZHANG & 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).

As indicated by the amounts of total RNA extractable per g of tissue, tissue RNA concentrations are increased in the presence of pyometra, probably due to increased transcription rates occurring during pyometra, being also characterized as cystic endometrial hyperplasia. When looking at the decreased AR mRNA per given amount of RNA in the diseased animals, the general increase is not true for this specific mRNA. When extrapolating the AR mRNA concentrations to a g of tissue basis, the highest levels are seen in samples from healthy animals. Considering the tissue reactions during 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 occurs independently from androgenic and possibly other endocrine control mechanisms.

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