Time related changes in luteal prostaglandin synthesis and steroidogenic capacity during pregnancy, normal and antiprogestin induced luteolysis in the bitch

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\textbf{A B S T R A C T}

In nonpregnant and pregnant dogs the corpora lutea (CL) are the only source of progesterone (P4) which shows an almost identical secretion pattern until the rapid decrease of P4 prior to parturition. For the nonpregnant dog clear evidence has been obtained that physiological luteal regression is devoid of a functional role of the PGF\textsubscript{2\alpha}/H\textsubscript{9251}-system and seems to depend on the provision of StAR. Yet in pregnant dogs the rapid prepartal luteal regression, coinciding with an increase of PGF\textsubscript{2\alpha}, may be indicative for different regulatory mechanisms. To assess this situation and by applying semi-quantitative Real Time (Taq Man) RT-PCR, expression patterns were determined for the following factors in CL of pregnant and prepartal dogs and of mid-pregnant dogs treated with the antiprogestin Aglepristone: cyclooxygenase 2 (Cox2), prostaglandin E\textsubscript{2} synthase (PGES), prostaglandin F\textsubscript{2\alpha} synthase (PGFS), its receptors (EP\textsubscript{2}, EP\textsubscript{4} and FP), the steroidogenic acute regulatory protein (StAR), 3\textbeta-hydroxysteroid-dehydrogenase (3\textbetaHSD) and the progesterone receptor (PR). Peripheral plasma P4 concentrations were determined by RIA. CL were collected via ovariohysterectomy from pregnant bitches ($n = 3\text{–}5$) on days 8–12 (Group 1, pre-implantation...
1. Introduction

In pregnant and nonpregnant bitches the corpora lutea (CL) are the only sources of progesterone (P4) (Concannon et al., 1989), with the course of P4-concentrations in peripheral blood being virtually identical shortly until parturition, when it declines around day 60 of pregnancy to basal levels preceding the onset of fetal expulsions (Concannon et al., 1978). In nonpregnant dogs P4-levels continue to decrease gradually and reach anoestrus levels <1 ng/ml between days 60 and 90 (Feldman and Nelson, 1987). During the first 20–30 days after ovulation the newly formed CL are independent of gonadotropic support (Concannon et al., 1987); thereafter in the second half of dioestrus or gestation, removal of pituitary gland support results in luteolysis as LH and in particular prolactin have become essential luteotropic factors (Concannon, 1980; Okkens et al., 1990). Availability of prolactin and LH increases during this period, commencing with the decline of P4 (Gräf, 1978; Hoffmann and Schneider, 1993). Hence luteal regression occurs in spite of an increased gonadotropic support (Hoffmann et al., 1996).

In the nonpregnant bitch luteal function is independent of a luteolysin of uterine origin since normal ovarian function was observed after hysterectomy (Hoffmann et al., 1992). In pregnant bitches, however, the immediate prepartal P4 decrease coincides with an increase of PGF2α (Concannon et al., 1988; Nohr et al., 1993) of unknown origin suggesting a functional role in relation to parturition, also because luteal life-span can be terminated by exogenous application of PGF2α, though relatively high dosages or repeated treatments are necessary and strong side effects can occur (Romagnoli et al., 1991; Concannon and Hansel, 1977).

As observed in other species among the many factors involved in the control of luteal function prostaglandins seem to be of particular importance. Thus in ruminants and the pig onset of cyclic luteolysis is triggered by a well timed release of PGF2α from the endometrium, while luteal PGF2α, which is expressed in many species (reviewed by Wilthbank and Ottobre, 2003), seems to contribute to structural luteolysis (Diaz et al., 2002; Hayashi et al., 2003). Small amounts of uterine PGF2α acting via induction of luteal Cox2 stimulate the intraluteal production of PGF2α (Diaz et al., 2002). On the other side, PGE2 has shown to be a very potent luteotropic factor in several species. It stimulates luteal P4 secretion via a CAMP-mediated pathway as was shown for cattle, rabbits and humans (Kotwica et al., 2003; Marsh and LeMaire, 1974; Boiti et al., 2001). Its luteotropic efficiency has been shown to be comparable to that of LH (Weems et al., 1997).
In a previous paper we addressed the capacity of canine CL from nonpregnant bitches to produce prostaglandins; luteal Cox1 and Cox2 expression was clearly shown with expression of Cox1 resembling the pattern of a housekeeping gene and that of Cox2 being strongly cycle related with a peak on both, the mRNA and protein level, at the beginning of dioestrus (Hoffmann et al., 2004; Kowalewski et al., 2006a). In a follow up study expression of the main downstream enzymes, PGES and PGFS, leading to the formation of PGE2 and PGF2α was investigated (Kowalewski et al., 2008a,b). While PGFS was not or only weakly expressed, expression of PGES resembled that of Cox2-mRNA; it was highest at the beginning of the CL-phase and dropped significantly thereafter (Kowalewski et al., 2008a).

Expression of mRNA encoding for the PGE2 receptors, EP2 and EP4, was colocalized with PGES to luteal cells (Kowalewski et al., 2008a) and the spatio-temporal expression pattern of Cox2, PGES, EP2 and EP4 in the canine CL during dioestrus suggested that the locally produced PGE2 might act as a luteotrophic factor during the initial period of formation of the canine CL, explaining its apparent independence of gonadotropic support (Kowalewski et al., 2008a). Other than for PGFS, expression of the prostaglandin F2α receptor (FP) was clearly demonstrated in the CL of nonpregnant bitches with the mRNA-expression levels increasing from early to late dioestrus, explaining the receptivity of canine CL to exogenous PGF2α (Kowalewski et al., 2008b). In order to further characterize the functional stages of the CL, expression of steroiogenic acute regulatory (StAR) protein and of 3β-hydroxysteroid dehydrogenase Δ4/5-isomerase (3βHSD) was assessed. StAR is responsible for transport of cholesterol from the outer to the inner mitochondrial membrane where it serves as substrate for the side-chain-cleavage enzyme (P450scc). 3βHSD catalyzes the synthesis of P4 from pregnenolone. The expression-pattern observed for both factors on both, the protein and mRNA-level, suggests that P4 production might be controlled by the provision of substrate at the level of cholesterol transfer to the inner mitochondrial membrane rather than the enzyme availability (Kowalewski et al., 2006b; Kowalewski and Hoffmann, 2008).

All these observations support our previous hypothesis that luteal regression in the nonpregnant dog is not an actively regulated process but rather a permissive one (Hoffmann et al., 2004; Kowalewski et al., 2008b). Other than that, the prepartal increase of PGF2α in pregnant dogs (Concannon et al., 1988; Nohr et al., 1993) is suggestive of an active luteolytic system which might originate in either the CL of pregnancy or the uterine/placental compartment or both. The present paper addresses a likely involvement of the CL of pregnancy in such a system. To get the respective information, expression of Cox2, PGFS, PGES and the respective PGF2α and PGE2 receptors (FP, EP2 and EP4) as well as the expression of the P4 receptor (PR), was assessed in CL of pregnant bitches.

Functional loss of P4 activity leads to abortion/parturition in the dog and can be induced by application of a P4 receptor blocker, e.g. Aglepristone (Fieni et al., 1996; Hoffmann et al., 1999; Hoffmann and Schuler, 2000). Interestingly this treatment also induces luteolysis and it was speculated that this effect might result from an interaction of the antiprogestin with P4 receptors expressed in the canine CL (Hoffmann et al., 2004).

Thus in order to test which steps within the cascade of endocrine changes leading to abortion/parturition would be affected by antiprogestin treatment, the same parameters as in normal pregnancies were assessed following treatment with Aglepristone.

2. Materials and methods

2.1. Animals, tissue sampling and preservation

All animal experiments performed were approved by the respective authorities [permit no II 25.3-19c20-15c GI 18/14 and VIG3–19c20/15c GI 18,14 (Gießen) and permit no Ankara 2006/06 (Faculty of Veterinary Medicine, University of Ankara)].

2.1.1. Normal pregnancy

Four groups (n = 3–5) of clinically healthy, pregnant bitches of various breeds, aged 2–8 years, were formed with ovariohysterectomy (OHE) scheduled for the following days of pregnancy:

Group 1, pre-implantation, days 8–12, \( n = 5 \);
Group 2, post-implantation, days 18–25, \( n = 5 \);
Group 3, mid-gestation, days 35–40, \( n = 5 \);
Group 4, prepartal progesterone decline, \( n = 3 \).

In Group 4 P4 was determined in 6 h intervals beginning on day 58 of pregnancy. OHE was performed when P4 levels continued to decrease in two consecutive measurements below the level of 3 ng/ml.

2.1.2. Induced abortion

In a second study 10 bitches were treated with the antiprogestin Aglepristone between days 40 and 45 of pregnancy, using the dose recommended for induction of abortion [10 mg/kg bw, 2 times 24 h apart]. OHE was performed 24 h (\( n = 5 \)) and 72 h (\( n = 5 \)) after the second injection.

In all bitches the day of insemination/mounting (day 0) had been recorded; pregnancy was confirmed by either ultrasound (Groups 2–4; dogs where abortion was induced) or detection of embryos in uterine flushings (Group 1).

Immediately after surgery the corpora lutea were trimmed off the surrounding tissue, incubated overnight in RNALater® (Ambion Biotechnologie GmbH, Wiesbaden) for RNA preservation and then stored at \(-80^\circ C\) until further use as described earlier (Kowalewski et al., 2006a).

2.2. Determination of progesterone (P4)

Determination was by an established in house radioimmunoassay as described (Hoffmann et al., 1973).

2.3. Reverse transcription (RT), semi-quantitative Real Time (TaqMan) polymerase chain reaction (PCR) and data evaluation

Expression profiles of Cox2, PGES, PGFS, FP, EP2, EP4, PR, 3βHSD and StAR were assessed using semi-quantitative Real Time (Taq Man) RT-PCR. Nucleotide sequences of previously published TaqMan-systems were applied (Kowalewski et al., 2006a,b, 2008a,b; Kowalewski and Hoffmann, 2008). The primers were ordered from MWG Biotech AG, the 6-carboxyfluorescein (6-FAM) and 6-carboxytetramethyl-rhodamine (TAMRA) labelled probes were from Eurogentec, B-4102 Serain, Belgium. For primers and TaqMan probes sequences, see Table 1. Total RNA was isolated using Trizol®-Reagent (Life Technologies, Karlsruhe, Roche Molecular Biochemicals, Mannheim, respectively). Our previously described protocol was applied (Kowalewski et al., 2006a) and the reactions were carried out in an automated fluorometer ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). Briefly: 100 ng of DNase-treated total RNA was reverse-transcribed using the GeneAmp Gold RNA PCR Kit (PerkinElmer Applied Biosystems GmbH, Weiterstadt, Germany). Samples were analyzed in duplicates. 25 µl of reaction mixture contained 12.5 µl TaqMan® qPCR MasterMix (Eurogentec, B-4102 Serain, Belgium), 300 nM of each primer and 200 nM TaqMan Probe and 5 µl of cDNA. Amplification was carried out as follows: denaturation for 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s.

Relative quantification was done by normalizing the signals of target genes with the GAPDH signal and using the comparative CT method (\( \Delta \Delta CT \) method) according to the instructions of the manufacturer of the ABI PRISM™ 7000 Sequence Detector.

2.4. Statistics

To test for an effect of the observational group on the mRNA-levels of the target genes, a parametric analysis of variance (ANOVA) was applied. Multiple comparison post-tests were performed in case of \( p < 0.05 \). Those were, Tukey–Kramer Multiple Comparison Test during the course of pregnancy and the Dunnett’s Multiple Comparison Test to test the effect of the antiprogestin treatment.
Table 1
List of primers used for Real Time (TaqMan) PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession numbers</th>
<th>Primer sequence</th>
<th>Product length (bp)</th>
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</thead>
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<tr>
<td>Cox2-for</td>
<td>AY044905</td>
<td>5′-GGA GCA TAA CAG AGT GTG TGA TGT G-3′</td>
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<td>Cox2-rev</td>
<td></td>
<td>5′-AAG TAT TAG CCT GCT GTG CTT GAA T-3′</td>
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</tr>
<tr>
<td>Cox2-Taq Man Probe</td>
<td></td>
<td>5′-CGC TCT ATG TCC CAT TCT GCG TGC T-3′</td>
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</tr>
<tr>
<td>PGFS-for</td>
<td>AY875970</td>
<td>5′-AGG GCT TGC CAA GTT TAT TGG-3′</td>
<td>74</td>
</tr>
<tr>
<td>PGFS-rev</td>
<td></td>
<td>5′-GCC TGT GCT TGC TCA GGA T-3′</td>
<td></td>
</tr>
<tr>
<td>PGFS-TaqMan Probe</td>
<td></td>
<td>5′-TCC AAC TTT AAC CGG CAG CTC G-3′</td>
<td></td>
</tr>
<tr>
<td>FP-for</td>
<td>DQ138060</td>
<td>5′-ACC AGT CGA ACA TCC TTT GCA-3′</td>
<td>86</td>
</tr>
<tr>
<td>FP-rev</td>
<td></td>
<td>5′-GGC CAT CAC ACT GCC TAG A-3′</td>
<td></td>
</tr>
<tr>
<td>FP-TaqMan Probe</td>
<td></td>
<td>5′-CAT GGT GTC TCC TCT GTC TCG C-3′</td>
<td></td>
</tr>
<tr>
<td>PGFS-for</td>
<td>EF063141</td>
<td>5′-CTG TCA TCA CGG GCC AAC A-3′</td>
<td>99</td>
</tr>
<tr>
<td>PGFS-rev</td>
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<td>5′-CCT GGT CAC TCC GGC AAT A-3′</td>
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<td>PGFS-TaqMan Probe</td>
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<td>5′-ACG CCC TGA GAC GAG GCC CTT-3′</td>
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<tr>
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<td>5′-CAC CCT GCT GTG CTC C-3′</td>
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<td></td>
<td>5′-CGG TGC ATG CCG ATG A-3′</td>
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<td>5′-TGG TGC CCT GCA ACT TCT AGC GTC-3′</td>
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<td>AF177934</td>
<td>5′-AAA TCA GCA AAA ACC CAG ACT TG-3′</td>
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<td>5′-ATC CGA ATT GCT GTG AAC CCT ATC C-3′</td>
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<tr>
<td>3βHSD-for</td>
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<tr>
<td>3βHSD-rev</td>
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<td>StAR-for</td>
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<td>5′-CGA GGC TCC ACC TGT GTG A-3′</td>
<td>65</td>
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<td></td>
<td>5′-CCT TTC TGC TCA GCC ATC TC-3′</td>
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<td>5′-CTG GCA TGG CCA CAC ATT TC-3′</td>
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</tr>
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<td>PR-for</td>
<td>AF177470</td>
<td>5′-CGA GTC ATT ACC TCA GAA GAT TTG TTT-3′</td>
<td>113</td>
</tr>
<tr>
<td>PR-rev</td>
<td></td>
<td>5′-CTT CCA TGG CCC TTT TAA AGA A-3′</td>
<td></td>
</tr>
<tr>
<td>PR-TaqMan Probe</td>
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<td>5′-AAG CAT CAG GCT ATG GTG CTC TAA AAT-3′</td>
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<tr>
<td>GAPDH-for</td>
<td>AB028142</td>
<td>5′-GCT GCC AAA TAT GAC GAC ATC A-3′</td>
<td>75</td>
</tr>
<tr>
<td>GAPDH-rev</td>
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<td>5′-GTA GCC CAG CAT GCT TTG GAG-3′</td>
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<tr>
<td>GAPDH-TaqMan Probe</td>
<td></td>
<td>5′-TCC TCT CGA TGC CTT CAC TAC CTT-3′</td>
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</tbody>
</table>

on the mRNA-expression of target genes. Due to the uneven distribution of the Real Time data for
the StAR mRNA-expression during the course of pregnancy the Kruskal–Wallis Test (a nonparametric
ANOVA) followed by Dunn's Multiple Comparisons Test was applied. Numerical data were presented
as the mean ± standard deviation. For all tests the statistical software program, GraphPad3 (GraphPad
Software Inc., San Diego, CA, USA) was used.

3. Results

3.1. Normal pregnancy

3.1.1. Expression studies

A significant effect of time was observed in the expression of Cox2 and PGES (p<0.0001 and
p<0.0022, respectively) with expression being highest during the pre-implantation period and signif-
icantly lower (p<0.001 and p<0.01) thereafter (Figs. 1A and 2A).

A significant effect of time was also observed for the expression of PGFS mRNA (p<0.04) which
was lowest at the pre-implantation period, highest (p<0.05) in the post-implantation and slowly
decreasing thereafter towards parturition (Fig. 1C).

A similar expression pattern was observed for FP, however, the changes were statistically not
significant (Fig. 1E).

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Expression of EP2 but not EP4 showed an effect of time ($p < 0.01$), with a constantly low expression until prepartal luteolysis when an upregulation was observed (Fig. 2C and E).

PR revealed a significant effect of time ($p = 0.04$) with a biphasic expression pattern showing an increased mRNA expression during the second half of gestation (Fig. 3A).

Expression of StAR and 3βHSD was time dependant ($p = 0.04$ and $p = 0.002$, respectively), showing a constant decrease from the pre-implantation period until prepartal luteolysis (Fig. 3C and E).

### 3.1.2. Progesterone concentrations

Mean progesterone concentrations were: 35.71 ± 7.9 ng/ml in the pre-implantation period, 29.73 ± 13.23 ng/ml in the post-implantation period, 13.32 ± 8.66 ng/ml at mid-gestation and 2.07 ± 0.99 ng/ml during the prepartal progesterone decline; the effect of time was highly significant ($p < 0.0019$).

### 3.2. Induced abortion

#### 3.2.1. Expression studies

For all parameters the expression patterns were compared with the mid-gestation group. Following treatment with the antiprogestin expression of StAR and 3βHSD decreased significantly ($p < 0.01$ and $p < 0.05$, respectively) resembling the situation in prepartal luteolysis (Fig. 3D and F). The expression...
Fig. 2. Expression of PGES, EP2 and EP4 as determined by Real Time (TaqMan) PCR during pregnancy and normal luteolysis (A, C, E) and during Aglepristone induced luteolysis (B, D, F; compared with the mid-gestation group.). RGE: relative gene expression (mean ± standard deviation); bars with different asterisks differ with $p < 0.01$ (A, C).

of PR, PGES, EP2, EP4, Cox2, and FP (Figs. 3B, 2B, D and F and 1B and F) was not affected; as in normal prepartal luteolysis but more pronounced ($p < 0.001$) was the decrease in PGFS (Fig. 1D).

3.2.2. Progesterone concentrations

The mean progesterone concentration before the first treatment was $15.11 \pm 6.7$ ng/ml. Mean concentration at the second Aglepristone treatment 24 h later was at $13.61 \pm 8.2$ ng/ml. It had decreased ($p < 0.01$) to $5.1 \pm 2.7$ ng/ml 24 h later and was at $2.33 \pm 1.44$ ng/ml, respectively $1.2 \pm 0.6$ ng/ml 48 and 72 h later.

4. Discussion

Establishment and maintenance of pregnancy in the dog depends on the secretion of P4 from the CL—the only source of this hormone in nonpregnant and pregnant animals. Luteotropic factors securing CL-function during the second half of pregnancy, respectively dioestrus are prolactin and LH (Concannon, 1980; Okkens et al., 1990) and only recently some evidence was obtained that luteal PGE2 acting via autocrine/paracrine mechanisms might support the developing CL during the first two to three weeks of dioestrus (Kowalewski et al., 2008a). On the other side our investigations in the nonpregnant, dioestric dog have revealed no hints of an active luteolytic principle which would involve uterine (Hoffmann et al., 1992) or CL-derived PGF2α (Kowalewski et al., 2008b). Clearly the decrease of P4-synthesis results from a decreased expression of StAR (Kowalewski and Hoffmann, 2008) and 3βHSD (Kowalewski et al., 2006b). Though the mechanisms governing StAR- and 3βHSD-expression in the CL of the bitch still remain open, the hypothesis was developed that the luteal regression in the...
nonpregnant dog is not an actively regulated but rather a passive, permissive process (Kowalewski et al., 2008b).

Other than in nonpregnant animals, there is no information available on the intraluteal expression of the prostaglandins system in the CL of pregnant dogs. Yet there is strong evidence, that in the pregnant animal prepartal luteolysis is actively regulated through PGF2α mediated mechanisms, since there is an immediate prepartal increase of PGF2α concomitant to the decline of P4 (Concannon et al., 1988; Nohr et al., 1993), which – apart from being responsible for the induction of labour – might also lead to luteolysis. The origin of this PGF2α increase is, however, unknown and both, the uterine/placental compartment as well as the CL might contribute. This would resemble the situation in the cattle, where luteal PGF2α is supposed to contribute to structural luteolysis (Diaz et al., 2002; Hayashi et al., 2003) in conjunction with the upregulation of placental Cox2 (Schuler et al., 2006).

The present study has clearly shown that Cox2 and PGES, similarly to the nonpregnant dog (Kowalewski et al., 2006a, 2008a), are upregulated at the phase of CL formation (pre-implantation period), with PGE2 possibly acting as a luteotropic factor. While the respective receptors for PGE2, EP2 and EP4, are expressed on a rather constant level from days 15 to 65 in nonpregnant dogs, expression of EP2, but not EP4, is upregulated in pregnant dogs during prepartal luteolysis. At present no functional interpretation can be given, however, an increased availability of EP2 might render the CL more susceptible for PGE2 of extra luteal origin. Other than in nonpregnant animals, where only weak or no PGFS expression was detectable in the CL during the dioestrus (Kowalewski et al., 2008b), luteal PGFS mRNA-expression was relatively high throughout pregnancy. The observed PGFS-levels were upregulated from the pre-implantation to the post-implantation period followed, however, by a gradual decrease towards parturition, making it most unlikely that the CL of late pregnancy is the source of the prepartal PGF2α increase. Similar changes though not significant, were observed for FP, an observation

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pointing towards a likely functional role of PGF2α as an autocrine/paracrine factor during early- and mid-gestation.

Following application of Aglepristone on days 40–45 of pregnancy, a time point distinctly apart from the onset of physiological prepartal luteolysis, a significant downregulation of StAR and 3βHSD was observed after 24, respectively 72 h after the second injection, paralleled by a concomitant decrease of P4 from 13.61 ± 8.2 ng/ml to 5.1 ± 2.7 and 1.2 ± 0.6 ng/ml and a significant decline in the expression of PGFS.

The mechanisms underlying these processes are still obscure; yet it might be speculated that interference of the antiprogestin with luteal P4-receptors, the expression of which is increased during mid-gestation and prepartal luteolysis (Fig. 3A), might be a triggering effect.

Thus, except for the upregulation of EP2 during prepartal luteolysis, the gross overall expression pattern of mRNA encoding for the prostaglandins system in antiprogestin treated dogs resembled that during the prepartal withdrawal of P4.

Thus our data do suggest that – as in nonpregnant bitches – also in pregnant bitches PGF2α of luteal origin is not a factor modulating luteolysis.

In conclusion, our data suggest that the blocking of PR by the antiprogestin Aglepristone triggered downregulation in the expression of StAR, 3βHSD and PGFS, pointing towards an involvement of luteal P4 as an autocrine factor within a positive loop feedback system. Similarly as in the nonpregnant bitch also in the pregnant bitch PGF2α of luteal origin seems not to be involved in the mechanisms leading to regression of the CL. However, as in the nonpregnant bitch the expression of FP renders the CL susceptible to extraluteal PGF2α. Our first and not yet published results make the placenta a likely candidate for the origin of the prepartal PGF2α increase. The high expression of PGES observed in the pre-implantation period agrees with previous observations in the nonpregnant bitch and stresses the role of CL derived PGE2 as an autocrine luteotropic factor during the period of CL formation in the dog.

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References


Dopamine agonists, anti-progestins, anti-androgens, long-term-release GnRH agonists and anti-estrogens in canine reproduction: A review

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Abstract

Over the last 10 years, new drugs have been applied to canine reproduction, widening the spectrum of therapeutic possibilities for diseases that were previously surgically treated, and facilitating better control of the estrous cycle and fertility. Some are not approved for use in dogs; their use is experimental and further clinical trials are necessary. Dopamine agonists such as cabergoline, bromocriptine or metergoline are ergoderivative alkaloids that exert an anti-prolactinergic effect via stimulation of D2 pituitary receptors or inhibition of central serotoninergic ones. Their main indication is suppression of lactation. Anti-prolactinergic compounds have also been successfully used for pregnancy termination and shortening of interestrous intervals. Anti-progestins, (e.g. mifepristone and aglepristone) are synthetic steroids that bind with high affinity to progesterone (P4) receptors, preventing P4 from exerting its biological effects. Anti-progestins have been indicated in P4-dependent conditions, such as pregnancy termination, induction of parturition and the medical treatment of pyometra. Several groups of drugs have been described to have anti-androgenic properties through different mechanisms of action: progestins, receptor binding anti-androgens (e.g. flutamide), competitive enzyme inhibitors (e.g. finasteride), aromatase inhibitors, and GnRH agonists. Their main application is medical treatment of benign prostatic hyperplasia. Long-term release formulations of GnRH agonists (e.g. leuprolide or deslorelin acetate) postponed puberty and reversibly suppressed reproductive function in male and female dogs for periods exceeding 1 year. Anti-estrogens (e.g. clomiphene and tamoxifen citrate) are synthetic non-steroidal type I anti-estrogenic compounds that competitively block estrogen receptors with a combined antagonist-agonistic effect. In dogs, their action is more agonistic than antagonistic.

Keywords: Dopamine agonist; Anti-estrogen; Anti-progestin; Anti-androgen; Long-term-release GnRH agonist; Dog

1. Introduction

Over the last 10 years, new drugs have been applied to canine reproduction. They have expanded the spectrum of therapeutic possibilities for diseases that were previously surgically treated, and they permit better control of the estrous cycle and fertility in general. Due to differences in availability of these drugs in various countries, the frequency of application and the general knowledge of these compounds vary considerably among practitioners.

Although most of these drugs have already been used for years in other species, only few are widely marketed or approved for use in dogs. The aim of this article is to briefly review the main pharmacological properties, reported trials and possible indications of new groups of drugs, e.g. dopamine agonists, anti-progestins, anti-estrogens, anti-androgens, and long-term-release preparations of gonadotrophin releasing hormone (GnRH) agonists for use in canine reproduction. Several of the
2. Dopamine agonists

Dopaminergic agonists are ergotine-derivative alkaloid compounds that exert an anti-prolactinergic effect. Two of the most widely used dopamine agonists in dogs are bromocriptine and cabergoline, which have a direct action on D₂-dopamine receptors of the lactotrophic cells of the anterior pituitary gland. Metergoline, another ergot alkaloid, is a serotonin antagonist, exerting dopaminergic effects at high doses [1,2]. The ability of dopamine agonists to inhibit prolactin (PRL) secretion makes them optimal for milk suppression, either during overt pseudopregnancy episodes or in the post-partum period [3–7] and they are marketed with that indication in several countries. It is well known that PRL is a required luteotropic hormone during the second half of canine luteal phase [8]. Therefore, anti-prolactins can also be used to suppress luteal function in progesterone (P₄) dependent conditions such as pyometra, unwanted pregnancy [9–11] and mammary tumors.

Different combinations of either natural or synthetic prostaglandins (PG) F₂α and dopamine agonists have been reported to efficiently terminate gestation from 25 days after the luteinizing hormone (LH) surge, without substantial side effects [12–14]. This drug combination was also used for medical treatment of various stages of spontaneous pyometra, with a success rate of 82% [15]. Dopamine agonists (e.g., cabergoline, 5 μg/kg/day p.o) have also been recommended for pre-surgery treatment of canine mammary tumors, and for reducing the incidence of mammary tumors by treating overt pseudopregnancy (associated with mammary tumors [16]), as well as for reducing mammary size before mammectomy [17]. Finally, treatment with PRL inhibitors shortened the interestrus intervals in bitches, either by advancing luteal regression or by reducing the anestrus period [7,18–20]. Although they were effective in inducing estrus in bitches with prolonged anestrus [21,22], precise mechanisms of action of dopamine agonists leading to estrus induction in anestrus bitches are not well understood. As estrous induction by dopamine agonists does not depend on decreasing serum PRL concentrations [7,23], perhaps they directly stimulate the hypothalamic-pituitary axis or exert a peripheral action on the ovaries.

In an early report, bromocriptine was used in four bitches (20 μg/kg bid p.o) from 1 to 5 days after the LH surge to the beginning of the next proestrus. The interestrus interval was significantly reduced in treated versus control bitches (123 ± 23 versus 245 ± 8 days, respectively) [18]. A pregnancy rate of more than 60% was reported [24] in bitches that were mated during bromocriptine-induced estrus. In an early study, using metergoline (12.5 mg im per bitch every 3 days, starting between 78 and 161 days after proestrus) the interestrus interval was significantly shorter than in the control group (144 versus 207 days, respectively). Ten treated bitches in this protocol ovulated and nine became pregnant, although the high dose of metergoline (1 mg/kg) provoked vomiting [25]. In another trial, metergoline (0.1 mg/kg bid p.o) administered to seven bitches 100 days after ovulation significantly decreased serum PRL concentrations, but did not affect the interestrus interval [26].

Oral treatment with cabergoline (5 μg/kg/day p.o) in bitches with prolonged anestrus resulted in estrous induction after 5–18 days of treatment and in pregnancy in all 28 bitches that were treated. [21]. Administration of the same dose of cabergoline from 30 days after the LH surge reduced the interestrus intervals from 216 to 66.5 days. However, none of these bitches became pregnant, probably due to insufficient uterine endometrial regeneration. Nevertheless, this study demonstrated that estrus cannot only be induced in anestrus, but also in diestrus [19]. The duration of cabergoline treatment was shorter in late versus early anestrus (means, 6 versus 20 days, respectively) and normal hormonal characteristics and fertility were obtained on induced cycles [20].

3. Anti-progestins

Anti-progestins are synthetic steroids that bind with great affinity to P₄ receptors, preventing P₄ from exerting its biological effects [27]. Some anti-progestins also have the ability to interact with different binding affinity for glucocorticoid receptors [27]. In dogs, the anti-progestins mifepristone (RU 486) and aglepristone (RU 534) have been used for experimental and clinical purposes, including pregnancy termination and management of pyometra. Aglepristone is available in the veterinary market of some American and European countries, with an indication for pregnancy termination. Aglepristone acts as a true P₄ antagonist at the uterine level, without initially decreasing serum P₄ concentrations. Mifepristone also terminated pregnancy in the bitch within 3–4 days, without side effects [28,29].
Aglepristone (10 mg/kg sc given twice, 24 h apart) was tested for pregnancy termination in 104 bitches at 15–55 days after mating. Treatment before 25 days resulted in resorption in all animals, whereas later administration induced abortion within 7 days in 96% of the cases [30]. Typical signs of parturition were associated with abortion, and vaginal discharge persisted for 3–5 days afterwards. Clinical monitoring over more than 18 months did not reveal any treatment sequelae [30]. In a more recent study in five beagle bitches receiving the same therapeutic protocol 30 days after ovulation, abortion was induced within 4–7 days after the start of treatment. Plasma PRL concentrations were elevated during treatment, whereas plasma P4 remained unchanged [31]. In 10 bitches treated in early or mid-pregnancy, aplepristone did not modify plasma concentrations of P4, PGF2α, oxytocin or cortisol within 24 h after administration, but increased PRL concentrations within 12 h of administration [32]. The interestrous intervals following treatments were shortened by 1–3 months in the three studies [30–32].

Other clinical applications described for anti-progestins are induction of parturition and medical treatment of pyometra. In four bitches, mifepristone induced signs of parturition after 50 days of pregnancy, although a caesarean section was necessary [33]. In another study, aplepristone (15 mg/kg sc) was administered 58 days of pregnancy in 10 bitches. Starting 24 h later, the bitches were injected sc with either oxytocin (0.15 IU/kg) or the PG alfaprostol (0.08 mg/kg) every 2 h until the end of parturition. On average, parturition started 32 h after aplepristone administration. Peripheral P4 concentrations increased after aplepristone injection such that bitches whelped at high P4 concentrations. Plasma concentrations of oxytocin and cortisol were unchanged during the first 24 h after aplepristone administration, whereas PG and PRL concentrations increased after 4 and 20 h, respectively [34]. Induction of parturition with aplepristone and PGs was also reported in one bitch with prolonged pregnancy [35].

In preclinical studies and early clinical trials, endometritis and pyometra complex were successfully treated with anti-progestins [36,37]. Aplepristone (10 mg/kg sc) was used successfully in bitches with pyometra that had P4 concentrations >3.2 nmol/L and normal ovarian function [38]. The combination of aplepristone and PGF2α has shown good results for the medical treatment of canine endometrial hyperplasia–pyometra complex [39,40]. In another combined-drug trial with the same dose of aplepristone and PGF analog cloprostenol (1 μg/kg sc every other day from days 3 to 15), clinical signs, blood parameters and uterine diameter consistently improved to normal values, independent of the initial P4 concentrations throughout days 15 or 28 of treatment. In 20% of bitches treated, pyometra recurred at the next estrous cycle [40]. In another study, the recurrence rate was 18.9% at 1 year after aplepristone treatment [41]. To prevent recurrent of pyometra, it is often suggested that successful medical treatment of pyometra should be followed by breeding at the subsequent cycle (in bitches intended for breeding).

4. Anti-androgens

Several groups of compounds have been described to have anti-androgenic properties (through different mechanisms of action), such as progestins, receptor binding anti-androgens, competitive enzyme inhibitors, aromatase inhibitors, and GnRH analogues. Estrogens and anti-estrogens have also been shown to have anti-androgenic effects in dogs [42,43]. The main clinical application of anti-androgens in dogs is benign prostatic hyperplasia. Other possible indications are management of testosteron (T) dependent behavioral problems and reversible suppression of fertility in male dogs.

Different progestins may have different anti-androgenic potency, depending on their anti-gonadotrophic and androgenic receptor binding effects. Administration of medroxyprogesterone acetate (3 mg/kg sc) decreased prostate size [44]. Doses ranging from 3 to 4.8 mg/kg reduced serum T concentrations for 5–13 weeks after treatment without affecting semen quality or libido [45]. Similar results have been found after megestrol acetate (2 mg/kg po for 7 days), whereas a higher dose (4 mg/kg) produced sperm abnormalities [46]. Cypionate acetate, a potent anti-androgenic progestin used for the treatment of prostatic disorders in men, has also been tested in dogs [27,47]. Other progestins, like delmadione [48] and clormadinone acetate are known to be effective in the treatment of benign prostatic hyperplasia in this species [49].

Flutamide, a pure androgen receptor blocker, inhibits androgen uptake and nuclear binding by binding to the androgen receptor [27]. Flutamide has yielded good results in treating prostatic hyperplasia without alteration of semen or libido [50]. Aromatase inhibitors, such as formestane, exert their anti-androgenic effect by inhibiting the conversion of androgens to estrogens in peripheral tissues [51]. Finasteride is a synthetic steroid that prevents conversion of T into dihydrotestosterone (DHT) by inhibition of the type II 5α-reductase [52]. Five beagle dogs with benign prostatic hyperplasia that were treated with finasteride (1 mg/kg po, q 24 h for 21 weeks) had decreased size
of the prostate (30% of the initial value) and complete lack of an ejaculate by the end of the treatment period. Fertility was restored after a 20–22 weeks recovery phase [53].

A dose–response study of finasteride (0.1, 0.25, or 0.5 mg/kg po every 24 h for 7 days) in three normal intact male dogs caused a significant decrease of DHT without changing T serum concentrations, libido and spermatogenesis [54]. More recently, using the same drug (0.1–0.5 mg/kg po every 24 h) for 16 weeks in nine client-owned dogs with spontaneous benign prostatic hyperplasia resulted in decreased serum DHT concentrations and a 43% reduction of prostatic volume. Finasteride treatment diminished ejaculate volume without affecting other semen characteristics, libido, serum T concentrations, or fertility [55].

5. Long-term-release GnRH agonists

Similar to the actions of native GnRH, synthetic GnRH agonists like nafarelin, leuprolide, deslorelin, buserelin, and goserelin, stimulate production and release of gonadotrophins from the pituitary. However, GnRH agonists, when used at sustained doses, reversibly inhibited the pituitary gonadal axis after the initial period of stimulation. Inhibition is produced by down-regulation of anterior pituitary GnRH receptors [56]. Continuous administration of, or long term release formulations of, GnRH agonists reversibly suppressed reproductive function in male and female dogs for periods exceeding 1 year in some studies [56–59]. The previous disadvantage of these compounds was the need for frequent administration over prolonged intervals; a substantial advance was development of slow-release formulations that can be easily implanted subcutaneously.

A single leuprolide acetate injection (1 mg/kg sc) administered to five male dogs decreased ejaculate volume and was accompanied by an increase in morphologically abnormal spermatozoa. An initial rise in LH and T serum concentrations, followed by a marked decline to below-normal concentrations for 6 weeks, was also described in this study. Twenty weeks after treatment, spermatogenesis had returned to normal [58].

In a more recent study, 30 mature dogs received sc implants of deslorelin acetate (doses ranged from 0.08 to 0.79 mg/kg), and 11 were re-implanted either before or after the suppression period. Their T concentrations decreased to <1 ng/mL within a mean of 17 days after implantation in all the treatment groups and remained at this level from 3 months to 2.7 years. The duration of the inhibitory effect seemed to be dose related and restoration to initial conditions became evident, based on scrotal circumference, T concentrations, semen quality, and fertility [60]. In another report, 0.5–1 mg/kg of the same GnRH agonist implanted in five dogs significantly decreased serum T and prostate size for 32 and 48 weeks, respectively [61]. Recently, in seven dogs that were implanted with 6.6 mg of buserelin, T and estradiol decreased to basal concentrations within 15 days of implantation and remained there for a mean of 233 days. Testicular and prostatic size were reversibly reduced and no semen could be collected 21 days after implantation [62].

The GnRH agonist [D-Trp⁶, des-Gly-NH₂¹⁰] GnRH ethylamide administered sc daily to prepubertal male and female dogs for 23 months decreased steroid hormone concentrations and delayed puberty, with normal fertility following a recovery period [63]. In three female dogs, sc implantation (in proestrus) of an osmotic pump releasing natural nafarelin acetate (18 µg/kg/day) suppressed estrous cycles for 18 months. Cyclicity was restored 3–18 weeks after cessation of treatment. When the same drug was administered to anestrous bitches, an infertile ovulatory estrus was induced, 1–2 week after the start of treatment. Postponement of pubertal estrus was obtained in three bitches in the same study during 18 months of treatment [56]. In a more recent report, pregnant, diestrous and anestrous bitches were treated with doses ranging from 0.1 to 2.4 mg/kg of deslorelin acetate in sc implants. Treatment prolonged interestrous intervals up to 27 months, independent of the stage of the cycle at implantation. When serum P₄ was <5 ng/mL, an estrous cycle was induced 4–8 days after implantation. Six of nine bitches that were mated after recovery of the treatment became pregnant [60]. In a study in which azagly-nafarelin was administered sc to six bitches, there was no estrous behavior and low concentrations of FSH and P₄ for 1 year of treatment [64].

In one trial, the expected initial estrous response induced in anestrous bitches after implantation of 6 mg deslorelin was suppressed by a 21 or 14-day treatment with 2.2 mg/kg megestrol acetate po, started 2 or 1 week before implantation, respectively [65]. Administration of the same dose of megestrol for 8 days, starting the day before deslorelin implantation, prevented estrous response in four of eight bitches [59].

Systematic re-implantation of long term release GnRH agonists could offer an option for dogs diagnosed with hormone-dependent diseases that have unacceptable risk factors for anesthesia or surgery. In this context, goserelin was used successfully for
12 months in the treatment of canine mammary gland tumors [66].

Considering the initial stimulating effect of GnRH agonists, these drugs have been used for estrus induction in bitches. A single sc injection of a sustained release formulation of the potent GnRH agonist, leuprolide acetate (100 μg/kg), followed by fertility in the first day of induced estrus, provoked behavioral estrus in 100% of bitches, with a pregnancy rate of 50–100% [67]. In a recent study with six bitches, removal of a 2.1 mg deslorelin implant at the time of the preovulatory LH surge induced 100% estrus and 50% pregnancy [68], whereas with the same protocol, one of the eight bitches remained pregnant to term if implants were not removed [69].

6. Anti-estrogens

Pharmacologic groups of compounds that inhibit or modify estrogens are classified as anti-estrogens. Some of these are GnRH analogs and aromatase inhibitors that inhibit estrogen synthesis. Another anti-estrogenic drug group is estrogen receptor blockers [70]. Clomiphene and tamoxifen citrate are synthetic non-steroidal type I anti-estrogen compounds which competitively block estrogen receptors with a combined antagonistic-agonistic effect. Type I estrogen antagonists partially inhibit the action of estrogen agonists, but due to their own agonistic properties they also induce, to some extent, estrogenic responses. The manifestation of these different actions depends on each species, organ, tissue and cell type considered [27]. Thus in women, tamoxifen has anti-estrogenic activities on the mammary gland and agonistic effects on the uterus [71]. The exact mechanism of this duality is incompletely understood, but may depend on the variable expression of specific cellular estrogen receptors [27]. In humans, estrogen receptor blockers have been used for treatment of male and female infertility and breast cancer [70–72].

Little is known about the effect of receptor blocker anti-estrogens in dogs. Most studies have been carried out using tamoxifen, which seems to act more like an agonist than antagonist [73–76], limiting its use in female dogs. In that regard, signs of proestrus were induced in two of four bitches after 10 days of clomiphene citrate (12.5–25 mg po) [77].

Although tamoxifen should be efficacious in preventing or terminating canine pregnancy when administered during proestrus, estrus or diestrus, it had estrogen-like side effects. Endometritis, pyometra and ovarian cysts developed in nine of 20 bitches treated with tamoxifen (1 mg/kg po bid) [73]. In one study in which tamoxifen was administered (2.5–10 mg po bid) to seven bitches with inoperable or metastatic mammary carcinoma, tamoxifen reduced tumor burden in five animals. Unwanted side effects were vulvar swelling, vaginal discharge, urinary incontinence, and pyometra [75]. In another report, 18 bitches diagnosed with mammary tumors were treated with tamoxifen in addition to mastectomy and ovariotomy. Ten bitches showed estrogenic effects and no anti-tumor activity could be documented [76]. Additional studies are needed to establish the potential benefit of anti-estrogen therapy for canine mammary tumors, particularly in combination with hormone receptors assays.

In a study in which seven male beagle dogs were treated with tamoxifen (2.5 mg po every 24 h for 28 days), the drug decreased testicular size and libido. During treatment, tamoxifen also decreased prostatic volume and T concentrations. One spermatogenic cycle after treatment, sperm count and volume decreased to low values, while motility and morphology deteriorated; all of these parameters returned to pre-treatment values the next spermatogenic cycle. No clinical or haematological side effects were observed and fertility was restored at the end of the study [74].

7. Conclusions

Progress in our understanding of physiology of canine reproductive has enhanced the possibility of utilizing pharmacological agents that were previously used only in other species. Although most of these drugs have already had an impact on canine reproductive therapy, only a few indications for dopamine agonists, long-term-release GnRH agonists and anti-progestins have been approved to date for use in dogs. Thus, further clinical randomized controlled trials are necessary to better refine indications and treatment regimens and to enhance the expected clinical outcome before the use of these drugs can be widely recommended.

References


Treatment of growth hormone excess in dogs with the progesterone receptor antagonist aglépristone

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Abstract

Acromegaly or hypersomatotropism in dogs is almost always due to progestin-induced hypersecretion of GH originating from the mammary gland. The aim of this study was to investigate whether aglépristone, a progesterone receptor antagonist, can be used to treat this form of canine acromegaly. In five Beagle bitches hypersomatotropism was induced by administration of MPA for over 1 year. Subsequently, aglépristone was administered. Blood samples were collected before MPA administration, immediately before, during, and 3.5 and 5.5 weeks after the last administration of aglépristone for determination of the plasma concentrations of GH and IGF-I. In addition, blood samples for the determination of the 6-h plasma profile of GH were collected before MPA administration, before aglépristone administration, and 1 week after the last aglépristone treatment.

MPA administration resulted in a significant increase of the mean plasma IGF-I concentration, whereas analysis of the pulsatile plasma profile demonstrated a trend (P = 0.06) for a higher mean basal plasma GH concentration and a higher mean AUC0 for GH. Treatment with aglépristone resulted in a significant decrease of the mean plasma GH and IGF-I concentrations. Analysis of the pulsatile plasma profile showed a trend (P = 0.06) for a lower mean basal plasma GH concentration and a lower mean AUC0 for GH 1 week after the last aglépristone treatment compared with these values before aglépristone administration. Three and a half and 5.5 weeks after the last aglépristone administration the mean plasma IGF-I concentration increased again.

In conclusion, aglépristone can be used successfully to treat dogs with progestin-induced hypersomatotropism.

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Keywords: Acromegaly; Hypersomatotropism; Insulin-like growth factor-I; Medroxyprogesterone acetate; Progesterone receptor blocker

I. Introduction

Acromegaly is characterized by bony and soft tissue overgrowth due to excessive growth hormone (GH) secretion. The syndrome is known to occur in humans, dogs, and cats. However, the pathogenesis differs among these species. Acromegaly in humans and cats is commonly caused by a somatotroph adenoma of the pituitary gland [1], whereas in dogs the GH excess usually originates from an extra-pituitary site [2]. In dogs, endogenous progesterone secreted during the luteal phase of the estrous cycle or exogenous progestins such as medroxyprogesterone acetate (MPA) used for estrus prevention may promote
hypersecretion of GH from foci of hyperplastic ductular epithelium of the mammary gland [2–4]. In contrast to the pulsatile secretion pattern of GH in healthy dogs [5–7], the plasma GH profile in bitches with progestin-induced acromegaly is not pulsatile [8]. In addition, progestin-induced GH hypersecretion cannot be stimulated with GH-releasing hormone (GHRH) and α-adrenergic agonists, nor can it be inhibited by somatostatin [8,9]. The progestin-induced increase in plasma GH concentrations are associated with increased plasma concentrations of insulin-like growth factor-I (IGF-I) [2].

The physical changes of progestin-related hypersomatotropism in dogs tend to develop gradually and consist of prominent skin folds, abdominal distension, and widening of the interdental spaces [10]. Due to the insulin-antagonistic action of GH, hyperglycemia and eventually diabetes mellitus may occur [11]. Ovariectomy is the treatment of choice in female dogs with spontaneous progesterone-induced acromegaly. Plasma GH concentrations rapidly return to normal after ovariectomy [12]. However, in dogs with acromegaly due to progestin administration the detrimental effects of the depot progestins may continue for a long time after cessation of administration [10,13].

Progestosterone receptor blockers such as aglépristone (RU 46534) and mifepristone (RU 38486) are competitive antagonists of the progesterone receptor [14,15]. Aglépristone is the first progesterone receptor blocker licensed for veterinary use and has been used efficiently to terminate pregnancy [16,17] and to induce parturition [18]. Furthermore, it is successfully used for the treatment of fibroadenomatous mammary hyperplasia in cats [19–21] and may be a useful adjunct in the medical treatment of endometritis and pyometra in the dog [22].

The presence of progesterone receptors in mammary gland tissue of dogs [23] allows for a targeted endocrine therapy with progesterone receptor blockers in dogs with progestin-induced hypersomatotropism. The aim of this study was therefore to investigate whether the progesterone receptor antagonist aglépristone can be used to treat canine acromegaly.

2. Materials and methods

2.1. Dogs

Five intact Beagle bitches were housed with outdoor access, fed on a commercial dog food once a day, and given water ad libitum. The ages and body weights of the dogs ranged from 3 to 9 years (mean 5 years) and 9.0 to 10.3 kg (mean 9.5 kg), respectively. The dogs were accustomed to the laboratory environment and procedures such as collection of blood samples.

2.2. Treatments

The five Beagle bitches were treated with the synthetic progestin depot preparation Depo-Promone® (medroxyprogesterone acetate (MPA), Pharmacia Animal Health, Puurs, Belgium). MPA treatment was started during anestrus and consisted of subcutaneous injections in a dosage of 10 mg/kg body weight at 4-week intervals for a total of 14 (three dogs) or 15 (two dogs) administrations.

Five (=day 0) and six days (=day 1) after the last MPA administration (=day −5), aglépristone (Alizin®, Virbac Animal Health, Barneveld, The Netherlands) was administered subcutaneously in a dosage of 10 mg/kg body weight. One (=day 8), two (=day 15), and three (=day 22) weeks later a single aglépristone treatment was given in the same dose. Three randomly chosen dogs received the first aglépristone treatment after the 14th MPA administration, and the other two dogs after the 15th MPA administration so that these two dogs could serve as control dogs for the three dogs that received the aglépristone treatment first.

2.3. Blood sample collection

Blood samples for determination of the plasma progesterone concentrations were collected 5 and 12 months after the start of the MPA treatment. Blood samples for determination of the plasma concentrations of GH and IGF-I were collected before MPA treatment, at days −9, −8, −7, −5, −3, −2, −1, and 0 (=immediately before aglépristone treatment and after MPA treatment for over 1 year), at days 1, 3, 5, 7, 8, 11, 13, 15, 18, 20, 22, and 25 (=during aglépristone treatment), and at days 46 and 60 (=3.5 and 5.5 weeks after the last aglépristone treatment). On days of treatment (MPA or aglépristone), blood samples were collected prior to the drug administration.

Blood samples for determination of the pulsatile plasma profiles of GH were collected at 15-min intervals between 08:00 and 14:00 h before MPA administration, before aglépristone administration, and 1 week after the last administration of aglépristone (at day 28).

All blood samples were collected by jugular venipuncture after an overnight fast, immediately transferred to ice-chilled EDTA-coated tubes and centrifuged at 4 °C for 10 min. Plasma was stored at −25 °C until assayed.
2.4. Assays

Plasma progesterone concentrations were determined with a previously validated radioimmunoassay (RIA) [24]. The intra-assay and inter-assay coefficients of variation were 8.8 and 7.1%, respectively. The sensitivity of the assay was 0.005 ng/L.

Plasma GH concentrations were measured using a commercially available RIA for porcine and canine GH (PGH-46HK; Linco Research, St. Charles, MS). The intra-assay coefficient of variation was 7.6% at a plasma concentration of 4.4 μg/L. The sensitivity of the assay was 1 μg/L.

Total plasma IGF-I was measured after acid–ethanol extraction to remove interfering IGF binding proteins. Plasma IGF was extracted using a mixture of 87.5% (v/v) ethanol and 12.5% 2 M formic acid. Tubes containing 100 μL plasma and 400 μL of the ethanol–formic acid mixture were mixed thoroughly and incubated for 30 min at room temperature. After centrifugation for 30 min at 5500 x g at 4 °C, a 50 μL aliquot of the supernatant was diluted 1:50 with assay buffer containing 63 mM Na2HPO4 (pH 7.4), 13 mM Na2EDTA, and 0.25% (w/v) BSA. The extraction efficiency amounted to 92.5 ± 5.7%. IGF-I concentrations were measured in a heterologous RIA validated for the dog [25]. The intra-assay coefficient of variation was 8.6% at a plasma concentration of 100 μg/L. The sensitivity of the assay was 10 μg/L. IGF-I antiserum AFP4892898 and human IGF-I for iodination were obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA).

2.5. Data processing and statistical analysis

To study the effect of MPA administration, the plasma GH and IGF-I concentrations before and after MPA treatment were compared using a mixed model with dog as random effect and period (two levels: before and after MPA treatment) as categorical fixed effect.

In order to assess the overall effect of aglégpristone on the plasma GH and IGF-I concentrations, a mixed model was fitted with dog as random effect and period (three levels: immediately before aglégpristone, during aglégpristone, and 3.5 and 5.5 weeks after the last aglégpristone treatment) as categorical fixed effect. The three periods were compared pair wise using Tukey’s multiple comparisons technique.

To study the evolution of the GH and IGF-I concentrations during the aglégpristone period, a mixed model was fitted with dog as random effect and time since start of aglégpristone treatment as continuous fixed effect at a global significance level of 5%.

The plasma GH and IGF-I concentrations before MPA treatment were compared with the concentrations 3 days after the last aglégpristone treatment (i.e. at day 25) using a mixed model with dog as random effect and period (two levels: before MPA treatment and 3 days after the last aglégpristone treatment) as categorical fixed effect.

To evaluate the effect of withdrawal of aglégpristone treatment, the two last measurements during aglégpristone treatment (days 22 and 25) were compared with the two measurements after aglégpristone treatment (days 46 and 60) using a mixed model with dog as random effect and period (two levels: days 22 and 25, and days 46 and 60) as categorical fixed effect.

The 6-h plasma profiles of GH were analyzed by means of the Pulsar program developed by Merriam and Wachter [26]. The program identifies secretory peaks by height and duration from a smoothed baseline, using the assay SD as a scale factor. The cut-off parameters G1–G5 of the Pulsar program were set at 3.98, 2.40, 1.68, 1.24, and 0.93 times the assay SD as criteria for accepting peaks 1, 2, 3, 4, and 5 points wide, respectively. The smoothing time, a window used to calculate a running mean value omitting peaks, was set at 5 h. The splitting cut-off parameter was set at 0.5 and the weight assigned to peaks was 0.05. The A-, B-, and C-values of the Pulsar program, used to calculate the variance of the assay, were set at A = 0, B = 7.2, and C = 5. The values extracted from the Pulsar analysis included the mean of the smoothed baseline, the pulse frequency, and the area under the curve (AUC). The AUC for GH was calculated above the zero-level (AUC0) as well as above the baseline (AUC_base). The difference in variables before MPA treatment, before aglégpristone administration, and 1 week after the last aglégpristone treatment (i.e. at day 28) were analyzed by the signed rank test with dog as block.

All values are expressed as mean ± S.E.M. or median. Statistical significance was defined at P ≤ 0.05. Analyses were performed with SAS Version 9.1 for Windows (Insightful Corp., Seattle, USA).

2.6. Ethics of the study

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

3. Results

During MPA administration none of the dogs showed signs of estrus and the mean plasma progesterone
concentration was low 5 months (0.2 ± 0.2 ng/L) and 12 months (0.2 ± 0.1 ng/L) after the start of the MPA treatment. The mean body weight of the dogs on the day of the last administration of MPA (12.4 ± 0.7 kg) was significantly higher (P < 0.02) than that on the day of the first MPA administration (9.5 ± 0.3 kg) (paired Student’s t-test). Signs of acromegaly became apparent in three of the five dogs after 6 months of MPA treatment and consisted of prominent skin folds especially on the head, an increase in the interdental spaces, inspiratory stridor, and snoring.

MPA administration for over 1 year resulted in a higher mean plasma GH concentration (2.3 ± 0.5 μg/L) compared to that before MPA treatment (1.9 ± 0.3 μg/L), although this difference did not reach statistical significance (Fig. 1a). However, the mean plasma IGF-I concentration after 1 year of MPA administration (146 ± 25 μg/L) was significantly (P = 0.003) higher compared to that before MPA treatment (36 ± 6 μg/L) (Fig. 1b). Analysis of the pulsatile plasma GH profiles after 1 year of MPA administration revealed a trend (P = 0.06) for a higher mean basal plasma GH concentration and a higher mean AUC 0 for GH compared to these values before MPA treatment (Table 1; Fig. 2).

The administration of aglépristone caused no side effects except a short-term skin irritation at the site of the injection in one dog. The mean plasma GH concentration immediately before aglépristone administration (2.3 ± 0.5 μg/L) was significantly higher than that during (1.7 ± 0.3 μg/L; P < 0.0001) and 3.5 and 5.5 weeks after (1.8 ± 0.3 μg/L; P = 0.018) the last administration of aglépristone (Fig. 3a). Also the mean plasma IGF-I concentration immediately before aglépristone administration (146 ± 25 μg/L) was significantly higher than that during (108 ± 27 μg/L; P < 0.0001) administration of aglépristone (Fig. 3b). In the weeks when aglépristone was administered, analysis of the course of the circulating hormone concentrations indicated a significant decrease in plasma GH (P = 0.005) and IGF-I (P < 0.0001) concentrations (Fig. 4a and b, respectively).

The plasma GH and IGF-I concentrations before MPA treatment did not differ significantly from these concentrations 3 days after the last aglépristone treatment (i.e. day 25).

Analysis of the pulsatile plasma GH profiles revealed a trend (P = 0.06) for a lower mean basal plasma GH concentration and a lower mean AUC 0 for GH compared to these values before MPA treatment. The AUCbase for GH increased again after the last aglépristone treatment compared with this concentration before aglépristone administration, although

<table>
<thead>
<tr>
<th></th>
<th>AUCbase (mean ± S.E.M.)</th>
<th>AUCO (mean ± S.E.M.)</th>
<th>GH pulse frequency (median)</th>
<th>Basal GH (mean ± S.E.M.)</th>
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</thead>
<tbody>
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<td>11.3 ± 1.1</td>
<td>1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Before A</td>
<td>0 ± 0.0</td>
<td>17.0 ± 3.6</td>
<td>0</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>After A</td>
<td>0.3 ± 0.2</td>
<td>8.8 ± 0.7</td>
<td>0</td>
<td>1.4 ± 0.1</td>
</tr>
</tbody>
</table>

Fig. 1. Mean (±S.E.M.) plasma concentrations of GH (a) and IGF-I (b) in five Beagle dogs before administration of MPA (before MPA) and after 1 year treatment with MPA (after MPA). Significant differences between periods are indicated with an asterisk.
this difference did not reveal statistical significance (Table 1; Fig. 2).

The mean plasma GH concentration at the end of the period of aglépristone administration (i.e. days 22 and 25) (1.5 ± 0.1 μg/L) did not differ significantly from that at 3.5 and 5.5 weeks (i.e. days 46 and 60) after withdrawal of aglépristone (1.8 ± 0.3 μg/L). However, the mean plasma IGF-I concentration at the end of the period of aglépristone administration (88 ± 22 μg/L) was significantly (*P < 0.0001) lower compared with that 3.5 and 5.5 weeks after withdrawal of aglépristone (124 ± 29 μg/L).

In the two control dogs that did not receive aglépristone after the 14th MPA administration, the mean plasma concentrations of GH and IGF-I before the 14th administration of MPA (1.6 ± 0.1 and 105 ± 28 μg/L, respectively) were not different from those before the 15th administration of MPA (1.7 ± 0.1 and 116 ± 26 μg/L, respectively).

4. Discussion

In three of the five Beagle dogs, signs of acromegaly became apparent after 6 months of MPA administration. In line with these changes, the mean plasma IGF-I concentrations were raised. Moreover, analysis of the pulsatile plasma profile showed a trend for a higher mean basal GH concentration and a higher mean AUC0 for GH in the five Beagle dogs. These findings are consistent with progestin-induced hypersecretion of GH [3,27–29].

Previous studies identified foci of hyperplastic ductular epithelium of the mammary gland as the site of origin of GH excess induced by progestins [2,4]. The expression of the gene encoding GH has been...
demonstrated in canine mammary gland tissue, and sequence analysis has revealed that this gene is identical to the pituitary GH gene [30,31]. Immunohistochemical studies have demonstrated the intracellular co-localization of both the progesterone receptor and GH in progesterone-exposed, hyperplastic canine mammary epithelial tissue, whereas immunoreactive GH could not be demonstrated in progesterone receptor-negative epithelial cells [23]. These observations are consistent with the central role of progestins in GH gene expression in the canine mammary gland and allow for a targeted endocrine therapy with progesterone receptor blockers in dogs with progestin-induced mammary-derived GH hypersecretion.

To the authors’ knowledge, treatment of acromegalic dogs with the progesterone receptor antagonist aglépristone (RU 46534) has not been reported before. The results of the present study demonstrate that progestin-induced elevations in circulating GH and IGF-I concentrations decrease significantly during treatment with aglépristone. At the end of the aglépristone treatment period the plasma GH and IGF-I concentrations did not differ significantly from those before MPA administration. Our findings are in agreement with those of Watson et al. [8] who found that administration of the antiprogestin mifepristone (RU 38486) resulted in a decrease of plasma GH concentrations and normalization of plasma IGF-I concentrations in bitches with progestin-induced acromegaly.

The mean basal plasma concentrations of GH and IGF-I in the two control dogs that did not receive aglépristone after the 14th MPA administration remained high and did not decrease. This indicates that indeed aglépristone is responsible for the lowering of the plasma GH and IGF-I concentrations in the dogs treated with the progesterone receptor blocker, and that this lowering is not due to, for example, a waning effect of MPA on GH and IGF-I secretion.

The 6-h pulsatile plasma profile of GH represents a more sensitive way of analyzing the effects of different treatments on the secretion of GH than the plasma GH concentration itself. Analysis of the plasma GH profiles revealed that the mean basal plasma GH concentration and AUCO for GH tended to decrease at the end of the treatment with the progesterone receptor blocker compared with these values before aglépristone administration. In addition, the AUC base for GH, i.e., the amount of GH secreted in pulses, increased again during aglépristone treatment, although this difference did not reveal statistical significance. Thus, treatment with aglépristone resulted in partial restoration of the normal pulsatile GH secretion. Higher dosages of aglépristone may result in complete normalization of the secretion pattern of GH.

Plasma IGF-I concentrations are generally regarded as more sensitive indicators of the GH status than plasma GH concentrations [32]. Consequently, the significantly higher plasma IGF-I concentrations at days 46 and 60 compared with those at days 22 and 25 therefore suggest increased GH exposure, despite the fact that analysis of the plasma GH concentrations did not reveal a significant increase. The recurrence of IGF-I hypersecretion after withdrawal of aglépristone treatment is not surprising as all dogs received a depot progestin preparation for a period of 1 year, and the progestin effect of this depot preparation is much longer than the duration of aglépristone treatment in the present study. Similarly, in a cat with fibroadenomatous mammary hyperplasia due to treatment with a depot progestin preparation hyperplasia recurred 8 days after discontinuation of aglépristone administration [19]. This indicates that treatment with an antiprogestin is required as long as the action of the synthetic progestin is present. Also in our three dogs with acromegalic signs, no physical changes were visible during or after treatment with aglépristone.

Due to the insulin-antagonistic action of GH, progestin-induced hypersecretion of GH may also result in hyperglycemia and eventually manifest diabetes mellitus may ensue [11,29]. Disappearance of these catabolic abnormalities depends on the functional status of the pancreatic β-cells. If an adequate population of functional β-cells is present at the time the progestin effect is blocked, hyperinsulinemia, carbohydrate intolerance, and hyperglycemia may be reversible after correction of the hypersomatotropism [13]. If the population of functional β-cells is severely decreased, permanent diabetes mellitus can be anticipated. Because the effects of depot progestins may persist for several months, prevention or reversal of the catabolic effects of progestin-induced GH excess is especially important when, in case of hyperglycemia, the depot progestin has been administered only recently. The results of the present study illustrate that in these cases aglépristone offers an effective treatment option. In conclusion, administration of aglépristone significantly decreases plasma GH and IGF-I concentrations in dogs with progestin-induced hypersomatotropism.

Acknowledgements

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References

Morphology of canine placental sites after induced embryonic or fetal death

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Abstract

Although spontaneous and medically induced canine embryonic or fetal death and “resorption” are clinically well documented, morphological studies of these processes are still missing. The objective of this study was therefore a detailed morphological investigation of canine placental sites after embryonic or fetal death. In five pregnant beagle bitches, embryonic or fetal death was induced by cloprostenol and cabergoline or by aglepristone. Two dogs served as untreated controls. Between Days 30 and 33 of gestation, the bitches were ovariohysterectomized, placental sites were fixed and examined by different methods.

Morphological features of placental sites after both treatments were similar, finally leading to a complete disappearance of the placental labyrinth. Although there was an increase in the number of cells in the glandular chambers (superficial endometrial glands) expressing lysozyme after induced fetal death, signs of phagocytosis were absent in these cells, and no increased infiltration of maternal stroma by macrophages (compared to normal placental sites at the same time of gestation) occurred. We inferred that fetal and placental tissues were lysed, but no phagocytosis by genuine or “functional” macrophages was detectable. Further investigations are needed for a more detailed understanding of the morphological processes occurring after embryonic or fetal death in the dog.

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Keywords: Dog; Fetal death; Resorption; Morphology; Placenta

1. Introduction

The administration of prostaglandin $F_{2\alpha}$ (or its analogue cloprostenol) has been used for decades to terminate unwanted pregnancies in dogs [1,2]. Since 1990, several reports were published about the administration of progesterone-receptor antagonists, such as mifepristone [3] or aglepristone [4,5] to induce embryonic or fetal death in dogs. Independent of the time of gestation at which such treatment starts, there are still different opinions whether the use of luteolytic agents (sometimes in combination with a dopamine agonist) or antigestagenic drugs results in abortion (defined as the expulsion of a conceptus incapable of independent life) [2,6] or in a so-called resorption (loss of the embryonic or fetal structure, without expulsion of embryonic or fetal or placental components) [7,8]. To our knowledge, there are no morphological studies on placental and/or endometrial changes after induction of embryonic or fetal death in dogs by those drugs. There is only one ultrastructural study of decidual alterations early after administration of mifepristone [9] and a number of morphological investigations into decidual
alterations after intra- \cite{10, 11} or extra-amniotic applications of prostaglandin \cite{12} in women. After induction of a second trimester abortion by extra-amniotic administration of prostaglandins in humans, no specific lesions of the placenta or decidua \cite{12} were observed.

Light-microscopic and ultrastructural morphology of the canine placenta during mid-gestation was described by several authors \cite{13–15}. The canine zonary placenta consists of the chorioallantoic membrane, the placental labyrinth (the area of direct fetomaternal interaction, with fetal chorionic villi contacting maternal blood vessels), the necrosis zone (which develops earlier during gestation due to erosion of the maternal epithelium and connective tissue by the terminal segments of the syncytiotrophoblast), the maternal glandular chambers (also named spongy zone, which develops from the superficial endometrial glands), the supraglandular layer (formed by connective tissue) and the deep endometrial glands (Fig. 1). A hemophagus zone develops at both edges of the placental labyrinth (Fig. 1).

The objective of the present study was to investigate the morphology of the canine placental labyrinth and the glandular chambers after experimentally induced embryonic or fetal death, in comparison to normal placental morphology at corresponding stages of gestation, and with special emphasis on the character of processes after embryonic or fetal death.

2. Material and methods

In five clinically healthy pregnant beagles (between 1 and 3 yr of age) embryonic/fetal death was induced by administration of luteolytic substances. Two dogs were given 1 µg/kg of cloprostenol subcutaneously on Days 24 and 27 (ovulation = Day 0) and 5 µg/kg cabergoline given per os once daily from Days 24 to 30. Three dogs were given 10 mg/kg of aglepristone (an antigestagenic drug) subcutaneously on Days 24 and 25. Two pregnant beagles served as untreated controls. Starting on Day 20, all dogs were submitted to daily sonographic examinations for assessment of embryonic or fetal development and death, respectively. The dogs were ovariohysterectomised between Days 30 and 34 of gestation. As most of the cell differentiation and organogenesis in the dog is complete by Day 30 of gestation, the conceptuses removed will be referred to as fetuses in this study. Numbers and sizes of placentaion sites and their localizations within the uterine horns were recorded. For light-microscopical investigations, placental sites \((n = 41)\) were fixed in 4\% neutral-buffered formalin and embedded in paraplast. Histological slides were stained with Hematoxylin–Eosin (H.–E.). Additionally, enzyme histochemistry \([\text{naphthol-AS-D chloracetate-esterase (CLAE) and alpha-naphthylacetate-esterase (ANAE), acidic and alkaline phosphatase}\] was conducted. For immunohistochemistry, primary monoclonal antibodies against Ki-67 antigen and myeloid histiocytes as well as polyclonal antibodies against lysozyme were used and routine immunohistochemical techniques with a peroxidase–anti peroxidase complex and diaminobenzidine (DAB) as chromogen were followed, counterstained with Papanicolaou’s technique. The antibodies used were as follows: anti-Ki-67 antigen, clone MM1, Medac GmbH, Hamburg, Germany; anti-myeloid histiocyte antigen, clone MAC 387, Dako Diagnostika GmbH, Hamburg, Germany; anti-lysozyme (Dako Diagnostika). Additionally, three samples from each placental site (placental labyrinth, glandular chambers and deep endometrial glands) were fixed in glutaraldehyde (3\%) and embedded in glycidether 100 for electronmicroscopy. In 0.3 µm (toluidinblue) slides, the area of interest was selected, from which 60 nm ultra-thin slides were cut, contrasted with uranyl-acetate and lead citrate and examined by transmission-electron microscopy.

3. Results

Fetal death, especially visible as lack of fetal heartbeat, was detected sonographically in 17 placentaion sites of the five treated dogs, whereas 10 fetuses in four of the dogs were alive on the day of ovariohysterectomy \((\text{Table 1})\). One of the two untreated controls showed spontaneous fetal death in 3 of 11 placentaion
sites (Table 1). In six placentation sites of treated dogs, no fetal structures were detectable macroscopically (Table 1); only slight to moderate amounts of a brownish, gelatinous material was present. Such areas were frequently neighbouring intact placentation sites with an apparently normal conceptus.

3.1. Normal placental morphology during mid-gestation

The histomorphology of the placentation sites in untreated controls (in Dog 7, only those with a living fetus at the day of ovariohysterectomy) corresponded to physiological histological placental appearance during mid-gestation, as described by other authors [13–15].

3.1.1. Placental labyrinth

Fetal capillaries with one or two thin endothelial cells and a few organelles were embedded in fetal connective tissue. In the single-layered flat to cuboidal cytotrophoblast (the inner layer of the fetal trophoblast with sparse cytoplasm and an oval, heterochromatin-rich nucleus), single cells prolifereated. Cells of the syncytiotrophoblast had moderate amounts of an eosinophilic cytoplasm and a heterochromatin-rich nucleus, sometimes with a distinct nucleolus. Very few cells showed signs of pyknosis. Between the cytotrophoblastic cells as well as the cytotrophoblast and the syncytiotrophoblast, desmosomes (approximately 6/intercellular contact) were present ultrastructurally. The intercellular spaces between cyto- and syncytiotrophoblast were narrow (150–500 nm). Maternal decidual cells were not evident ultrastructurally.

3.1.2. Glandular chambers

The epithelial cells of the glandular chambers were high columnar with light to moderate intracytoplasmatic vacuolisation. Single epithelial cells were degenerated and desquamated into the necrosis zone especially in the apical region and in the periphery of chambers. Additionally, some syncytial cells and single giant cells with one or two large nuclei occurred within the epithelial layer. Some macrophages in the interstitium of the glandular chambers contained reaction products of enzymes investigated histochemically (acidic and alkaline phosphatase, CLAE, ANAE). In single epithelial cells of the glandular chambers, only reaction products of acidic phosphatase were detected. Intracytoplasmatically, they expressed lysozyme moderately. Regarding ultrastructure, some epithelial cells of the glandular chambers had apical protrusions covered by fine, short microvilli. These protrusions contained some round to oval (cristae-type) mitochondria and a few mucous vesicles, some of which fusing with the surface cell membrane. There were some well-developed Golgi complexes and rough endoplasmatic reticulum (rER), and some lipid droplets in the infra- and supranuclear cytoplasm. The euchromatin-rich nucleus was round to slightly oval. Individual cells, mainly in apical regions of the glandular chambers

<table>
<thead>
<tr>
<th>Placentation site number</th>
<th>Cloprostenol and cabergoline</th>
<th>Aglepristone</th>
<th>Untreated controls</th>
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<tbody>
<tr>
<td></td>
<td>Dog 1 Dog 2</td>
<td>Dog 3 Dog 4</td>
<td>Dog 5</td>
</tr>
<tr>
<td>1</td>
<td>23&lt;sup&gt;a&lt;/sup&gt; 34</td>
<td>29 Alive 27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Alive 21&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Alive 31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28&lt;sup&gt;a&lt;/sup&gt; Alive 33</td>
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<tr>
<td>3</td>
<td>33 Alive</td>
<td>28 Alive 32</td>
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<tr>
<td>4</td>
<td>32 Alive</td>
<td>29 30 Alive</td>
<td>– Alive</td>
</tr>
</tbody>
</table>
| 5                       | Alive –                     | 28 28<sup>a</sup> 26<sup>a</sup> | – 24<sup>a</sup>
| 6                       | – –                         | 28 Alive Alive | – Alive |
| 7                       | – –                         | – – – – –     | – – – 28          |
| 8                       | – –                         | – – – – –     | – – – – – –        |
| 9                       | – –                         | – – – – –     | – – – – – –        |
| 10                      | – –                         | – – – – –     | – – – – – –        |
| 11                      | – –                         | – – – – –     | – – – – – –        |
| Day of ovariohysterectomy | 33 34                       | 32 30 33      | 31 30               |

<sup>a</sup> No fetus or remnants of fetal material were visible at hysterectomy.
showed moderately electron-dense cytoplasm with high apical protrusions, filled with mucus containing vacuoles.

3.1.3. Endometrial stroma, deep endometrial glands and myometrium

Variable (slight to moderate) infiltration of the placental stroma by macrophages occurred in all placental sites. There were no differences between controls and treated dogs in endometrial stroma, deep endometrial glands and myometrium, as assessed with light microscopy.

3.2. Alterations of placental morphology in treated dogs

The number of macrophages infiltrating the maternal stroma did not increase after treatment.

3.2.1. Placental labyrinth

Quality and degree of alterations in the placental sites of treated dogs depended on the time after fetal death. Morphological alterations, however, after both treatments were similar, but they occurred somewhat earlier in dogs that had received aglepristone. In most placentaion sites \( (n = 3) \), where fetal death had been detected sonographically on the day of ovariohysterectomy, the syncytiotrophoblast detached multifocally from the cytotrophoblast and fibrinous extravasations occurred in the resulting spaces (Fig. 2a). Within these areas of detachment, the number of cytotrophoblast cells expressing Ki-67 increased slightly (from 4/high power field (HPF) in the normal placenta to 8/HPF). In two of these altered placentaion sites, extended areas of coagulation necrosis were also observed, but no thromboses were found in fetal or maternal blood vessels. Under light microscopy, those parts of the placental labyrinth being not affected by detachment corresponded to that of the normal placenta; only the number of proliferating cytotrophoblastic cells (Ki-67 antigen) increased (to 6 per HPF). However, ultrastructurally, intercellular spaces between cyto- and syncytiotrophoblast were widened (up to 2 \( \mu \)m; Fig. 2c). They were empty or contained small amounts of slightly electron-dense granular material. Additionally, no or only a few desmosomes occurred between the two cell types. Cytoplasm, organells and nuclei of the endothelial cells of the maternal capillaries were moderately swollen. In those placentaion sites where fetal death was diagnosed by sonography 2–4 d before ovariohysterectomy, the number of cytotrophoblast cells expressing Ki-67 antigen obviously increased (up to 20/HPF), and the cytrotrophoblast was multifocally multilayered (Fig. 2b). The cytoplasm of the underlying syncytiotrophoblast contained moderate amounts of very small eosinophilic clods and vacuoles, nuclei were hyperchromatic with signs of pyknosis or karyorrhexis. Due to degeneration and necrosis of the syncytiotrophoblast, the placental labyrinth was thinned. Because of

Fig. 2. (a) Detachment of syncytiotrophoblast (arrows) from cytotrophoblast (arrowheads) in a canine placental site where the fetus had died on the day of ovariohysterectomy (H.–E.-stain; magnification 10×); (b) proliferation of cytotrophoblast (arrowheads) and degeneration of syncytiotrophoblast 3 d after fetal death (H.–E.-stain; magnification 20×); (c) loss of desmosomes and extended intercellular spaces (arrow) between cyto (CT) and syncytiotrophoblast (ST) in a placental site where the fetus had died on the day of ovariohysterectomy (transmission electron microscopy; magnification 3000×).
excessive cellular shrinkage in two of the placentation sites, the two trophoblast cell populations were not distinguishable. These phenomena were accompanied by an increased amount of cellular debris in the necrosis zone, reaching deep into the lumina of glandular chambers. In three of four placentation sites where fetal death had been diagnosed more than 5 d before ovariohysterectomy and in two placentation sites 4 d after fetal death, no placental labyrinth was detectable. However, great amounts of cellular debris from the necrosis zone were noted on and within the lumina of glandular chambers.

3.2.2. Glandular chambers

In placental sites with a living or recently deceased fetus, epithelial cells of the glandular chambers had a moderate increase in cytoplasmic vacuolisation and higher numbers and quantities of apical protrusions (visible already by light microscopy). As for ultrastructure, the cytoplasm of glandular epithelia contained very distinct rER and very numerous apical vacuoles, mostly filled with mucous material, as well as many small lipid droplets. Compared to controls, the number of epithelial cells containing lysozyme increased very slightly. In addition, the enzyme appeared within the apical protrusions of several cells. Glandular epithelia of those placentation sites, where the fetus had died 2 d or more before ovariohysterectomy, had a distinct pleomorphy and the number of cells degenerating and desquamating into the necrosis zone had clearly increased (from 20/HPF in the controls up to 50/HPF; Fig. 3). Several large intracytoplasmatic vacuoles, ultrastructurally corresponding to intracytoplasmatic lumina, lined by short microvilli, represented a special finding in the epithelia of the glandular chambers (Fig. 3). Lysozyme was distinctly expressed at the cytoplasmatic borders of the vacuoles, and in total, the number of lysozyme expressing cells increased obviously compared to the controls. After the complete disappearance of the placental labyrinth (i.e., after about 5 d or more after fetal death), the number of cells with large intracytoplasmic vacuoles decreased again. No secondary and/or tertiary lysosomes were detectable (besides single myelinosomes) in some of the epithelial cells. By enzyme histochemistry, there were no differences between the glandular chambers of treated and untreated control dogs. The morphology of the placentation sites of Dog 7 (control) with spontaneous occurring fetal death corresponded to that described after induced fetal death, but the alterations seemed to proceed at a slower rate.

4. Discussion

Both protocols used for pregnancy termination in this study caused similar morphologic changes of the placentation sites. In humans, it is suggested that antiprogestins induce an increased production of prostaglandins in the decidua (due to reduced concentrations of prostaglandin dehydrogenase in these cells) [9,16,17]. Taking this into account, similar placental alterations observed in this study might have been caused by comparable mechanisms, i.e. a decreased blood supply due to prostaglandins inducing hypoxic degeneration of the syncytiotrophoblast. Coagulation necrosis observed in two of the placentation sites early after fetal death was morphologically consistent with infarcts, but no thromboses were visible within placentation sites investigated, neither in fetal nor in maternal blood vessels. The edematous swelling of the maternal endothelium detected in this investigation was the only morphological alteration of blood vessels that might contribute to hypoxia. The first placental morphological lesion after treatment (before fetal death) was a loosening of intercellular contacts between cyto- and syncytiotrophoblast. Afterwards, if cells do not separate immediately (and undergo coagulation necrosis), the syncytiotrophoblast degenerates, and reactively, the cytotrophoblast proliferates. Those parts of the placental labyrinth that are not desquamated into the necrosis zone had degeneration with cellular shrinkage, finally leading to vanishing of the labyrinth. The distinct rER, the high number of secretory vacuoles and the

Fig. 3. Pleomorphic glandular chambers in a canine placenta 3 d after fetal death, with numerous epithelial cells being desquamated (arrows) into the necrosis zone (N) that extends into the lumina of the glandular chambers (H.–E.-stain, magnification 20×). Inset: intracytoplasmatic lumen (L) lined by short microvilli (arrow) within epithelial cells of glandular chambers after fetal death (transmission electron microscopy, magnification 3000×).
Intraepithelial lumina in the epithelial cells of the glandular chambers are signs of increased secretory activity in treated dogs (beginning early after treatment, before fetal death) compared to findings in the normal canine placenta during mid-gestation [15] and to the controls. Grether et al. [15] assumed that mucus, secreted by glandular chambers, might be harmful or even toxic to the trophoblast. Increased production of glandular secretions after medication might thus be conducive to the destruction of the placental labyrinth. Enzymes secreted by the epithelial cells of glandular chambers may contribute to this. Enzyme histochemistry, however, did not reveal any differences between treated dogs and controls. Only the production of lysozyme (a ubiquitous enzyme splitting up mucopolysaccharides and mucopeptides) rises in glandular chambers after medication. The functional relevance of the lysozyme secretion in the controls and in the treated dogs as well remains unclear, because this enzyme specifically destroys the murein in bacterial cell walls. As bacterial infection appears to be impossible during our experiments, the secretion of lysozyme may represent just a nonspecific endometrial reaction during gestation with an increase after induced fetal death. It remains to be clarified, whether other enzymes that might destroy fetal and/or placental material are co-secreted with lysozyme.

That the numbers of macrophages infiltrating the placental structures do not increase after fetal death, may lead to the conclusion that phagocytosis by genuine or functional macrophages does not play a major role in the disappearance of the dead fetuses. Additionally, no indications of active phagocytosis (i.e. lysosomes) or any other kind of uptake were detected (i.e. intercellular absorption) in the placental and glandular cells investigated. However, abortion, i.e. the expulsion of the whole conceptus (fetus together with fetal membranes) can probably be excluded, because affected placentaion sites were usually neighbouring intact ones.

In summary, active secretion of glandular epithelia and degeneration of the placental structures, not phagocytosis, appeared to be the major morphological incidents after experimentally induced and spontaneous fetal death in dogs. However, additional immunohistochemical and electronmicroscopical investigations have to be carried out for a more detailed understanding of morphological processes after fetal death in dogs.

References

Receptor blockers — general aspects with respect to their use in domestic animal reproduction

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Abstract

Receptor blockers compete with the respective agonist for binding to a given receptor without inducing complete signal transduction. In recent years, major interest has focused on sex-steroid hormone receptor blockers (antagonists). Indications have been obtained that inadequate changes in receptor conformation and subsequent failure of transcriptional activation are major events preventing hormonal activity. However, various subtypes and variants of receptors and receptor mutations have also been identified. Expression of antihormonal effects may vary depending on the type of receptor the blocker is bound to. Hence, receptor blockers may also have an inherent agonistic activity. Aglepristone™ is the first antiprogestin registered for veterinary use with the indication “interruption or prevention of pregnancy”; similarly, these types of compounds were successfully used for induction of parturition in the dog and cat and for conservative treatment of pyometra in the dog. Moreover, application of antiprogestins has clearly demonstrated the role of progesterone as a major factor controlling overt pseudopregnancy in dogs. With respect to farm animals, parturition was induced in cows without an increased incidence of retained fetal membranes. Other than antiprogestins, antioestrogens and antiandrogens are still in a more experimental phase. In particular for use in humans, high-affinity blockers binding to the oxytocin/vasopressin receptor are in development; they exert distinct tocolytic activities. Also, the release of GnRH can be inhibited by respective antagonists; however, their use in reproduction is still hampered by the high dose requirement and the side effects observed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Hormone antagonists; Receptors; Sex steroids; Domestic animals

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1. Introduction

The removal of endocrine organs and study of the resulting effects has been a classical approach in endocrine research. However, by such an operation in general not only one but a whole array of hormonal factors is removed. Thus the observed responses may be of multifactorial origin. In order to overcome this problem, more specific approaches were followed in the past, like active and passive immunisation against specific hormones, the development of competitive enzyme blocking agents, development of neurotransmitter-like agents blocking the release of a distinct hormone (see Concannon et al., 1987), development of competitive receptor blockers (see below) and — as the latest addition — the targeted disruption of genes encoding for mediators or the respective receptors in knockout models.

This paper deals with receptor blockers and their use in domestic animal orientated basic research, biotechnology and therapy. By taking into account data from own studies, main attention will be given to those agents blocking the activity of sex steroid hormones. However, reference will also be made to those agents blocking the activity of peptide hormones of hypothalamic origin.

2. Steroid hormone receptor blockers

Knowledge about the mechanisms of action of receptor blockers for steroid hormones is still incomplete. They exert their activity by binding to the steroid hormone receptor without inducing transcription. The understanding of this phenomenon is based on the knowledge of the processes induced by hormone–receptor interaction. Steroid hormones are small lipophilic hormones able to pass the membranes of target cells. They bind to intracellularly located receptor proteins and after decades of controversial discussion there is now overwhelming evidence that these receptors are basically located in the nucleus (Yamashita, 1998). Nuclear hormone receptors represent an evolutionary conserved class of transcription factors being present from flies to mammals (for review see Beato et al., 1995). According to the hormone they bind to, these receptors can be classified into those binding to steroids (e.g. progestins, androgens, oestrogens), steroid derivates (vitamin D3), nonsteroids (e.g. thyroid hormone) and receptors for which no ligands have been found yet (orphan receptors). Hormone binding leads to a conformational change, resulting in specific binding to palindromic DNA sequences (hormone responsive elements, HREs) as liganded receptor dimers (Gronemeyer, 1992; Beato et al., 1995; Tenbaum and Banaihmad, 1997). Thus, one role of the hormone is to induce DNA binding (see Fig. 1).

In general nuclear receptors possess a highly conserved DNA binding region separating the receptor into a variable amino (N) terminal and a higher conserved carboxy (C) terminal part. The DNA binding region is essential for sequence-specific recognition of the hormone response element of the target gene by the receptor. It consists of 66 amino acids containing two zinc fingers. The N terminals of the receptor are the least conserved parts. Length and sequence composition varies strongly among receptors.
However, a constitutionally active transcriptional activation function (AF-1) was demonstrated to be located in the N terminals for most of the analyzed receptors. The C terminals of nuclear receptors harbour multiple functions such as hormone binding (ligand binding), transcriptional activation (AF-2), transcriptional repression (silencing), nuclear translocation and dimerisation (see Fig. 2). C-terminal receptor functions are modelled upon hormone binding. Thus, in contrast to AF-1, the second transactivation function is dependent on ligand binding (Evans, 1988; Gronemeyer, 1992; Tenbaum and Baniahmad, 1997).

There is now ample evidence that a ligand may interact with various isoforms or variations of a nuclear receptor. Thus progesterone may be bound to either progesterone receptor A, B or C, most likely resulting in different transcriptional activities (Kastner et al., 1990; Gronemeyer et al., 1991; Vegeto et al., 1993; Wen et al., 1994; Wei et al., 1996; Giangrande et al., 1997). In particular for the classical oestrogen receptor (ERα) the formation of many variants and mutants has been described, mostly resulting from the deletion of one or several complete exons by alternative splicing (Hu et al., 1996; Pfeffer et al., 1996; Friend et al., 1997; Murphy et al., 1997; Okada et al., 1998). Cloning of another oestrogen receptor from a rat prostate cDNA library (Kuiper et al., 1996) led to the definition of the oestrogen receptor β, which has been demonstrated in an increasing list of mammalian (Mosselman et al., 1996; Tremblay et al., 1997; Pau et al., 1998; Rosenfeld et al., 1999) and nonmammalian species (Lakaye et al., 1998;
Tchoudakova et al., 1999). In man, the genes for the two oestrogen receptors are located on different chromosomes (Gosden et al., 1986; Enmark et al., 1997). However, homogeneity of the DNA binding domain between the two receptors is high (96%); it is distinctly less in the other domains (Enmark et al., 1997, Mosselman et al., 1996). For the oestrogen receptors α and β, a different but overlapping distribution pattern has been described (Enmark et al., 1997; Kuiper et al., 1997, 1998; Shugrue et al., 1998; Hiroi et al., 1999). Thus, in many cases they may occur simultaneously in the same target cell, possibly together with other receptor variants. In addition it has been shown that one ligand may be bound with different affinities to the various receptor variants (Kuiper et al., 1997) or may exhibit different agonist/antagonist activities (Barkhem et al., 1998). Moreover, it must be assumed that heterologous dimerisation products may form following binding of the ligand (Cowley et al., 1997; Pace et al., 1997; Pettersson et al., 1997). Thus, depending on the expression of receptors in a target tissue, different responses may be induced by the same ligand

Also steroid hormone antagonists show a high affinity for the respective nuclear receptors with dissociation constants in the picomolar to lower nanomolar range (Caponi and Rochefort, 1978; Katzenellenbogen et al., 1981; Hurd and Moudgil, 1988; Terakawa et al., 1988). As with agonists, this binding induces dimerisation, which is followed by binding to the HREs of the respective genes, however, without or only slightly inducing transcription. This latter phenomenon is still poorly understood. Recent investigations point to the role of a coactivator in the case of inducing transcription and a corepressor in case of steroid hormone antagonists (Baniahmad et al., personal communication, see Fig. 1). In addition, the steroid hormone antagonist leads to conformational changes of the receptor. Since inhibition of transcriptional activity by the steroid hormone antagonist may vary, most antihormones have a partial agonistic activity. In addition, expression of agonistic and antagonistic activity may vary between hormone dependent tissues, depending on the spectrum of locally available nuclear receptors. Based on these observations it can be explained why tamoxifen, an anticancer
drug used in human therapy, acts predominantly as a strict anti-oestrogen in the mammary gland (Gottardis et al., 1988; Jordan, 1992, 1997) while it shows clear oestrogenic activity on the uterine endometrium (Malfetano, 1990; Daniel et al., 1996).

In conclusion, the characteristics of steroid hormone antagonists may be summarised as follows.

- They bind to the ligand-binding domain of a receptor.
- Binding is competitive and reversible.
- They induce a different conformation of receptors.
- They reduce hormone dependent gene activation and/or other receptor mediated effects.

3. Observations following the use of hormone antagonists in laboratory and domestic animal species

Steroid hormone antagonists exhibiting a competitive binding to the oestrogen, androgen and progesterone receptor have been developed and are presently in use for therapy in human medicine. To the knowledge of the authors, there is so far only one registration of a sex hormone antagonist in veterinary medicine (Aglepristone®, Virbac, France). However, apart from the application in laboratory animal species, there are data from experimental use in large and small animals with apparent emphasis on the application of antiprogestins. In view of the multitude of the experiments performed, this paper will restrict itself to observations in domestic animal species.

4. Antiprogestins

The chemical structure of three antiprogestins, mifepristone (RU38486), aplepristone (RU46534) and onapristone (ZK98299), is shown in Fig. 3. They exhibit a high structural similarity and it has been proposed that the hydrophobic side chain at C17 is responsible for the high-affinity receptor binding while the additional aromatic ring at C11 with a dimethyl-amino- group is responsible for the changes in receptor conformation leading to a suppression of transcription (Baulieu, 1985, 1987). As shown in Table 1 for RU38486, apart from binding to the progesterone receptor, binding to the glucocorticoid receptor and — to a lesser extent — also to the androgen receptor has been observed. Binding affinity may vary between species with no binding observed to the progesterone receptor of the chicken oviduct (Sakiz et al., 1984). No binding was observed to sex-hormone-binding globulin and transcortin in the human, monkey and chicken, confirming own observations in the dog (Gerres, 1991). Thus it must be expected that the effects observed after treatment with an antiprogestin will vary depending on receptor expression, the affinity to the receptor and the dose applied.
Moreover, effects will vary depending on the role of progesterone as an endocrine and paracrine factor at a given stage of the reproductive cycle.

Table 1
Binding of RU38486 to steroid receptors and plasma proteins in different species (from Baulieu, 1985)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Species</th>
<th>Rat/Mouse</th>
<th>Man/Monkey</th>
<th>Chicken</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone receptor</td>
<td>rat/rabbit</td>
<td>(+ + +) (∝ P)</td>
<td>(+ +) (∝ P)</td>
<td>(-)</td>
</tr>
<tr>
<td>Glucocorticoid receptor</td>
<td>rat/mouse</td>
<td>(+ +) (∝ D)</td>
<td>(+ +) (∝ D)</td>
<td>(+ +) (∝ D)</td>
</tr>
<tr>
<td>Androgen receptor</td>
<td>rat (+1/4 T)</td>
<td>(+ + +) (∝ D)</td>
<td>(+ + +) (∝ D)</td>
<td>(-)</td>
</tr>
<tr>
<td>Mineralocorticoid receptor</td>
<td>rat (−)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Sex-hormone-binding globulin</td>
<td>man/mouse</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Transcortin</td>
<td>man/mouse</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*In comparison to the affinity of the corresponding agonist.
5. Application of antiprogestins in the dog and cat

Different from other domestic animal species the course of the progesterone concentrations in peripheral plasma is virtually identical in pregnant and nonpregnant dogs. They begin to increase prior to ovulation, reach a maximum during early dioestrus and decrease thereafter, reaching baseline levels (below 1 ng ml⁻¹ plasma) about 70–90 days after onset of dioestrus. In pregnant animals parturition is preceded by a change from the more gradual to a precipitous decline prior to parturition (length of pregnancy 63 ± 2 days). Luteolysis in the dog is independent of a uterine luteolysin (Hoffmann et al., 1992). However, depending on the dose regimen selected, application of prostaglandin F₂₀ (PGF₂₀) during the second half of pregnancy may lead to a temporary or permanent depression of luteal function resulting in abortion (Romagnoli et al., 1993). Also in the cat, an induced ovulator, ovarian function is independent of the uterus. After sterile matings cats become pseudopregnant and the resulting corpora lutea pseudograndititates show a life span of about 40 days (Tsutsui and Stabenfeldt, 1993).

5.1. Effects of antigestagen treatment during early pregnancy and prior to parturition

Observations after treatment during early pregnancy are limited to the dog. There, implantation occurs about 14–15 days after ovulation. As was shown by Fiéni et al. (1996), parenteral treatment prior to implantation with two times 10 mg aglepristone kg⁻¹ bw 24 h apart completely blocked establishment of pregnancy. None of the 35 mated bitches became pregnant, confirming our own observations (Hoffmann et al., unpublished data).

Also during the second half of pregnancy treatment with the antigestagen interfered with pregnancy. Oral application of 20 mg RU38486 kg⁻¹ bw induced abortion in two bitches; no reactions were observed in two other dogs (Linde-Forsberg et al., 1992). Concannon et al. (1990) report about abortions or resorptions in conjunction with vulval discharge after oral application of 2–5 mg RU38486 kg⁻¹ bw over several days. Interestingly, in these studies luteolysis was observed after 40–45 days. Following parenteral application of two times 10 mg aglepristone 24 h apart, Fiéni et al. (1996) report about a 97.1% success rate in inducing resorption/abortion up to day 55 post mating.

In our own experiments repeated subcutaneous injections of 6 mg RU38486 kg⁻¹ bw after day 56 of pregnancy led to an onset of parturition in the presence of unchanged high progesterone concentrations. Otherwise, initial changes (drop in body temperature, onset of cervical opening, maternal behaviour) resembled a normal parturition. However, concerning the further cascade of events leading to expulsion of the fetuses, the prepartal increase of PGF₂₀ was missing in the treated dogs and parturition had to be finished by cesarean section (Nohr et al., 1993; Hoffmann et al., 1996). From these observations a combined treatment with aglepristone and PGF₂₀ was developed to initiate parturition in the dog (Hoffmann et al., 1999; Riesenbeck et al., 1999). Using a similar type treatment regimen abortion could be induced in a 5-week pregnant cat suffering from fibroadenomatosis (see below). These data clearly show that in dogs and cats progesterone is the
dominating factor throughout the entire length of pregnancy controlling uterine and cervical function.

5.2. Effect of antigestagen treatment on gynaecological disorders

Pseudopregnancy is an inherent phase of the reproductive cycle of the nonpregnant bitch. Depending on the appearance of clinical symptoms, it may be classified as covered or overt (Chakraborty, 1987). Clinical symptoms associated with overt pseudopregnancy are mammary gland hyperplasia with or without secretion, increased aggressiveness, nesting behaviour and acceptance of dummy puppies. Symptoms may appear as early as 30 days after ovulation and can last 30–90 days (Arbeiter and Winding, 1977). The underlying endocrine mechanisms are still poorly understood and in order to test for the involvement of progesterone, in a controlled clinical study overtly pseudopregnant dogs were treated with the antiprogestin RU38486 (Gerres and Hoffmann, 1994). Treatments commenced on days 24, 35 and 43 after onset of prooestrous bleeding, the antiprogestin was given by subcutaneous injections in a dose of 2 mg kg\(^{-1}\) bw in 2–3 day intervals until progesterone concentration had reached levels between 1 \(\text{ng ml}^{-1}\) (3–6 nmol l\(^{-1}\)) plasma. Treatment with the antigestagen led to an earlier onset of overt pseudopregnancy while clinical symptoms were less. Furthermore, duration of pseudopregnancy was shortened when treatment commenced during the first and second third of dioestrus (Table 2). These results implicate a role for progesterone in the onset and maintenance of overt pseudopregnancy. The advanced onset of overt pseudopregnancy in the treatment group was interpreted as a result of the mimicked progesterone withdrawal induced by treatment with the antiprogestin. The other observations of reduced clinical symptoms and duration suggest that maintenance of pseudopregnancy at least partly depends on progesterone.

Another gynaecological problem in the dog is the development of a pyometra (endometritis purulenta). In respect to the importance of progesterone in controlling uterine and cervical function and based on the observation that over 60% of the dogs presented with pyometra are during the state of dioestrus with progesterone levels higher than 1 \(\text{ng ml}^{-1}\) plasma (> 3.18 nmol l\(^{-1}\)), in a series of experiments the hypothesis was tested that progesterone might be an important regulatory factor involved. An initial pilot study (Blendinger et al., 1997) clearly demonstrated that treatment with RU38486 at a dose of 6 mg kg\(^{-1}\) bw twice on day 1 of treatment and once on days 2, 3 and 4 led to a complete evacuation of uterine contents. These observations were confirmed in an open clinical study comprising 41 bitches (Lemmer, 1999). However, successful treatments were bound to an undisturbed ovarian function and progesterone levels above 1 \(\text{ng ml}^{-1}\) plasma at onset of treatment. As far as reported, dogs regained their breeding capacity.

Fibroadenomatosis is a noncarcinogenic tumour of the mammary gland in cats occurring spontaneously during pregnancy or pseudopregnancy. The enlargement of the mammary gland is predominantly due to a gestagen-dependent proliferation of its stromal component. Common therapy is ovariohysterectomy and/or mastectomy. Treatment with the antiprogestin RU38486 given daily by subcutaneous injection over 5 days not only resulted in abortion (after additional treatment with PGF\(_{2\alpha}\), see above), but also
Table 2
Effects of the antiprogestin RU38486 on onset and duration of overt pseudopregnancy and mammary gland development; values expressed as $\bar{x} \pm SD$

<table>
<thead>
<tr>
<th>Onset of treatment (day after onset of pro-oestrous bleeding)</th>
<th>Control cycle</th>
<th>Treatment cycle</th>
<th>Treatment cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Onset$^a$</td>
<td>Duration (days)</td>
<td>Score$^b$</td>
</tr>
<tr>
<td>24</td>
<td>$48.2 \pm 7.5$</td>
<td>$48.8 \pm 10.8$</td>
<td>$3.8 \pm 0.4$</td>
</tr>
<tr>
<td>35</td>
<td>$62.0 \pm 4.5$</td>
<td>$40.2 \pm 21.2$</td>
<td>$3.4 \pm 0.9$</td>
</tr>
<tr>
<td>43</td>
<td>$62.5 \pm 4.5$</td>
<td>$43.7 \pm 25.3$</td>
<td>$3.3 \pm 0.8$</td>
</tr>
</tbody>
</table>

$^a$Day after pro-oestrous bleeding.
$^b$Mean maximum score of mammary gland development (0–4).
in a reduction of mammary gland hyperplasia. After further treatments on days 9, 14, and 20 mammary gland enlargement was completely regressed on day 28 (Blendinger et al., 1994). These observations clearly confirm that fibroadenomatosis is a progesterone-mediated disease in the cat and that blocking of progesterone at the receptor level is an effective therapy. That was demonstrated in several clinical cases and also a male cat which developed severe fibroadenomatosis after application of a depot progestagen for hormonal castration was successfully treated with the antigestagen (Hoffmann, unpublished data).

5.3. Effect on luteal function in the dog

Following treatment with RU38486 for induction of abortion Concannon et al. (1990) and Linde-Forsberg et al. (1992) reported about an accelerated luteal regression. Similarly, in our own studies when treating dogs with pyometra, progesterone declined significantly during treatment from 18.3 ng ml\(^{-1}\) on day 1 to 6.1 ng ml\(^{-1}\) on day 6 (Blendinger et al., 1997) and from 16.8 nmol l\(^{-1}\) on day 1 to 6.82 nmol l\(^{-1}\) on day 14 and 4.9 nmol l\(^{-1}\) on day > 21 (Lemmer, 1999). On the other hand, in a hysterectomized dog luteal function was not affected by subcutaneous applications of 2 mg RU38486 kg\(^{-1}\) bw in 2-day intervals over the entire cycle (Gerres, 1991). This raises the question whether the observed accelerated luteal regression in “intact” dogs is a direct or rather indirect effect of the antiprogestin. The conclusion that it is a rather indirect effect is supported by observations of Li et al. (1991a) in gilts, where it was shown that the acute luteolytic effect of RU38486 depended on the presence of the uterus and/or conceptuses.

5.4. Other observations

Antiglucocorticoid effects of RU38486 were clearly demonstrated in the human and laboratory animals. Thus in both species prior treatments with RU38486 inhibited the activity of dexamethasone (Philibert et al., 1981, Baulieu, 1985). In the dog, Wade et al. (1988) report about significantly increased cortisol and ACTH concentrations following oral application of 20 and 50 mg RU38486 kg\(^{-1}\) bw, respectively. However, as it was indicated by unchanged cortisol levels when treating pseudopregnant dogs (Gerres and Hoffmann, 1994) and dogs with pyometra (Lemmer, 1999) in our own studies using a different dose regimen no interference with adrenal function became obvious. The only side effects observed were labour-like pains after a dog with pyometra had been treated with a single dose of 20 mg kg\(^{-1}\) bw.

6. Application of antiprogestins in large animals

Application of RU38486 induces premature parturition in cattle and sheep. In cattle treatment with 2 mg RU38486 kg\(^{-1}\) bw at 8 h on days 277 and 278 resulted in parturition 55 h later, whereas in vehicle-treated control cows parturition occurred after 210 h (\(p < 0.01\)). Different from induction of parturition with glucocorticoids or PGF\(_{2\alpha}\)
no increase in retained fetal membranes was observed (Li et al., 1991b). In sheep the interval from the first treatment to the onset of lambing was $31 \pm 2$ h in animals given $4 \text{ mg RU38486 kg}^{-1}$ bw on day 144 while it was $121 \pm 27$ h in the vehicle-treated controls ($p < 0.01$) (Gazal et al., 1993). In both animal species progesterone levels started to decline following treatment with the antiprogestin, though the prime source of progesterone in the sheep is the placenta while it is the corpus luteum in the late pregnant cow (Hoffmann, 1994). Also in pigs oral treatment with $4 \text{ mg RU38486 kg}^{-1}$ bw on days 111 and 112 of pregnancy resulted in parturition on day 112.7 compared to day 114.7 in the control group ($p < 0.01$). In treated animals progesterone decreased abruptly from a pretreatment mean of 11 to less than 0.6 ng ml$^{-1}$ during the second day of RU38486 treatment; preterm parturition coincided with an advanced relaxin peak with a maximum on day 112.1. Also in this study, hysterectomized gilts carrying persistent corpora lutea were submitted to treatments with RU38486. Rather than a decrease of progesterone concentration an abrupt increase of progesterone and prolactin secretion was observed leading to the conclusion that the acute luteolytic effect of RU38486 observed in pregnant pigs depends on the presence of the uterus and/or conceptuses (Li et al., 1991a). Thus, it may be concluded that luteal regression in cattle and the decrease of placental progesterone synthesis in sheep following treatment of pregnant animals with RU38486 may also be an effect depending on the presence of the uterine/fetal compartment. Gonçalves et al. (1997) studied the effect of an antiprogestin (naprostone) on in vivo and in vitro fertilization. Studies were performed in oestrus synchronised, superovulated ewes. Treatment with the antiprogestin 3 and 15 h after sponge removal had no effects on synchronization of oestrus, ovulation and oocyte maturation; however, in vivo fertilization rate decreased significantly from 41% in the control group to 2–3% in the treatment group ($p < 0.001$) due to sperm arrest in the cervix. Also fertilization of bovine oocytes in vitro decreased significantly from 62.6% in the control group to 48% in the treatment group. These observations clearly point to the role of progesterone in respect to control of cervico-uterine (tubal) functions and to sperm–oocyte interaction.

7. Antiandrogens and antioestrogens

The antioestrogen tamoxifen and the antiandrogen cyproterone acetate (see Fig. 4) are established compounds used in human therapy. Indication for treatment with tamoxifen is mammary cancer therapy; similarly, an indication for the application of cyproterone acetate is a nonsurgically accessible prostate carcinoma. Other indications are male hypersexuality and hyperandrogenisation in females. The phenylethylene derivatives tamoxifen or clomifen (see Fig. 4) are mixed antagonists–agonists of oestrogen action and belong to the group of type I antioestrogens (Clark, 1994; Klein-Hitpaß et al., 1998). Type I oestrogen antagonists partially inhibit the action of agonists, but, due to their own inherent weak agonistic properties, they also induce to some extent oestrogenic responses. The degree of agonistic or antagonistic activity depends on the species, organ, tissue, or cell type that is being examined. Thus in the human tamoxifen exerts distinct antioestrogenic activities in the mammary gland while it exerts agonistic activities on the
uterus (Gottardis et al., 1988; Jordan, 1992, 1997). The underlying mechanisms are not yet fully understood; however, they may relate to the expression of cell-type-specific oestrogen receptor variants, cell-type-specific arrays of cofactors or to different ways of receptor–DNA interactions. Moreover, in the dog tamoxifen acts rather like an agonist than antagonist (Morris et al., 1992), suggesting also species specific variations with respect to the agonistic activities of type I antioestrogens.

The 7α-alkyl-amide analogues ICI 164,384 and ICI 182,780 may be considered pure antioestrogens and are classified as type II antioestrogens. They lack agonistic activity for uterine growth as shown in rats and mice and they are unable to induce the progesterone receptor in the immature rat. Similarly with type I antioestrogens, and as shown in experiments with transiently transfected mammalian cells, these type II antioestrogens also induce receptor dimerisation and DNA binding. Since there seems to be no significant reporter gene activation, they obviously prevent the activity of both AF-1 and AF-2 (for review see Klein-Hitpaß et al., 1998).
Type I antioestrogens have been applied in domestic animals to further explore the endocrine functions of estradiol-17β. Thus, Jacobs et al. (1988) could demonstrate that the oestrogen provoked preovulatory LH release in heifers is inhibited by concomitant treatment with tamoxifen. The same experiment showed that the blocking of endogenous estradiol-17β prevented the endometrial PGF₂α-release leading to luteolysis. However, Cloprostenol-induced luteolysis was not prevented. Janowski et al. (1996) treated prepartal cows with a dose of 360 mg tamoxifen in 4-h intervals starting on day 268 of pregnancy. They observed no changes in respect to course of parturition and oestrogen synthesis, raising the question on the biological role of prepartal oestrogen production in cattle.

Cyproterone acetate (see Fig. 4) is the 1,2-cyclopropal derivative of chlormadinone acetate. It exerts antiandrogenic and gestagenic activities with an inherent androgenic activity. In laboratory animals cyproterone acetate inhibits development of accessory sexual organs but has little effect on neuro-gonadal feedback mechanisms and hence testosterone biosynthesis. Other than cyproterone acetate, cyproterone and flutamid, a nonsteroidal receptor-binding antiandrogen (see Fig. 4), inhibit the negative feedback of androgens which in consequence leads to increased LH and testosterone values (Neumann et al., 1984; Fuhrmann et al., 1998). In rats, guinea pigs or mice no inhibiting effect of cyproterone acetate on male sexual activity was observed, yet inhibition was observed in dogs, rabbits and in ewes in which a male behaviour had been induced by repeated treatment with testosterone (reviewed by Fabre-Nys, 1982). Daily treatment of boars with 200 mg cyproterone acetate completely suppressed development of boar taint (Horst and Bader, 1969). However, based on the intrinsic hormonal activities of cyproterone acetate, this effect is probably a result of the progestagenic rather than antiandrogenic activity. Also in the dog it has been shown that cyproterone acetate has an antagonadotrophic effect (van Sluijs, 1997). This inherent diversity of hormonal and antihormonal effects of cyproterone acetate makes it difficult to relate experimental observations to distinct hormonal activities, which may explain the paucity of information on the use of these type antiandrogens in domestic animal species.

8. Peptide hormone receptor blockers

In respect to reproduction GnRH and oxytocin antagonists have been developed. Briefly these two hormones comprise 10 (GnRH) and 8 (oxytocin) amino acids, their main synthesis is by hypothalmic neurons though synthesis in periphal organs like the ovary (oxytocin) and placenta (GnRH-like peptide) have been described (Döcke, 1994a,b). They bind to membrane receptors inducing second messenger mediated intracellular signal cascades. The GnRH and oxytocin receptors are members of the large family of G-protein-coupled receptors and have seven transmembrane domains (Kimura et al., 1992; Flanagan et al., 1997; Salvatore et al., 1998). They have been identified in various organs and their specific inhibition would provide further information on the biological role of these two hormones in addition to the opening of new therapeutic approaches. In conjunction with dog reproduction, Vickery et al. (1989) refer to GnRH antagonists with different relative potencies measured on the inhibition of LH release. Side effects observed concerned the mast cell degranulating activity of these
compounds and the side effects to be expected from the release of histamine and other mediators. Together with the high dose requirements, this asks for the development of more potent and safer analogues. However, since similar effects may be observed by downregulation of GnRH receptors following application of a continuously high dose of GnRH, there is only a relative need for these type compounds to interfere with small animal reproduction.

The oxytocin receptor gene is expressed in the myometrium, endometrium and cervical epithelium and blocking the activity of oxytocin by an appropriate antagonist might prove to be a valuable approach to control gynaecological or obstetrical disorders (reviewed by Goodwin and Zograbyan, 1998). Concerning their application in domestic animals, Fuchs et al. (1997) report about the affinity and specificity of the antagonist [1-D(CH₂)₃, Tyr(ME)², Thr⁴, Tyr-NH₂⁻] ornithine vasotocin and its use in late pregnant cows. The antagonist showed a similarly high binding to endometrial and myometrial oxytocin binding sites and a 40-fold molar excess of antagonist inhibited the release of prostaglandin induced by oxytocin in the bovine endometrium prior to parturition. Similar observations in the sheep (Jenkin et al., 1994) point to the role of these compounds as tocolytic agents. Thus the oxytocin and vasopressin antagonist atosiban has reached the phase III of clinical development for human use (Bossmar, 1998).

9. Conclusions

As was demonstrated during the past 20 to 30 years hormone antagonists blocking the activity of reproductive hormones have shown to be powerful tools in endocrine research, therapy and biotechnology. Of particular interest are compounds which block agonists by competitive binding to the respective receptor, thereby inducing complete loss of receptor functions (pure antagonists). In respect to sex-steroid hormone antagonists the effects observed not only depend on the molecular structure of the antagonist but also on the structure of the respective receptor. Thus for example, for the oestrogen and progesterone receptor various subtypes, variants and mutations have been identified by adequate cloning experiments and their distribution varies between cell types, tissues and species. In spite of their highly conserved DNA-binding domain transcriptional activation seems to be regulated differently, leading to a tissue (cell)-specific expression of — for example — oestrogenic activity. Accordingly, the phenomenon of antagonists exhibiting a partial agonistic activity may at least partly be explained on interactions with different subtypes of receptors. However, in spite of recent data explaining in more detail the regulation of receptor activity, still only little is known about the receptor inactivating mechanisms of action of hormone antagonists. Further insight into these mechanisms together with a comprehensive mapping of receptors and receptor subtypes are necessary to develop more specific and selective hormone antagonists.

In spite of the still existing limitations research and also therapeutical approaches in domestic animal reproduction have benefited from the availability of hormone receptor antagonists. This particularly concerns progestagen antagonists and their use in small and large animals. There are also distinct indications for the application of androgen, oestrogen and GnRH antagonists; however, particularly in respect to therapy further research is necessary.
References


