Effects of aglépristone, a progesterone receptor antagonist, administered during the early luteal phase in non-pregnant bitches

S. Galac\textsuperscript{a,b,*}, H.S. Kooistra\textsuperscript{b}, S.J. Dieleman\textsuperscript{c}, V. Cestnika, A.C. Okkens\textsuperscript{b}

\textsuperscript{a}Veterinary Faculty, University of Ljubljana, Ljubljana, Slovenia
\textsuperscript{b}Department of Clinical Sciences of Companion Animals, Utrecht University, Yalelaan 8, P.O. Box 80154, TD Utrecht 3508, The Netherlands
\textsuperscript{c}Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Received 7 July 2003; received in revised form 28 October 2003; accepted 1 November 2003

Abstract

Aglépristone, a progesterone receptor antagonist, was administered to six non-pregnant bitches in the early luteal phase in order to determine its effects on the duration of the luteal phase, the interestrous interval, and plasma concentrations of progesterone and prolactin. Aglépristone was administered subcutaneously once daily on two consecutive days in a dose of 10 mg/kg body weight, beginning 12 ± 1 days after ovulation. Blood samples were collected before, during, and after administration of aglépristone for determination of plasma progesterone and prolactin concentrations. The differences in mean plasma concentration of progesterone and of prolactin before, during, and after treatment were not significant. Also, the duration of the luteal phase in the six treated bitches (72 ± 6 days) did not differ significantly from that in untreated control dogs (74 ± 4 days). However, the intervals during which plasma progesterone concentration exceeded 64 and 32 nmol/l were significantly shorter in the six treated bitches than in untreated control dogs. The interestrous interval was significantly shorter in beagle bitches treated with aglépristone (158 ± 16 days) than in the same group prior to treatment (200 ± 5 days).

It is concluded that administration of aglépristone during the early luteal phase in the non-pregnant bitch affects progesterone secretion, but not sufficiently to shorten the luteal phase. The shortening of the interestrous interval suggests that aglépristone administered in the early luteal phase influences the hypothalamic–pituitary–ovarian axis.

© 2003 Elsevier Inc. All rights reserved.

Keywords: Progesterone receptor antagonist; Luteal phase; Prolactin; Interestrous interval; Dogs
1. Introduction

Aglépristone, a progesterone receptor antagonist, has proved to be safe and effective in early and midgestation pregnancy termination in the bitch [10,11,13]. Progesterone receptor antagonists terminate pregnancy by binding with high affinity to the uterine progesterone receptor, thereby preventing progesterone from exerting its biological effect [5,14]. In addition to pregnancy termination, endocrine effects such as temporary elevation of plasma prolactin concentration, premature cessation of the luteal phase, and shortening of the interestrous interval have been observed after administration of progesterone receptor antagonists [10,11,13,16]. However, depending on the time of administration, differences in the regulation of the luteal phase might cause other endocrine effects. So far none of the studies concerning the effects of progesterone receptor antagonists have been carried out early in the luteal phase in the cyclic non-pregnant dog.

The regulation of canine luteal function in both cyclic and pregnant bitches differs between the first and the second parts of the luteal phase. Between ovulation and Days 24–28 of the luteal phase, the corpora lutea function independently of pituitary support [18]. During the second part of the luteal phase pituitary luteotropic factors, such as prolactin and possibly LH, are necessary to maintain the luteal function [18–20]. The role of LH as a luteotropic factor remains unclear. It was reported in 1987 that LH may be necessary to sustain progesterone synthesis [3], but more recent studies failed to confirm a direct luteotropic function for LH in the bitch [19,21].

It was assumed that a progesterone receptor antagonist would have different endocrine effects early in the luteal phase in the non-pregnant cyclic bitch than it would in early pregnancy or midgestation and for this reason the effects of aglépristone on plasma concentrations of progesterone and prolactin were studied in the early luteal phase, together with its effects on the length of the luteal phase and the interestrous interval.

2. Materials and methods

2.1. Animals, treatment, and collection of blood samples

Six healthy beagle bitches, 3–6 years of age, were housed singly or in pairs in indoor–outdoor runs, fed a standard commercial dry dog food once daily, and given water ad libitum. All dogs were examined three times per week for the presence of vulvar swelling and serosanguinous vaginal discharge, which were considered to signify the onset of proestrus. The first day of the luteal phase (Day 1) was defined as the day subsequent to the onset of proestrus on which plasma progesterone concentration exceeded 16 nmol/l, which is when ovulation is assumed to occur [2,28,17]. The luteal phase was considered to be terminated when plasma progesterone concentration was found to be below 3 nmol/l for the first time. The interestrous interval was defined as the period between the onset of one proestrus and that of the next.

The bitches were treated with aglépristone (Alizine®, Virbac Laboratories, Carros, France) in a dose of 10 mg/kg body weight subcutaneously, once daily on two consecutive days beginning 12 ± 1 days after ovulation. Blood samples for measurement of plasma
concentrations of progesterone and prolactin were collected at least three times weekly from the onset of proestrus to the end of the luteal phase. Blood samples were also collected immediately before and at 1, 2, 4, and 8 h after administration of aglépristone. Blood samples were collected by jugular venipuncture, immediately placed in chilled heparin-coated tubes, and centrifuged. Plasma was stored at −20°C until assayed. In three untreated control beagle bitches blood samples were collected once daily between the onset of proestrus and the 90th day of the luteal phase [19].

2.2. Radioimmunoassays

Plasma concentrations of progesterone were measured by a previously validated radioimmunoassay [6,17]. The intra-assay and inter-assay coefficients of variation were 11 and 14%, respectively, and the lowest detectable level of progesterone was 0.3 nmol/l.

Plasma concentrations of prolactin were measured by a previously validated heterologous radioimmunoassay [17]. The intra-assay and inter-assay coefficients of variation were 3.5 and 11.5%, respectively. The lowest detectable level of prolactin was 0.8 µg/l.

2.3. Calculations and statistical analysis

The mean plasma concentrations of progesterone and prolactin 5 days before, during, and after treatment, until Day 21 of the luteal phase, were compared by ANOVA for repeated measures. The mean intervals during which plasma progesterone concentration exceeded 64 and 32 nmol/l in the group of bitches treated with aglépristone were compared with those intervals in the control dogs using a one-tailed Student’s t-test. A two-tailed Student’s t-test was used to compare the mean duration of the luteal phase in the treated and control bitches and the mean interestrous interval in bitches before and after treatment with aglépristone. Values are expressed as mean ± S.E.M. and/or range. P ≤ 0.05 was considered significant.

3. Results

The administration of aglépristone caused no side effects except a painless inflammation at the injection site in three bitches, which disappeared within 1 month. In the other three bitches the area was massaged after the injection and no inflammation was observed.

The mean duration of the luteal phase in the six bitches treated with aglépristone (72 ± 6 days) did not differ from that in the untreated control bitches (74 ± 4 days). Mean plasma progesterone concentration remained unchanged during the 2 days of treatment with aglépristone. The mean plasma progesterone concentrations before treatment (106 ± 13 nmol/l, range 70–140 nmol/l), during treatment (124 ± 13 nmol/l, range 76–156 nmol/l), and after treatment (130 ± 16 nmol/l, range 89–184 nmol/l) did not differ. The mean interval during which plasma progesterone concentration exceeded 64 nmol/l in the treated bitches was 28 ± 2 days, which was significantly shorter (P < 0.05) than that in the control bitches (34 ± 3 days). The mean interval during which plasma progesterone concentration exceeded 32 nmol/l in the treated bitches was 39 ± 2 days, which was
significantly shorter ($P = 0.05$) than that ($47 \pm 5$ days) in the control bitches (Fig. 1). The interestrous interval in bitches treated with aglépristone ($158 \pm 16$ days) was significantly shorter ($P < 0.05$) than in the same bitches before treatment ($200 \pm 5$ days).

The profile of plasma prolactin concentrations in bitches treated with aglépristone was characterized by a fluctuating baseline with occasional distinct elevations (Fig. 1). The differences in mean plasma prolactin concentration before treatment ($3.2 \pm 1.1$ µg/L, range
1.3–8.7 µg/l), during treatment (3.2 ± 0.8 µg/l, range 1.6–7.1 µg/l), and after treatment (3.2 ± 0.8 µg/l, range 1.4–5.9 µg/l) were not significant.

4. Discussion

Administration of aglépristone in the early luteal phase did not influence plasma progesterone concentrations during or immediately after treatment. The duration of the luteal phase in the non-pregnant bitches did not differ significantly from that in control dogs, as opposed to the premature cessation of the luteal phase when aglépristone is administered in midgestation [11,13]. However, the intervals during which the plasma progesterone concentration exceeded 32 and 64 nmol/l were significantly shorter than in untreated bitches. The administration of aglépristone during the early luteal phase does therefore affect progesterone production but not sufficiently to shorten the luteal phase. Shortening of the luteal phase in bitches after administration of aglépristone in midgestation is associated with increased plasma levels of PGFM, the main metabolite of prostaglandin F$_{2\alpha}$, probably induced by fetal expulsion [11,16]. An increased level of prostaglandin F$_{2\alpha}$ could not have caused the effects on the progesterone secretion in the bitches of our study since they were not pregnant. Moreover, early termination of pregnancy would not lead to abortion but rather to resorption of the fetuses, which would not induce an increase in plasma PGFM concentrations [7,11]. It is also questionable whether in this part of the luteal phase prostaglandin F$_{2\alpha}$ release could have caused premature luteolysis, since the rapidly growing cyclic and pregnant corpus luteum is relatively resistant to prostaglandin F$_{2\alpha}$ [1,22,23]. Alternatively, the affected progesterone secretion in our bitches may be ascribed to the fact that occupation of the progesterone receptors by aglépristone may have reduced the autoregulatory positive feedback of progesterone on its own secretion during the early luteal phase [24].

In the present study, administration of aglépristone in the early luteal phase resulted in shortening of the interestrous interval. Since the duration of the luteal phase was unaffected, this can only be explained by an early termination of anestrus. In the bitch, increased release of GnRH by the hypothalamus [25] and increased sensitivity of the pituitary to GnRH from early to late anestrus [26] have been reported to be important determinants of the initiation of a new follicular phase. Furthermore, progression from early to late anestrus is associated with a rise in basal plasma FSH without a concomitant rise in basal LH [15]. The shortening of the interestrous interval by aglépristone is presumably due to an effect on the hypothalamic–pituitary–ovarian axis.

In contrast to the effects of aglépristone when administered in midgestation [11,13], its administration in the early luteal phase in the cyclic dog did not induce a significant change in plasma prolactin concentration. Similar observations have been noted in early pregnancy termination [11]. Elevated plasma prolactin concentrations after administration of progesterone receptor antagonist in mid-gestation occur as a result of the occupation of the progesterone receptors by aglépristone, which may mimic a sudden decline of plasma progesterone concentration and consequently induce enhanced prolactin release. This could be similar to the pathogenesis of pseudopregnancy, in which a rapid decrease in plasma progesterone concentration induces a rise in plasma prolactin concentration [4,12].
The absence of a rise in prolactin after administration of aglépristone in our study cannot be explained by the inability of the pituitary to increase prolactin secretion in this part of the luteal phase, for Okkens et al. [17] observed a significant increase in plasma prolactin concentration in bitches following hysterectomy at 8–16 days after ovulation, i.e., early in the luteal phase. However, the number of pituitary progesterone receptors may be lower in the early luteal phase than in midgestation. The expression of progesterone receptors in the uterus and ovary in the early luteal phase is similar to that in midgestation [9,27], but quantitation of progesterone receptors in the canine pituitary has not been reported. Another explanation for the absence of a rise in prolactin after administration of aglépristone could be that early in gestation, when the embryo is very sensitive to changes in its environment, the action of the progesterone receptor antagonist is sufficient only to induce pregnancy termination but not to increase plasma prolactin concentration.

Aglépristone has been shown to be very effective in the early termination of pregnancy [11]. In cases of early pregnancy termination because of unwanted mating, aglépristone is administered shortly after ovulation, when the diagnosis of pregnancy is not yet possible. Feldman et al. [8] reported that only 40% of bitches conceived after an unwanted mating, e.g., mating without fertility control. This implies that when aglépristone is used as an abortifacient soon after mating, a large proportion of the dogs treated will not be pregnant. This study therefore used non-pregnant bitches to study the effects of aglépristone administered during the early luteal phase.

In conclusion, this study has shown that administration of aglépristone in the first part of the luteal phase affects plasma progesterone concentration but not sufficiently to shorten the luteal phase. Plasma prolactin concentration was not affected. The interestrous interval in bitches treated with aglépristone was shortened, suggesting that in the early luteal phase aglépristone influences the hypothalamic–pituitary–ovarian axis.

Acknowledgements

The authors are grateful to Mrs. D.M. Blankensteijn for technical assistance. We thank Mr. S. Woitkowiak from Virbac Laboratories for the generous gift of Alizine™.

References

TERMINATION OF MID-GESTATION PREGNANCY IN BITCHES WITH AGLEPRISTONE, A PROGESTERONE RECEPTOR ANTAGONIST

S. Galac,1,2 H.S. Kooistra,2a J. Butinar,1 M.M. Bevers,3 S.J. Dieleman,3 G. Voorhout4 and A.C. Okkens*2

1 Small Animal Clinic, Veterinary Faculty, University of Ljubljana, Slovenia
2 Department of Clinical Sciences of Companion Animals, 3 Department of Farm Animal Health and 4 Division of Diagnostic Imaging, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

Received for publication: May 18, 1999
Accepted: August 4, 1999

ABSTRACT

Six pregnancies were terminated in mid-gestation with aglepristone, a progesterone receptor antagonist, in 5 beagle bitches in order to determine the effects of aglepristone on plasma concentrations of prolactin and progesterone, the duration of the luteal phase, and the interestrous interval. In addition, the effects of aglepristone on the condition of the uterus and fetuses were examined by ultrasonography. After confirmation of pregnancy by ultrasonography, the dogs received 10 mg, sc aglepristone per kg body weight on 2 consecutive days at about 30 d post ovulation. Before, during and after treatment with aglepristone, plasma samples were collected for determination of the concentrations of prolactin and progesterone. The condition of the uterus and fetuses was assessed by ultrasonography the day before and at least 3 times a week for at least 2 wk after aglepristone administration. Termination of pregnancy occurred within 4 to 7 d after the start of aglepristone treatment, which was well tolerated, with no side-effects except slight vaginal discharge. The results of ultrasonographic examination indicated that aglepristone leads to abortion but not to fetal resorption. Elevated plasma concentrations of prolactin were observed during aglepristone treatment, while plasma progesterone levels remained unchanged. Pregnancy termination with aglepristone resulted in premature cessation of luteal function. In addition, the interestrous interval was shortened. The latter effects may be the consequence of actions of the progesterone receptor antagonist at the hypothalamus-pituitary level. In conclusion, aglepristone proved to be a safe and effective abortifacient in mid-gestation in the bitch. The results of the present study also indicated that aglepristone directly or indirectly influences pituitary function.

Key words: abortion, progesterone receptor antagonist, prolactin, dogs

Acknowledgments: The authors thank Mrs. D.M. Blankenstein, Mr. A.V.P. van de Poll and Mrs. Y.W.E.A. Pollak for technical assistance and Prof. Dr. A. Rijnberk, Dr. M. Kosec and Dr. B.E. Belshaw for critical reading of the manuscript.

Correspondence and reprint requests: H.S. Kooistra@vet.uu.nl

© 2000 by Elsevier Science Inc.
INTRODUCTION

For many decades unwanted pregnancy in dogs has been avoided early in gestation by relatively large doses of estrogens (37). Administration of estrogens prolongs the oviductal transport time and tightens the utero-tubular junction, resulting in implantation failure and embryonic death (19). However, the use of estrogens can result in side-effects such as cystic endometrial hyperplasia (CEH), pyometra, and bone marrow suppression (1), although the occurrence of these side-effects was lower when relatively low doses of estradiol benzoate were administered on Days 3, 5 and 7 after mating (41).

Maintenance of pregnancy in the bitch is dependent on ovarian secretion of progesterone by the corpus luteum (CL) throughout gestation (5,39,40). During the second part of the luteal phase, luteotropic factors originating from the pituitary such as prolactin and possibly luteinizing hormone are essential for the maintenance of the CL (7,25,26,32). Consequently, unwanted pregnancy in dogs can be terminated during mid-gestation by pharmacological agents which suppress prolactin release or interfere with the action or synthesis of progesterone. However, use of many of the currently available abortifacients is accompanied by unwanted and sometimes severe side-effects (2).

Repeated administration of prostaglandin F2α and its analogues during mid-term pregnancy in the bitch results in luteolysis (5,13). The narrow margin between a lethal dose (LD50) and a therapeutic one; side-effects such as vomiting, diarrhea, hyperpnea and ataxia; and the need for repeated administration are important factors limiting the use of prostaglandins in veterinary practice (12,13). Dopamine agonists, such as bromocriptine and cabergoline, cause luteolysis by suppressing pituitary prolactin secretion. Reports on their efficacy differ, depending on the dose and the day of pregnancy at which administration is begun (18,29,46). High doses of bromocriptine frequently cause vomiting, nausea, lethargy and anorexia (7). Cabergoline has been reported to be better tolerated (32,35). The combined use of cabergoline and prostaglandins has also been described (30). When used in combination, they are generally effective at lower doses than with single administration and result in fewer side-effects (31).

Because of the undesirable side-effects of the above mentioned drugs, attention has been given to agents that prevent the action of progesterone, i.e., progesterone receptor antagonists. Mifepristone (RU 486) has proven to be an effective abortifacient in the dog (8,24,42), but is not available for clinical use. Recently, another progesterone receptor antagonist, aglépristone, has been marketed in France as an abortifacient for veterinary use. Administration of aglépristone effectively terminated pregnancy in 66 of 69 bitches in which pregnancy had been confirmed by ultrasonography (14).

The purpose of the present study was to determine the effects of aglépristone on plasma concentrations of prolactin and progesterone, the duration of the luteal phase and the interestrous interval in bitches in which it was given to terminate pregnancy at mid-gestation. In addition, the effects of aglépristone on the condition of the uterus and fetuses were examined by ultrasonography.
Materials and Methods

Animals, Treatment and Collection of Blood Samples

Five clinically healthy beagle bitches, 4 to 6 yr of age, were housed singly or in pairs in indoor-outdoor runs, fed a standard commercial dry dog food once daily, and given water ad libitum. The dogs were examined 3 times a week for the presence of vulvar swelling and serosanguinous vaginal discharge, which were considered to signify the onset of proestrus. The day on which the plasma progesterone concentration reached 5 μg/L for the first time after the onset of proestrus was defined as the day of ovulation, i.e., the first day of the luteal phase (Day 1). The luteal phase was considered to be terminated when the plasma concentration of progesterone fell below 1 μg/L for the first time. The interestrous interval was defined as the period from 1 ovulation to the next. All beagle bitches were mated 1 or 2 d after the estimated day of ovulation, and all became pregnant. One bitch was mated and became pregnant during 2 successive estrous cycles. Pregnancy was confirmed by ultrasonographic examination at about Day 28. After confirmation of pregnancy the dogs were treated subcutaneously with aglëpristone (Alizine® Hoechst Roussel Vet, Romainville, France) on 2 consecutive days at a dose of 10 mg/kg body weight, once a day, starting approximately 30 days after ovulation. Following the administration of aglëpristone, ultrasonography was performed 3 times a week to assess the condition of the fetuses by determining cardiac activity. In addition, the architecture of the uterus was studied for at least 2 wk. Pregnancy was considered to be terminated on the day on which fetuses could no longer be detected in the uterus. All ultrasonographic examinations were performed through the ventral abdominal wall, with the dogs in dorsal recumbency, using a high definition ultrasound system (HDI 3000, Advanced Technology Laboratories, Woerden, The Netherlands) equipped with a 10-5 MHz broadband linear array transducer. Plasma concentrations of progesterone and prolactin were determined 3 times per week from the start of proestrus until the end of the luteal phase. Additionally, on the days of administration of aglëpristone the plasma concentrations of progesterone and prolactin were determined immediately before and at 1, 2, 4 and 8 h after treatment. During the 10 d after administration of aglëpristone plasma concentrations of progesterone and prolactin were determined once daily. Blood samples were collected by jugular venipuncture into evacuated heparinized tubes. The samples were centrifuged, and plasma was stored at -20 °C until levels of progesterone and prolactin were analyzed.

Radioimmunoassays

Concentrations of progesterone in the peripheral blood were measured by a previously validated radioimmunoassay (9,27). The intra-assay and inter-assay coefficients of variation were 11 and 14%, respectively, and the limit of detection was 0.1 μg/L.

Concentrations of prolactin in the peripheral blood were determined by a previously validated heterologous radioimmunoassay (27). The intra-assay and inter-assay coefficients of
variation were 3.5 and 11.5%, respectively. The lowest detectable concentration of prolactin was 0.8 μg/L.

Statistical Analysis

Plasma prolactin concentrations ≥ 50 μg/L were considered to be 50 μg/L. For each dog mean plasma prolactin concentrations before treatment, during treatment and after treatment (Day 36 until the end of the luteal phase) were compared by an ANOVA for repeated measures. Subsequently, multiple comparisons were performed using the Student-Newman-Keuls test. The mean duration of the luteal phase and mean interestrus interval in the dogs treated with aglepristone were compared with these same variables in untreated beagle dogs in our colony using the Student’s t-test (two-tailed). Values are expressed as a range or as mean ± SEM. Values were considered to be significantly different at P ≤ 0.05.

RESULTS

Pregnancy was confirmed in all dogs at 27 to 31 d following mating. The fetuses were alive and the number ranged from 2 to 6 (mean 4.5). The bitch that was mated during 2 successive estrous cycles conceived normally with 5 fetuses the first time and 6 fetuses the second time.

Aglepristone treatment was well tolerated and no side-effects were observed except for a small amount of mucoid vaginal discharge during the week after the start of treatment. Two days after the start of the administration of aglepristone both living and dead fetuses were observed in 2 dogs, while in the remaining dogs all the fetuses were alive. One to two days later all the fetuses in 5 dogs were dead, with whole fetuses or remnants of fetuses visible in 3 of the dogs. In 1 of these 3 dogs contractions of the uterine wall were observed, with expulsion of a dead fetus from the placental site. In the remaining dogs all fetuses were still alive. Again, 3 to 4 d later no fetuses or remnants of fetuses were observed anymore.

Ovoid placental sites of the uterus were broader than the interplacental zones in all the dogs and had echogenic luminal contents with very poor distinction between wall and contents. During the next 6 to 11 d the placental sites became smaller but were still present in all the dogs 14 to 18 d following the administration of aglepristone. In one 5-yr-old bitch vaginal discharge was observed 4 wk after initiation of the aglepristone treatment. Ultrasonographic examination of the uterus at the same time revealed cystic and hyperplastic changes compatible with cystic endometrial hyperplasia (CEH).

Plasma progesterone levels did not change during treatment with aglepristone, and all pregnancies were terminated despite the presence of relatively high plasma concentrations of progesterone. However, after treatment, plasma progesterone concentrations declined to less than 1 μg/L within 8 to 34 d (mean 19 ± 4 d). Thus, the mean duration of the luteal phase in which the beagles were treated with aglepristone (52±4 d) was significantly shorter (P=0.005) than that of untreated beagle dogs (75 ± 4 d; ref. 27). The interestrus interval in which the
beagles were treated with aglépristone (155±10 d) was significantly (P<0.05) shorter than that in another group of 6 beagle bitches in our dog colony (199±15 d).

Plasma prolactin concentrations were clearly elevated within 24 h after the start of the treatment with aglépristone and returned to basal levels in 2 to 4 d (Figure 1). Mean plasma prolactin concentrations in all bitches during treatment (range: 26.4 to 47 µg/L) were significantly higher (P<0.001) than those before (range: 3.3 to 13.8 µg/L) and after (range: 2.7 to 13.2 µg/L) treatment (Figure 2). However, high plasma prolactin concentrations (>10 µg/L) were also occasionally observed before and after treatment with aglépristone.

Figure 1. Plasma concentrations of prolactin (*) and progesterone (O) in a 4-yr-old beagle bitch, starting from the day of ovulation (Day 1) to the end of the luteal phase. On Days 30 and 31, the bitch was treated subcutaneously with aglépristone at a dose of 10 mg/kg body weight, once each day.

Figure 2. The average (± SEM) of the mean plasma prolactin concentrations in 6 beagle bitches before, during and after treatment with aglépristone. Asterisks indicate significant difference.
DISCUSSION

The results of this study demonstrate that aglépristone, a new progesterone receptor antagonist, is a safe and effective abortifacient for use during mid-gestation in the bitch. All pregnancies were successfully terminated 4 to 7 d after initiation of treatment with aglépristone. Rapid and effective termination of pregnancies is consistent with the results of Fiéni et al. (14) who employed aglépristone, and of Concannon et al. (8) and Linde-Forsberg et al. (24) who used mifepristone. In humans, combined use of progesterone receptor antagonists with prostaglandins has proven to be more effective than the use of progesterone receptor antagonists alone (4). Taking into account the results of the present study and the reported high effectiveness rate of aglépristone (>95%) in terminating pregnancy during mid-gestation as well as the side-effects of prostaglandins (12,13), the combination of aglépristone and prostaglandins is not recommended in dogs.

Aglépristone can be used effectively as an alternative to estrogens to avoid unwanted pregnancy early in gestation in the bitch (14). In our study we used only bitches with confirmed pregnancy. It may be advisable to use aglépristone only after pregnancy is confirmed, since Feldman et al. (12) reported that only 40% of the bitches conceived after an unwanted mating (i.e., mating without fertility control). When mating occurs within a fertility program a pregnancy rate higher than 90% can be expected (45).

The appearance of the uterus following the disappearance of whole fetuses or of remnants of fetuses resembles that of a postpartum uterus, as described by Pharr and Post (34), and differs from that of a uterus following fetal resorption (11). This appearance together with the observation of contractions of the uterine wall with the expulsion of a dead fetus from the placentation site indicate that the administration of aglépristone leads to abortion and not to fetal resorption.

The only side-effect of aglépristone treatment observed in this study was some mucoid vaginal discharge in the first week after administration. In one 5-yr-old bitch vaginal discharge was also observed 4 wk after the start of treatment with aglépristone. In this dog cystic and hyperplastic changes compatible with CEH were detected by ultrasonography of the uterus. Considering the central role of progesterone in the pathogenesis of CEH (10,22,43) and the relatively long interval between treatment with aglépristone and ultrasonographic detection of CEH, the latter should probably not be regarded as a side-effect of aglépristone treatment. However, the possibility that aglépristone treatment might reinforce the development of an already existing mild case of CEH cannot be excluded.

One bitch was mated twice and became pregnant with 6 fetuses after having been treated with aglépristone during the previous pregnancy. Similar results have been reported in studies on RU 486 (23,24). These observations suggest that progesterone receptor antagonists do not impair future fertility, an important prerequisite for an abortifacient.

The still high plasma progesterone concentrations at the time of fetal death indicate that pregnancy was not terminated by a decline in plasma progesterone concentrations, as
suggested by Concannon and Hansel (5), but probably by a direct effect of aglépristone at the level of the uterine progesterone receptor (8,24). Following binding to the progesterone receptor, a progesterone receptor antagonist stabilizes the receptor’s structure by its high affinity and prevents progesterone from exerting its biological effect (8,44).

Termination of pregnancy with aglépristone resulted in premature cessation of luteal function. Luteolysis secondary to abortion or resorption can be related to uterine release of luteolytic amounts of prostaglandin F\(_{2\alpha}\) (8). Similar to this findings administration of prostaglandin F\(_{2\alpha}\) to induce abortion in bitches is also associated with a shortened luteal phase (36). Pregnancy termination by the progesterone receptor antagonist mifepristone (RU 486) causes increases in peripheral concentrations of PGFM, the main metabolite of prostaglandin F\(_{2\alpha}\) (24). Such an increase can also be expected to occur when aglépristone is used as an abortifacient. Studies of RU 486 in humans have suggested another possibility for the early cessation of the luteal phase. Based on the observation that there is a decrease in LH pulse amplitude, LH pulse frequency and LH secretion in humans during treatment with RU 486, it has been speculated that premature luteolysis may be an effect of the progesterone receptor antagonist at the hypothalamic-pituitary level (15,38). Detailed studies in dogs on the effect of progesterone receptor antagonists on the hypothalamic-pituitary-ovarian axis are lacking. However, the observed shortening of the interestrous interval associated with aglépristone treatment also suggests an influence on the hypothalamic-pituitary-ovarian axis. The aglépristone-induced shortening of the interestrous interval in the bitches in this study cannot be explained by the shortening of the luteal phase alone and thus may be due to shortening of anestrus. Progression from early to late anestrus in the bitch is associated with an increase in circulating FSH without a concomitant rise in basal LH concentration (21). This indicates that an increase in circulating FSH is a critical event required for the initiation of ovarian folliculogenesis and consequently for the termination of anestrus. Therefore, it may be hypothesized that the aglépristone-induced shortening of the interestrous interval is directly or indirectly the result of enhanced pituitary FSH release.

There are also other indications that aglépristone has central effects in the bitch. Treatment with aglépristone resulted in a pronounced increase in plasma prolactin concentrations within 24 h. Elevated plasma prolactin concentrations have also been reported in bitches with overt pseudopregnancy (28). A rapid decline in the plasma concentration of progesterone may be a precipitating factor in the pathogenesis of pseudopregnancy (16,17). Correspondingly, ovariection performed in the luteal phase of the estrous cycle often induces overt pseudopregnancy. The rapid decline in plasma concentrations of progesterone is assumed to cause prolactin release, which in turn would give rise to pseudopregnancy. In addition, at the time of parturition plasma prolactin levels in the bitch rise in response to the sharp decline in the plasma progesterone concentration (6). Occupation of the central progesterone receptors by the antagonist aglépristone may mimic a sudden decline in progesterone concentration and consequently result in increased prolactin release. According to our findings, progesterone receptor antagonist-induced elevation of plasma prolactin values cannot be explained by intrauterine fetal death, as proposed by Linde-Forsberg et al. (24), because fetal death occurred at least 2 d after the rise in plasma prolactin concentration.
High plasma prolactin concentrations were also observed occasionally before and after treatment with aglépristone. Studies in which plasma prolactin concentration was measured daily have also demonstrated such peak values (6,27,33). This might indicate pulsatile secretion of prolactin in the bitch, as has been found for several other pituitary hormones (3,20,21). However, studies to determine whether the secretion of prolactin is pulsatile in the bitch have not yet been performed.

In conclusion, aglépristone proved to be a safe, reliable and effective abortifacient during mid-gestation in the bitch. Aglépristone treatment was associated with a shortening of the luteal phase and the interstrous interval, and resulted in temporarily highly elevated plasma prolactin levels.

REFERENCES

Safety and efficacy of mid-term pregnancy termination using aglepristone in dogs

OBJECTIVES: To investigate effects and side effects of aglepristone in terminating pregnancy in bitches.

METHODS: Twenty-two bitches were treated in mid-pregnancy with subcutaneous injections of aglepristone at a total dose of 20 mg/kg. Short-term follow-up (one to two weeks after treatment) included clinical examination and abdominal ultrasonography in 18 of the dogs. Long-term telephone follow-up was recorded for all 22 dogs.

RESULTS: Pregnancy was terminated in 21 bitches (95 per cent). Signs of abortion occurred one to eight days after treatment. Vaginal discharge was evident in 17 (77 per cent) dogs. Obvious signs of parturition were seen in nine (41 per cent) dogs. Eight dogs (36 per cent) developed anorexia, and in two (9 per cent) of the dogs a local reaction at the injection site was evident. Two dogs developed pyometra two and four years after treatment, respectively.

CLINICAL SIGNIFICANCE: Aglepristone, when administered in mid-gestation, is effective in terminating pregnancy. Side effects are few and transient.

The purpose of this study was to investigate the effects and side effects of the progesterone receptor antagonist aglepristone in bitches in mid-pregnancy.

MATERIALS AND METHODS

Medical reports at Albano Animal Hospital in Stockholm, Sweden from June 2002 to June 2006 were examined. Abdominal ultrasound was performed on 82 bitches between day 23 and day 42 (median 30 days) after suspected mismating using a 5 to 12 MHz transducer (HDXE11; Philips).

Twenty-eight (34 per cent) dogs were judged as pregnant on ultrasonographic examination. Nineteen (51 per cent) of a total of 37 dogs, in which mating had been observed, were pregnant. Twenty-two bitches were treated with the injectable oil-based solution aglepristone (Alizin; Virbac).

About 20 mg/kg aglepristone was the total dose. In 19 of the 22 dogs, the dose was divided and given on two consecutive days according to the label. Three of the dogs were given the total dose on a single occasion. A 1-2 × 40 mm (18G × 1/2 inch) needle was used. The subcutaneous injection site in the neck was massaged immediately after the injection. Doses exceeding 10 ml were divided between two different injection sites.

A follow-up examination was performed, in 18 of the 22 bitches, one to two weeks after treatment with aglepristone. Three owners were interviewed by telephone at this time and one owner was interviewed one month after treatment. The examination included inspection of the injection site and the vulva for any visible vulvar discharge. The uterus was examined by ultrasound, and diameter of uterine horns measured. All owners completed a questionnaire, including questions on the presence of vulvar discharge, loss of appetite, vomiting, diarrhoea or other abnormalities, and the timing and duration of these signs.
All 22 owners were interviewed by telephone during October 2007 (1-3 to 5-3 years after treatment) using a questionnaire that included the subsequent questions (1) the present health of the bitch, (2) the timing of first oestrus after aglepristone treatment and intervals between the following oestrus cycles, (3) whether the bitch had undergone ovariohysterectomy, the reason for this and the timing after treatment, (4) whether the bitch had been successfully bred after the aglepristone treatment and (5) whether or not the owner was satisfied with the treatment of mismating.

**Statistical methods**

Statistical calculations were performed by use of a computerised statistical program (JMP 3.2, SAS Institute Inc.). Data are presented as median and range.

**RESULTS**

The 22 dogs ranged in age from nine to 101 months (median 43 months). The dogs weighed between 3 and 47 kg (median 12.8 kg). Six (27 per cent) of the dogs were mixed breeds, two (9 per cent) were papolons, two were American cocker spaniels and two were cavalier King Charles spaniels. There was one (5 per cent) of each of the following breeds: shih-tzu, Groenendael, Löwchen, Tibetan spaniel, Irish red and white setter, Terveuren, Estrela mountain dog, Shetland sheepdog, Slovensky kopov and rottweiler. Pregnancy was terminated in 21 bitches (95 per cent). Seventeen of the 22 dogs (77 per cent) were treated between day 28 and day 30. In two of these dogs, the owners detected parts of foetuses in the discharge. Three of the remaining dogs were treated between day 23 and day 26, and in these dogs no vaginal discharge was detected. An abortion with noticeable foetuses occurred in two dogs treated on day 41 and day 42 after mating, respectively.

Eighteen of the 22 treated dogs were evaluated by ultrasound seven to 16 days after treatment. The size of the remnants of the embryonic cavities varied from undetectable to 2.2 cm in diameter (Table 1). Viable foetuses were seen in one (5 per cent) dog. Vaginal discharge was observed in 17 of the 22 (77 per cent) dogs. In 16 of these dogs, the discharge was detected from one to two days after treatment. In one dog, the discharge was minimal, brownish and evident six to 11 days after treatment. In most of the dogs, the discharge was still visible at the time of the first follow-up examination. Signs of parturition, such as shivering, panting or nesting behaviour, were seen in nine of the 22 (41 per cent) dogs. These clinical signs were detected over one to two days after treatment, except in one dog where the signs lasted for seven days (from day 2). The dog, in which the treatment failed showed signs of parturition but had no visible vaginal discharge.

Eight (36 per cent) of the dogs were anorexic for several days (four to 10 days). One of the dogs lost 3 kg (10 per cent of its bodyweight) within 14 days after treatment. One dog (5 per cent) had polydipsia for five days from one day after treatment. Two dogs (9 per cent) developed a local interoestrus interval, three (15 per cent) had a prolonged interval and two (10 per cent) a shortened oestrus interval the first oestrus after the treatment, according to their owners. In the remaining five dogs, fever, vaginal discharge and an enlarged uterus (1 to 2 cm in diameter). Although no sample of the vaginal discharge was submitted for bacterial culture, this dog was prescribed enrofloxacin (Baytril Vet; Bayer Healthcare AB) at a dose of 5 mg/kg bodyweight once daily orally for 10 days. At re-examination six days later, the body temperature had normalised and the diameter of the uterus measured less than 1 cm. The bitch was considered clinically healthy.

Long-term follow-up was performed, between 1-3 and 5-3 years after treatment, by telephone using a questionnaire. One (5 per cent) of the dogs was euthanased six months after treatment because of pre-existing epilepsy. The remaining 21 (95 per cent) dogs were considered healthy. One of the 22 dogs underwent ovariohysterectomy because of treatment failure. Seventeen of the remaining 20 (85 per cent) bitches (including the bitch that was treated because of a suspected endometritis) were, according to the owners, still cycling with normal oestrus at the time of follow-up. Ten (50 per cent) of these bitches had a normal interoestrus interval, three (15 per cent) a prolonged interval and two (10 per cent) a shortened oestrus interval the first oestrus after the treatment, according to their owners. In the remaining five dogs,
no information was available concerning
time of first oestrus cycle after treatment.
Two bitches developed pyometra and
underwent ovariohysterectomy, two and
four years after treatment, at nine and seven
years old, respectively. In one dog, the
owner had elected for ovariohysterectomy
because of the presence of a male dog in the
household. In conclusion, four of 22
(18 per cent) bitches underwent ovario-
hysterectomy, one because of treatment
failure, one because of having a male
dog in the family and two because of de-
veloping pyometra. Four of 20 (20 per cent)
dogs, excluding the dog that was eutha-
nased because of epilepsy and the dog
ovariohysterectomised because of treat-
ment failure, were bred after the treatment
with aglepristone. Three bitches conceived
when bred and had normal litters. One of
them was bred twice, one and two years
after treatment and had two normal litters.
A second dog gave birth to one normal lit-
ter one year after treatment. The third dog
had two litters (two and three years after
treatment). One dog did not conceive
when bred at the first oestrus after treat-
ment, but later had two normal litters
(one and three years after treatment).

The owners of all the bitches in which
the treatment was successful said that they
were pleased with the method used to ter-
minate pregnancy.

DISCUSSION

In the present retrospective study, aglepris-
tone was shown to be effective at terminat-
ing pregnancy in mid-gestation in dogs
with few and reversible side effects. Agle-
pristone, as is the case with some other
agents to terminate or prevent pregnancy
in dogs (51 per cent) in which mating had
been confirmed in untreated bitches. Prostaglandins have
effects on many different organs and have
also been used to induce abortion. Their
efficacy as an abortive drug results from
induction of uterine contractions and
luteolysis (Feldman and others 1993).
However, prostaglandins also have many
side effects such as hyperventilation, vom-
iting, defecation, urination and pad swear-
ing. Natural prostaglandins must be given
two or three times daily for four to seven
days. Synthetic prostaglandins have fewer
side effects and a longer duration of action,
but increase the risk of death because of
inadvertent overdose. Hospitalisation is
advised for the duration of treatment on
account of side effects and the necessity
of frequent injections (Fieni and others
1989, Fieni and others 1997).

The corpus luteum in the bitch is depen-
dent on prolactin to maintain progesterone
levels during late pregnancy. Treatment
with dopamine agonists that exert antipro-
lactinergic effect via stimulation of D2
pituitary receptors or inhibition of central
serotoninergic receptors from day 35 to 40
can result in abortion (Concannon and
others 1987, Post and others 1988, Onclin
and others 1993). There are also other
methods of terminating pregnancy such
as combining prostaglandins and dopa-
mine agonists, studies of which have shown
good results and fewer side effects (Onclin
and others 1993, Onclin and others 1995,
Onclin and Verstegen 1996, Gobello and
others 2002). Dexamethasone has also
been used to terminate pregnancy in
bitches (Zone and others 1995, Wanke
and others 1997). However, the use of corticosteroids to induce abortion is
unsuitable, as high doses are required for
several days resulting in pregnancy termi-
nation from around day 40.

It is interesting to note that only 19 of 37
dogs (51 per cent) in which mating had
been observed were pregnant. This indi-
cates that early use of pharmaceutical
agents to terminate or prevent pregnancy
may be unwarranted in many cases. Own-
ers should be informed of these facts and
have the option of choosing what treatment
may be most suitable for their bitch.

In accordance with this study, other
studies reported effective abortion in at
least 95 per cent of bitches treated in
mid-pregnancy (Galac and others 2000,
Fieni and others 2001). It has been recom-

mended that the total dose of aglepristone
be divided into two injections given on
consecutive days. Three of the 22 dogs
were given the total dose as a single injection
because of problems with owner compli-
ance. The only bitch which did not abort,
was one of these three dogs. It is possible
that the use of a single dose caused treat-
ment failure in this case.

Side effects previously reported for agle-
pristone included local pain and occasional
swelling at the injection site, vaginal dis-
charge, discomfort, depression, anorexia
and a shortened inter-oestrus interval at
first oestrus after the treatment (Galac
and others 2000, Hubler and Arnold
2000). In the present study, local reactions
were seen in two (9 per cent) of 22 dogs.
Behavioural changes, such as discomfort
and depression, are expected in conjunc-
tion with parturition. Anorexia was seen
in eight (36 per cent) dogs. This has also
been observed in other studies when the

treatment was initiated in early pregnancy
(Hubler and Arnold 2000, Galac
and others 2004).

The size of the uterus after treatment dif-
fered between dogs treated at the same stage
of gestation (Table 1) and did not correlate
with bodyweight. The differences might be
because of the fact that the examination was
performed on different days (days 6–15)
after treatment and by different veterinary
surgeons.

In conclusion, aglepristone is an effec-
tive drug for pregnancy termination, when
used in mid-pregnancy, and is reasonably
well tolerated by dogs. Although treatment
with aglepristone does not seem to nega-
tively influence fertility and does not gen-
erally seem to generate long-term uterine
or oestrus abnormalities, more work is
Aglepristone and mid-term pregnancy

required to specifically examine whether aglepristone treatment influences fertility.

References


Induction of abortion with aglepristone significantly changed the expression of progesterone and estrogen receptors in canine endometrial stromal cells

H. Kanca a,*, I. Walter b, S. Schäfer-Somi c, S. Budik c, S.S. Ay d, I. Kucukaslan a, A.R. Agaoglu a, H. Izgur a, S. Aslan a

a Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, University of Ankara, 06110 Ankara, Turkey
b Institute for Histology and Embryology, University of Veterinary Science, Veterinärplatz 1, 1210 Vienna, Austria
c EU Centre for Artificial Insemination and Embryo Transfer, University of Veterinary Science, Veterinärplatz 1, 1210 Vienna, Austria
d Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, University of Ondokuz Mayis, Samsun, Turkey

Received 19 December 2007; received in revised form 23 April 2008; accepted 24 April 2008

Abstract

In the present study, resorption/abortion was induced between days 25 and 45 of gestation with aglepristone (group IRA, n = 10). The aim was to observe the change in the distribution of progesterone (PR) and estrogen receptors (ER), in comparison to a group of spontaneous resorptions/abortions (group SRA, n = 5), and a further group of normal healthy pregnant animals, ovariohysterectomized between days 25 and 45 of gestation (controls, n = 7). The receptors were assessed by means of immunohistochemistry (IHC) and RT-PCR, at the placental and interplacental sites of the uterine horn as well as in the corpus uteri. Significant differences were observed between the controls on one side and the groups of resorption/abortion on the other side. The total scores of the progesterone receptors (TPR) in the placental and interplacental part of the uterine horn, was significantly lower in the endometrial stromal cells (ESC) of the control group than in those of the SRA- and IRA-group, respectively (placenta: 5.8 vs. 6.5 and 6.7, $p < 0.01$; interplacental sites: 5.6 vs. 6.6 and 6.6, $p < 0.05$). In contrary, the total scores of the estrogen receptors (TER) at interplacental sites and the corpus uteri, respectively, was significantly higher in the myometrial smooth muscle cells (MSMC) and the ESC ($p < 0.05$) of the controls. We therefore conclude, that the here observed differences between groups point to an up-regulation of TPR- and a down-regulation of TER-scores in endometrial stromal cells at different uterine sites during resorption/abortion, which indicates a special role of these cells.

© 2008 Published by Elsevier Inc.

Keywords: Aglepristone; Bitch; Endometrium; Estrogen receptor; Pregnancy termination; Progesterone receptor

1. Introduction

In the bitch, several medication schemes for the induction of abortion have been investigated, however, information about the abortion inducing mechanisms are widely lacking, especially after application of the antiprogesterone aglepristone. When compared to other medication schemes using dexamethasone [1], prostaglandins [2–6] or combinations of prostaglandins with dopamine agonists [7–9], antigestagens proved to be comparably reliable at all stages of pregnancy and had virtually no side effects [10–16]. Antigestagens are synthetic steroids that bind with strong affinity to
progesterone receptors and competitively displace endogenous progesterone [17]. In dogs, two antiprogestins have been studied, namely mifepristone (RU 468) [11,13] and aglepristone (RU 534), but only aglepristone is available for veterinary use and meanwhile has been tested during numerous scientific studies. The early administration of aglepristone between days 0 and 25 after mating (\(n = 5\)) always prevented pregnancy. Administration of aglepristone between days 26 and 45 after mating (\(n = 5\)) induced resorption or abortion within 7 days in 96% of cases without side effects [15]. During an own study, aglepristone was administered at day 30 after mating which induced abortion in all of cases (5/5) within 10.6 days. After this study we concluded that the use of aglepristone is a safe method for the induction of abortion, however, the termination of abortion can be delayed when application started after the end of placentation [18].

It has been supposed, that aglepristone induces abortion mainly via its direct effect on the uterus [14], after competitively displacing P4 and suppressing its biological effect [19]. Several studies have been performed, to investigate the presence and distribution of estrogen receptors (ER) and PR during physiological canine estrous cycle and pregnancy [20–22]. However, there are no publications available in the relevant literature about the change in the number and distribution of PR and ER after application of aglepristone, in the course of resorption and abortion, respectively. Thus, the aim of the present study was to describe and compare the distribution of uterine progesterone and estrogen receptors in aglepristone-induced and spontaneous pregnancy termination in bitches.

2. Materials and methods

2.1. Animals

A total of 22 animals from different breeds (Doberman, Husky, Terrier, Labrador Retriever, Golden Retriever, Beagle, German Shepherd, Anatolian Shepherd, Irish Setter and Bastard), aged 3.6 ± 2.6 years, was included into this study. Most bitches (\(n = 17\)) were introduced to the Clinic of Obstetrics and Gynecology, Faculty of Veterinary Medicine, University Ankara (Tr) because of mismating. In 10 out of 17, abortion was induced between days 25 and 45 of gestation (group of induced spontaneous resorption/abortion = IRA), the other seven bitches served as controls. The controls were healthy pregnant animals and ovariohysterectomized between 25 and 45 on the request of owners. These animals had no history of abortion or resorption. Additionally, bitches in IRA group were divided into two subgroups (Group I, \(n = 5\), days 25–35 of pregnancy; Group II, \(n = 5\), days 36–45 of pregnancy). Gestational age was assessed sonographically according to Luvoni and Grioni [23]. Five further bitches were introduced during the same stage of gestation because of beginning spontaneous resorption and abortion, respectively (group of spontaneous resorption/abortion = SRA; see Table 1). A detailed history was taken from all bitches, then clinical and ultrasonographical examinations of the uteri revealed no specific infectious or non-infectious causes.

2.2. Induction and observation of resorption/abortions

In the IRA groups, abortion was induced by application of 10 mg/kg body weight of the antiprogestrone aglepristone (Alizine, Virbac, F) s.c. at 2 subsequent days. The start of abortion was diagnosed when bloody vaginal discharge was seen and ultrasonography revealed characteristic signs of beginning resorption/abortion. Then clinical examinations and ultrasonography [24] were performed daily until the end of abortion, when no more fetuses were detected sonographically (6.0–8.0 MHz; linear, Pie-Medical, Falco 100).

<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>Day of gestation</th>
<th>Medication</th>
<th>Time of OHE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induction of resorption/abortion (IRA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>5</td>
<td>25–35</td>
<td>Aglepristone s.c.</td>
<td>Immediately after resorption/abortion</td>
</tr>
<tr>
<td>Group II</td>
<td>5</td>
<td>36–45</td>
<td>Aglepristone s.c.</td>
<td>Immediately after resorption/abortion</td>
</tr>
<tr>
<td>Spontaneous resorption/abortion (SRA)</td>
<td>5</td>
<td>25–45</td>
<td>–</td>
<td>Immediately after resorption/abortion</td>
</tr>
<tr>
<td>Control group</td>
<td>7</td>
<td>25–45</td>
<td>–</td>
<td>During gestation</td>
</tr>
</tbody>
</table>

IRA: induced resorption/abortion; SRA: spontaneous resorption/abortion; OHE: ovariohysterectomy.

Table 1
Overview over the experimental and control groups
2.3. Operations and sampling

Animals were ovariohysterectomized under general anesthesia. Immediately after extirpation of the organs, tissue samples were cut off from different sites of the uterus: placental sites, interplacental sites and corpus uteri. One half of the samples was covered with Tissue Tek™, then stored in liquid nitrogen at −196 °C for later RNA isolation. The other half was fixed in a 4% formalin solution, then embedded in paraffin and histosections prepared. In all groups, before the operation, blood samples were taken once, serum produced and stored at −18 °C until analyses for estradiol and progesterone concentrations.

2.4. Immunohistochemistry (IHC)

For the immunohistochemical examinations, the following antibodies were used: Anti-ER (1:200 Rabbit monoclonal, Zymed Laboratories, South San Fransisco, USA); Anti-PR (1:50 Rabbit monoclonal SP2, NeoMarkers, Fremont, CA, USA) and staining prepared as follows: serial paraffin sections were cut at 3 μm thickness and placed onto APES (3% 3-aminopropyl-triethoxysilane, Sigma A3648, dissolved in 100% acetone) coated slides. After deparaffinizing in xylene and rehydration, endogenous peroxidase activity was inhibited by 0.6% methanol/perhydrol for 15 min. Microwave pretreatment (3–5 min in 0.1 M EDTA solution, pH 8.0) was performed in the aim of antigen retrieval. All following incubation steps were performed in an immunostaining center (Sequenza; Shandon, Pittsburgh, PA, USA). After rinsing with PBS, the sections were incubated with normal goat serum (1.5%) for 30 min at room temperature, to prevent nonspecific binding reactions. Subsequently, primary antibodies were used by incubating overnight at 4 °C. A secondary antibody with a horseradish peroxidase (HRP)-conjugated dextran polymer to enhance the signal was used (PowerVision Poly-HRP anti-rabbit IgG; Immunovision Technologies, Daly City, CA, USA). Finally, sections were washed with PBS and peroxidase activity detected with DAB substratum (10 mg 3,3′-diaminobenzidine in 50 ml 0.1 M Tris buffer, pH 7.4) and 0.03% (v/v) H2O2 for 10 min at room temperature. After that, sections were counterstained with hemalum, dehydrated and mounted with DPX (Fluka, Buchs, Switzerland). Negative controls included the replacement of the primary antibody by PBS and an IgG control by using unspecific rabbit serum instead of the primary antibody.

The expression of progesterone (PR) and estrogen receptors (ER) in uterine tissue was evaluated in a light microscope at 400× magnification according to Vermeirsch et al. [21,22]. For the assessment of the total and proportional scores (PR and ER), 100 positive cells per slide were evaluated and grouped into an intensity and proportional score. For the PR, the method after Vermeirsch et al. [22] was used (intensity score: 0 = no staining, 1 = weak, 2 = moderate, 3 = strong, 4 = extremely strong staining; proportional score: 0 = no positive nuclei, 1 = <1%, 2 = 1–9%, 3 = 10–32%, 4 = 33–65%, 5 = >65% positive nuclei). For the ER, the method according to Vermeirsch et al. [21] was used (intensity score: 0 = no staining, 1 = weak, 2 = moderate, 3 = strong staining; proportional score: equal). The intensity score indicates the subjective intensity of the staining, the proportional score the percentage of positively stained cells. Both scores were taken together and a total score calculated. Prior to the cell count, the thickness of the endometrium and myometrium was measured in four different microscopic fields in each histological section using the computer image analyzing program (Eclipse; Nikon, Austria). The samples were obtained from placental and interplacental sites of the uterus and the corpus uteri.

2.5. Polymerase chain reaction (RT-PCR)

For qualitative RT-PCR, pieces of ovaries and uteri (placental sites, interplacental sites and corpus uteri) were homogenized using TRI Reagent (T 9424, Sigma–Aldrich, A) and subsequently treated as recommended by the manufacturer. With TRI Reagent, it is possible to isolate mRNA, protein and DNA of any parameter chosen.

For the detection of PR-genes, primers were designed using the sequences available from the Nucleotide database (PR: sense 5′ CAG GTG TAC CAG CCG TAC CT 3′, antisense 5′ ATT TCG AAA ACC TGG CAATG 3′, 618 bp, accession no. AF177470). The products were visualized after separation by ethidium bromide containing agarose gels using UV-light. The bands of the expected size were cut out of the agarose gel and sent to the sequencing service (IBL, Vienna, A) using one of the primers used for amplification for the sequencing procedure. The sequence obtained was compared with the databases using BLAST analysis. The mastermix served as negative control.

2.6. RNA quality control

In each sample, the quality of the isolated RNA was assessed via measurement of the RNA integrity number (RIN). The RIN values were measured with the Agilent
2100 bioanalyzer on microfabricated RNA Lab Chip kits (Agilent technologies GmbH, Vienna, A). Among others, the bioanalyzer software generates an electropherogram which provides a detailed picture of the size distribution of the RNA fragments and allows thus a visual assessment of the quality of an RNA sample.

2.7. Statistical analysis

Data are given as means ± standard deviations (X ± S.D.). All statistical comparisons were performed using the SPSS software® (Version 11 for Windows, SPSS Inc., Chicago, IL, USA). Comparisons of two mean values within one group were performed using the Wilcoxon test (respectively, Fisher exact test) and comparisons between groups with the Mann–Whitney U-test. Comparisons of more than two independent groups were performed using the Kruskal–Wallis Test. A p-value of <0.05 was considered statistically significant. Since the total score contains the subjectively assessed intensity score, each calculation was repeated using the number of positively stained cells solely and the results compared.

3. Results

3.1. Histological findings

After spontaneous and induced abortions, in the glandular chambers of the endometrium and the placenta, accumulations of secretions within the glandular chambers and degenerative alterations of the tissue were detected (Fig. 1).

Measurement of the uterine tissue revealed, that the endometrium in the IRA group was 1260.9 ± 165.8 μm, which was significantly thicker than in the control group (873.5 ± 114.8 μm; p < 0.05). In the SRA group, the endometrium was also thicker than in the controls, however, the difference was non significant (SRA: 1129.6 ± 515.7 μm).

3.2. Progesterone receptor

Comparison of total scores with the number of positively stained cells revealed no statistical significant differences. Therefore, in the present study, results are expressed using the total score.

In the placental part of the uterine horns, brown nuclear staining demonstrating the presence of PR was detected in myometrial smooth muscle cells (MSMC), endometrial stroma cells (ESC), endometrial glandular epithelia (EGE) and glandular chamber epithelial cells (GCE). However, in the blood vessels, no PR were detected (Fig. 2A–C).

In all groups (IRA, SRA and controls), the average total scores of the progesterone receptors (TPR) in the ESC and the MSMC were significantly lower than those in the EGE and the GCE (Table 2; p < 0.05). In addition, the TPR in ESC was significantly lower in the control group than in the SRA- and IRA-group, respectively (5.8 vs. 6.5 and 6.7; p < 0.01; Fig. 3).

At the interplacental sites of the uterine horn and the corpus uteri, the distribution of PR resembled the placentation sites. In the control group, the average TPR was significantly lower in ESC (5.6) than in all other cell types (SRA: 6.6; IRA: 6.6; p < 0.05), with the lowest value in the corpus uteri (control: 5.3, IRA: 6.3, SRA: 6.6; p < 0.01). In the control and IRA group, the average TPR were highest in epithelial cells (EGE, ESE, GCE; see Table 2).

Groups I and II (IRA), in which pregnancy was terminated on different days, did not differ significantly concerning any cell population in any part of the uterus, except the MSMC in the interplacental part of the uterus (Group I: 7.0; Group II: 6.5).
3.3. Estrogen receptors

Like the PR, the estrogen receptors (ER) were detectable in the placental (Fig. 4A–C) and interplacental part of the uterine horn and in the glandular chambers. Positive immune reactions for ER were detected in all cell types investigated (MSMC, ESC, EGE, GCE).

In the placental part of the uterine horn and in all groups, the average score for TER was significantly lower in MSMC and ESC than in EGE and GCE (p < 0.05 and p < 0.01, see Table 3). Comparison between groups revealed no significant differences concerning any cell population (p > 0.05).

Similarly, at the interplacental part, the TER was significantly lower in MSMC and ESC (5.7 and 3.8) than in EGE- and ESE-cells (6.3 and 6.4; p < 0.05 and p < 0.01), in all groups investigated (Table 3). However, in MSMC (5.7) and ESC (3.8 and 5.0) of the SRA-group, the TER was significantly lower than in MSMC and ESC of the controls (6.2 and 6.0, p < 0.05, Fig. 5), whereas no difference between groups was detectable concerning the epithelial cells.

Table 2
Total scores of the progesterone receptor (TPR) in (a) placental and (b) interplacental part of the uterine horn—all groups

<table>
<thead>
<tr>
<th>Group</th>
<th>MSMC</th>
<th>EGE</th>
<th>GCE</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRA (n = 5)</td>
<td>6.7 ± 0.2a (6.2–6.9)</td>
<td>7.2 ± 0.1b (7.0–7.4)</td>
<td>7.0 ± 0.1b (6.9–7.5)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IRA (n = 10)</td>
<td>6.8 ± 0.4a (6.0–7.6)</td>
<td>7.2 ± 0.2a,c (6.9–7.8)</td>
<td>7.1 ± 0.2c (6.8–7.5)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Controls (n = 7)</td>
<td>6.8 ± 0.3a (6.1–7.1)</td>
<td>7.1 ± 0.1a,c (6.9–7.2)</td>
<td>7.1 ± 0.0c (7.0–7.2)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRA (n = 5)</td>
<td>6.6 ± 0.3a (5.9–6.8)</td>
<td>7.1 ± 0.1b (6.8–7.4)</td>
<td>7.0 ± 0.1b,c (6.9–7.2)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IRA (n = 10)</td>
<td>6.7 ± 0.4a (6.0–7.1)</td>
<td>7.1 ± 0.2b (6.7–7.4)</td>
<td>7.1 ± 0.0b (7.0–7.6)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Controls (n = 7)</td>
<td>6.8 ± 0.3a (6.1–7.0)</td>
<td>7.1 ± 0.1a (6.9–7.2)</td>
<td>7.1 ± 0.1a (6.8–7.2)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

MSMC: myometrial smooth muscle cells; EGE: endometrial glandular epithelia; GCE: glandular chamber epithelial cells; ESE: endometrial surface epithelia; SRA: spontaneous resorption/abortion; IRA: induced resorption/abortion; controls: normal pregnant bitches. Values with different indices (a–c) within one line differ significantly (p < 0.05).

3.3. Estrogen receptors

Like the PR, the estrogen receptors (ER) were detectable in the placental (Fig. 4A–C) and interplacental part of the uterine horn and in the glandular chambers. Positive immune reactions for ER were detected in all cell types investigated (MSMC, ESC, EGE, GCE).

In the placental part of the uterine horn and in all groups, the average score for TER was significantly lower in MSMC and ESC than in EGE and GCE (p < 0.05 and p < 0.01, see Table 3). Comparison between groups revealed no significant differences concerning any cell population (p > 0.05).

Similarly, at the interplacental part, the TER was significantly lower in MSMC and ESC (5.7 and 3.8) than in EGE- and ESE-cells (6.3 and 6.4; p < 0.05 and p < 0.01), in all groups investigated (Table 3). However, in MSMC (5.7) and ESC (3.8 and 5.0) of the SRA-group, the TER was significantly lower than in MSMC and ESC of the controls (6.2 and 6.0, p < 0.05, Fig. 5), whereas no difference between groups was detectable concerning the epithelial cells.
In the corpus uteri, like in the interplacental part of the uterine horn, the TER for MSMC and ESC (4.9 and 4.1, SRA; 5.0 and 4.3, IRA; \( p < 0.01 \)) were significantly lower than for EGE- and ESE (5.9 and 5.4, SRA, \( p < 0.05 \); 6.1 and 6.1, IRA, \( p < 0.01 \)). However, in the control group, significantly higher TER for MSMC and ESC were detectable than in all other groups (MSMC: 5.6, ESC: 5.2; \( p < 0.05 \)).

3.4. RT-PCR (progesterone receptors)

Quality assessment of the samples of the IRA and SRA group revealed very low RIN values, with an average of 2.25/C60.72 (0–2.6). In the group of induced abortions (IRA), no mRNA for PR was found by means of RT-PCR, neither in the placental and interplacental part of the uterine horn, nor in the corpus uteri. Only in the group of spontaneous abortions (SRA), in one bitch there was still PR-mRNA detectable. In the control group, mRNA for PR was assessed in all bitches and at all sites investigated. The RIN value of the mixed sample from the control group was 10.

3.5. Serum concentrations of progesterone and estradiol-17\( \beta \)

In normal pregnant bitches, who had been ovariohysterectomized between days 25 and 45 of gestation, the average P4 value was 78.8 nmol/l, which was significantly higher compared to SRA and IRA (13.5 and 24.3 nmol/l, respectively, \( p < 0.01 \) each). In the controls, the average serum E2 concentration was 18.6 pg/ml; in the abortion groups, the respective values were 24.8 (SRA) and 33.4 pg/ml (IRA, \( p > 0.05 \)).

![Fig. 4. Immunohistochemical demonstration of ER in the normal placenta (A) and after induced (B) and spontaneous (C) abortion. MM: myometrium; UG: uterine glands. Scale bar = 50 \( \mu \)m.](image)

<table>
<thead>
<tr>
<th>Table 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total scores of the estrogen receptor (TER) in (a) placental and (b) interplacental part of the uterine horn</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>MSMC</th>
<th>ESC</th>
<th>EGE</th>
<th>GCE</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRA (( n = 5 ))</td>
<td>5.7 ± 0.7a* (5.1–6.5)</td>
<td>4.8 ± 0.6a,b* (4.0–5.3)</td>
<td>6.5 ± 0.0a,c* (6.5–6.5)</td>
<td>6.6 ± 0.4c* (5.7–6.8)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IRA (( n = 10 ))</td>
<td>5.8 ± 0.6a* (4.5–6.6)</td>
<td>5.0 ± 0.5b* (4.1–5.6)</td>
<td>6.5 ± 0.2c* (6.0–7.0)</td>
<td>6.6 ± 0.4c* (5.8–6.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Controls (( n = 7 ))</td>
<td>5.9 ± 0.6a* (5.0–6.6)</td>
<td>5.3 ± 0.5a,b* (4.5–6.2)</td>
<td>6.4 ± 0.1a,c* (6.3–6.6)</td>
<td>6.7 ± 0.1c* (6.5–6.9)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>( p )</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRA (( n = 5 ))</td>
<td>5.7 ± 0.7a* (5.1–6.5)</td>
<td>3.8 ± 1.1a,b* (2.4–5.1)</td>
<td>6.3 ± 0.4a,c* (5.6–6.6)</td>
<td>6.4 ± 0.5c* (5.8–6.8)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IRA (( n = 10 ))</td>
<td>5.7 ± 0.4a* (5.1–6.3)</td>
<td>5.0 ± 0.3b** (4.4–5.4)</td>
<td>6.5 ± 0.1c* (6.4–6.8)</td>
<td>6.6 ± 0.3c* (5.8–6.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Controls (( n = 7 ))</td>
<td>6.2 ± 0.3a** (5.6–6.5)</td>
<td>6.0 ± 0.4a,b** (5.6–6.5)</td>
<td>6.2 ± 0.1a,c* (6.0–6.4)</td>
<td>6.5 ± 0.3c* (5.8–6.7)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>( p )</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

MSMC: myometrial smooth muscle cells; ESC: endometrial stroma cells; EGE: endometrial glandular epithelia; GCE: glandular chamber epithelial cells; ESE: endometrial surface epithelia; SRA: spontaneous resorption-abortion; IRA: induced resorption-abortion; controls: normal pregnant bitches.

Values with different indices (a–c) within one line differ significantly and highly significantly, respectively (\( p < 0.05 \) and \( p < 0.01 \)).

*,**Values with different indices within one row differ significantly (\( p < 0.05 \)).
4. Discussion

4.1. Embryonic/fetal death

Maternal endocrine abnormalities, immune factors, genetic factors, environmental factors, and nutritional factors have to be considered as causes for embryonic/fetal death in dogs [25]. Whether the fetus is resorbed or aborted depends on the cause of pregnancy loss, stage of gestation, and maternal and fetal responses [26]. Spontaneous, non-infectious resorptions can be caused by large sized puppies [26] or uneven distributed puppies in the uterine horns [24]. In addition, inhomogeneous distribution of estrogen receptors (ER) in the uterine tissue has been described as a possible cause. In these cases, nidation and placentation can take place; however, the number of receptors might not be sufficient for the maintenance of pregnancy [27,28]. In the present study, the bitches were introduced to the clinic immediately after abortion, information about size and distribution of puppies is therefore lacking. After both induced and spontaneous resorption/abortions we found accumulations of secretions and degenerative alterations in the glandular chambers of the endometrium and the placenta; these morphological changes might have contributed to embryonic/fetal death [29].

4.2. Regulation of receptor expression in endometrium and placenta

In the present study, the total progesterone receptors (TPR) and total estrogen receptors (TER) were assessed according to the method of Vermeirsch et al. [21,22]. These authors used a score, calculated of cell number and subjectively assessed staining intensity. Since results might differ considerably between investigators, we additionally evaluated the cell number without staining intensity and found no significant difference between the two methods. Our results, presented as scores, are thus reliable.

In accordance to Galabova-Kovacs et al. [30] and Vermeirsch et al. [22] we detected PR and ER in the luminal and glandular epithelium, in stromal cells, and in the myometrial smooth muscle cells, as reported in other species [31–33]. Biochemical [34,35] and immunohistochemical [21,22,36] studies in the dog revealed cyclic changes in the PR and ER expression throughout the estrous cycle. Generally, during the estrus cycle, stromal cells were found to have higher staining scores for PR than epithelial cells, whereas stromal cells were found to show more fluctuations in the ER expression [21,22]. Regulation of cyclic endometrial changes is believed to be mediated by steroid hormones, namely the estradiol-17ß: progesterone ratio, and their effects on stromal cells. During the cycle, staining scores for PR and ER in the uterine horns were found to be high during proestrus, and expression of both receptors decreased through estrus to early metestrus. During late metestrus and anestrus, while serum P4 concentrations already decreased or were basal, staining scores for ER and PR in the stromal and smooth muscle cells increased again [21,22]. In humans, ER and PR expression in the endometrium changed similarly during the menstrual cycle [42,43]. The relative intense expression of PR in stroma in the late secretory phase indicates its role for the support of decidualization during early pregnancy [43].

In another study, in normal pregnant bitches, as during the cycle, stromal cells stained more frequently positive for ER-alpha and PR than epithelial cells [44]. However, in the present study, in all groups at placental as well as interplacental sites, the PR-score was significantly lower in MSMC and ESC than in the EGE and GCE (p < 0.05). The difference could be due to different phases of gestation at the time of sampling since this was not indicated by the cited authors.

Concerning the comparison between groups, we found significantly higher TPR scores in ESC after resorption/abortion than in normal pregnant animals, indicating an up-regulation of PR during resorption/abortion. To our knowledge, this is the first report on the effects of progesterone antagonists on the abundance and the distribution of uterine steroid hormone receptors in canine species. Similarly, in pregnant women, the expression of PR and ER in endometrium and decidua increased in the endometrial stroma and glandular epithelium after mifepristone application [37,38]. In contrary, application of different agents with anti-progesterone effect decreased PR and ER in the uterus...
from early pregnant mice [39]. This points to species-specific receptor regulation during induced resorption/abortion. Whether this is dependent on phase of gestation, uterine site and/or progesterone and estradiol concentration, is not proven.

The present results revealed that the TER score in ESC at interplacental sites was significantly lower in the SRA group than in the IRA and control group (p < 0.05), this difference was non-significant at placental sites, however, the loss of ER might have contributed to embryo/fetal loss [27,28]. In the normal pregnant controls, the TER values in MSMC and in ESC were significantly higher than in the abortion groups. This was also the case in ESC of the corpus uteri (p < 0.05), indicating a general down-regulation during resorption/abortion, however, this was more pronounced in the spontaneous cases. In contrast to these results, other authors found an increase in the number of ER in the endometrium of pregnant women and rhesus monkeys after application of mifepristone [40,41]. This confirms our hypothesis, that in the bitch, ER and PR expression during spontaneous and induced resorption/abortion is regulated in a species-specific way and independent of the time of induction since no differences were assessed between Group I (induction between days 25 and 35) and Group II (induction between days 36 and 45). It is known that aglepristone induces abortion by blocking progesterone receptors inside the uterus [17,45], however, the local effect on the steroid receptors has not been described yet. The results of the present study indicate that aglepristone enhances the expression of stromal PR in the uterus and the maternal placenta, which might be due to the decrease in the biological effect of P4, since results were similar in the IRA and SRA group.

In the present study, detection of PR mRNA in tissues obtained from animals after resorption/abortion was not possible. According to the sample quality assessment via measurement of the RNA integrity number (RIN), the mRNA content and quality of the samples of the IRA and SRA groups was inferior. As the mRNA content and quality of the control group was good, we can exclude a methodological error during sampling and subsequent RNA preparation. We suppose, that due to advanced stages of resorption/abortion the mRNA in the uterine tissue was too degenerated for the here used RT-PCR method.

4.3. Serum concentrations of progesterone and estradiol-17β

In the present study, the average serum P4 concentrations were significantly higher in the controls than in the abortion groups IRA and SRA (p < 0.01 each); the E2 concentrations did not differ between groups (p > 0.05). Vermeirsch et al. [21] detected a negative correlation between the serum P4 concentration and the expression of ER in the corpus uteri in pregnant bitches. In addition, a positive effect of E2 and a negative influence of P4 on the respective receptors has been reported in the dog and other species [20,31]. The down regulating effect on the PR is absent when P4 concentrations in serum are low or serum concentrations of E2 are increasing [35]. The markedly lower serum P4 concentration observed in abortion groups compared to the normal pregnant controls is a well described phenomenon after application of PR antagonists [11,14,18] supposed to be caused by fetal death. This hypothesis is confirmed in the present study, since the significant decrease in P4 and fetal death occurred at the same time. However, it is well known that after application of P4 antagonists, serum P4 concentrations are not decreased within 24–48 h after its administration, due to delayed luteolysis [15,18]. In addition, the serum E2 concentrations did not change significantly after application of aglepristone but corresponded to normal pregnancy as described before [18].

5. Conclusions

In the present study, we found that induction of abortion had a significant effect on the expression of PR and ER in endometrial stromal cells. Consequently, even though higher numbers of receptors were found in epithelial cells, endometrial stromal cells may be more important in the progress of resorption/abortion after induction with aglepristone. However, the factors regulating uterine steroid hormone receptor expression during normal pregnancy and resorption/abortion, respectively, have to be further investigated in the bitch.

References


Repeated induction of abortion in bitches and the effect on plasma concentrations of relaxin, progesterone and estradiol-17β

S. Schäfer-Somi a,*, O.A. Aksoy b, H.B. Beceriklisoy b, A. Einspanier c, H.O. Hoppen d, S. Aslan b

a Clinic for Obstetrics, Gynecology and Andrology, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria
b Clinic for Obstetrics and Gynecology, Faculty of Veterinary Medicine, University Ankara, 06110 Diskapi, Turkey
c Veterinary-Physiological-Chemistry Institute, University Leipzig, An den Tierkliniken 1, 04103 Leipzig, Germany
d Section for Chemical Analytical and Endocrinology, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, Haus 123, 30173 Hannover, Germany

Received 6 February 2007; received in revised form 14 June 2007; accepted 14 July 2007

Abstract

The aim of the present study was to investigate the effects of two medications on two subsequent abortions and plasma hormone concentrations of dogs. For this purpose, two groups of bitches (n = 5 each), received the antiprogesterone aglepristone (Alizine®) at 10 mg/kg body weight on two subsequent days around day 30 after mating. In group II, the antiprolactin cabergoline (Galastop®) was additionally administered po at 5 μg/kg body weight until the start of abortion. The plasma concentrations of relaxin, progesterone (P4) and estradiol-17β (E2) were measured before, during and after each abortion. During the next cycle after the abortion, the same bitches were mated again and in pregnant animals, induction of abortion was performed as before. During the third cycle, pregnant bitches were allowed to whelp. Termination of first pregnancy occurred significantly earlier after the combined treatment (6.8 versus 10.6 days, p < 0.05). In both groups and during both abortions, relaxin varied between individuals; however, there was a continuous decrease after the abortions and no significant differences between groups (p < 0.05). In one bitch with high relaxin concentrations before treatment (11.6 ng/ml), a cystic endometrial hyperplasia was diagnosed. In the aglepristone only group, P4 concentrations increased significantly after the first application (p < 0.05), then decreased continuously until day 45 after the beginning of abortion. In the combined group, there was a continuous decrease until day 45 (p > 0.05). At this time, P4 concentrations between 0.47 and 84.9 nmol/l were measured in both groups. The level of E2 over time was not influenced by any medication. We therefore note that the two medications mainly influenced plasma concentrations of P4 in different ways, probably due to specific treatment-hormone interactions. However, all measurements fell within the range considered normal.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Dogs; Pregnancy termination; Fertility; Hormones

1. Introduction

Termination of pregnancies in bitches is often required, especially in cases of mismating and miscarriage. A single mismating and induced abortion usually has no harmful consequences, provided the abortion is professionally carried out and carefully
observed [1,2]. However, no data are available concerning fertility after two or more successive abortions and whether or not fertility relates to the medication scheme and/or endocrine changes during and after the abortions.

Several methods exist for the induction of abortions in dogs. As one of the first trials, dexamethasone was administered orally twice daily for 10 days from day 30 of pregnancy on. This resulted in abortion in the two treated bitches [3]. However, side effects after the use of dexamethasone were polydipsia, polyuria, vaginal discharge, restlessness, anorexia and emesis [4]. Later natural and synthetic prostaglandins were used during all stages of pregnancy, at different dosages and with different application regimens [5–9]. To further improve the effect and to enhance abortion, natural and synthetic prostaglandins have been combined with the dopamine agonist cabergoline [10–12] or with bromocriptine mesylate [13]. Cabergoline suppresses pituitary prolactin secretion and is supposed to antagonize the luteotrophic effect of prolactin. However, strong side effects like vomiting, diarrhea, and decrease in blood pressure have been described after the use of prostaglandins. These side effects were less pronounced with synthetic prostaglandins [10]. However, antigestagens proved to be most reliable at all stages of pregnancy and had virtually no side effects [14–21]. Antigestagens are synthetic steroids that bind with strong affinity to progesterone receptors and competitively displace endogenous progesterone [22]. In dogs, two anti-progestins have been studied, namely mifepristone (RU 486) [15,17] and aglepristone (RU 534), but only aglepristone is available for veterinary use. In one study, the early administration of aglepristone between days 0 and 25 after mating always prevented pregnancy. Administration of aglepristone between days 26 and 45 after mating induced resorption or abortion within seven days in 96% of cases without side effects [20]. This suggested that the use of aglepristone is a safe method for prevention of pregnancy and induction of abortion before day 45 of gestation. During the second half of pregnancy, antigestagens can be combined with prostaglandins, and this has proved to be effective for inducing parturition in dogs [23,24]. However, a combination of a local receptor blocker (aglepristone) with an indirect luteolytic drug (cabergoline) might terminate pregnancy in a comparable time or even quicker while avoiding the side effects usually seen when prostaglandins are used.

Thus, in the present study, we induced abortions repeatedly with two different treatment schemes, namely, with aglepristone and a combination of aglepristone and cabergoline. In addition, we monitored the effects of this combination on the course of plasma concentrations of P4, E2 and relaxin, and observed the course of consecutive cycles and abortions.

2. Animals and methods

2.1. Animals

Ten dogs of different breeds (two German shepherds, two Anatolian shepherds, five malinois, one dalmatian), aged 2–6 years, with an average body weight of 20.7 ± 4 kg (17–28 kg), were randomly allotted to two experimental groups. The dogs were housed in the kennel of a military working dog breeding unit in Gemlik (Tr). Each bitch was housed in a box (3 m²) with a paddock (9 m²). They all received commercially available canned food and water ad libidum.

2.2. Treatments, blood sampling and observation of abortions

Group I (n = 5): estrus was detected by means of clinical symptoms, vaginal cytology and P4 measurements (enzyme immunoassay, EIA). A progesterone value between 15 and 30 nmol/l indicated the day of ovulations. All dogs were mated one or 2 days after ovulations and pregnancy was confirmed by ultrasonographic examination around day 30 after mating. Immediately afterwards, the dogs were treated subcutaneously with aglepristone (Alizine®) at a dose of 10 mg/kg body weight on two consecutive days. Blood samples were collected before aglepristone administration and every second day until the termination of abortion. The start of abortion was diagnosed when bloody vaginal discharge was seen and ultrasonography revealed characteristic signs of beginning resorption/abortion. Then clinical examinations and ultrasonography were performed daily until the end of abortion, when no more fetuses were detected sonographically. Further blood samples were collected at days 1, 2, 15, 30 and 45 after the abortions. The same dogs were mated during the following cycle and another abortion induced with the same treatment and procedure as before. During the subsequent third cycle, dogs were mated again and pregnant animals were allowed to whelp.

Group II (n = 5): Bitches were examined, mated and pregnancy confirmed as described for group I. Immediately after confirmation of pregnancy, dogs were treated with a combination of 10 mg/kg body
weight aglepristone (Alizine®) subcutaneously and 5 μg/kg body weight cabergoline (Galastop®) orally on two consecutive days. The treatment was continued with cabergoline alone until the beginning of abortion. Blood sampling and the observation of abortion was performed as in group I. The whole procedure was repeated during the following cycle. During the third cycle, pregnant bitches were allowed to whelp.

2.3. Hormone measurements

For the production of plasma, blood samples were centrifuged three minutes at 3000 × g. Samples were stored at −18°C until analysis. The concentrations of P4 and E2 in peripheral blood plasma were measured using radioimmunoassay [25]. The concentrations of relaxin in peripheral blood plasma were measured using a modified species-specific EIA system [26].

2.4. Statistical analyses

Data are given as means ± standard deviations (x ± S.D.). All statistical comparisons were performed using the SPSS software® (Version 14 for Windows, SPSS Inc., Chicago, IL, USA). Comparisons of two mean values within one group were performed using the Wilcoxon test (resp. Fisher exact test) and comparisons between groups with the Mann–Whitney–U-test. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Clinical data

Clinical data are displayed in Table 1. In both groups, the time between start of medication and termination of abortion, as well as the interestrous interval, did not differ significantly between the first and second abortion (p > 0.05). However, after induction with alizine and cabergoline, the first abortion terminated significantly quicker than after induction with alizine alone (6.8 versus 10.6 days, p < 0.05).

We note the following additional information. In group I, after two abortions, one bitch was mated during the following three cycles and never became pregnant (interestrous intervals: 93, 112 and 195 days). This bitch was ovariohysterectomized and a cystic endometrial hyperplasia diagnosed pathologically and histologically. In group II, during the second abortion, one bitch aborted all puppies except one, that had to be delivered by caesarean section; this bitch was not mated again.

3.2. Relaxin

In group I, relaxin concentrations ranged between 0.21 and 1.33 ng/ml before treatment. Only one bitch had an exceptional high value (11.6 ng/ml); in this bitch, a cystic endometrial hyperplasia was diagnosed later. In group II, the average relaxin concentrations tended to be higher (0.59–13.25 ng/ml before treatment), however, at no time did they differ significantly between groups (p > 0.05). The course of relaxin was similar in both groups and during both abortions and showed a more or less continuous decrease after the abortion; at day 45 after the termination of abortion, relaxin concentrations ranged between 0.15 and 10.8 ng/ml. In both groups, the average relaxin concentrations did not differ significantly between first and second abortion (p > 0.05; Fig. 1a and b).

3.3. Progesterone and estradiol-17β

The average P4 and E2 concentrations did not differ significantly between groups (p > 0.05; Figs. 2 and 3). However, in group I, the average E2 and P4 concentrations tended to be higher before and during the first abortion than during the second abortion (Figs. 2a and 3a). During both abortions, in group I, the average plasma concentrations of P4 increased significantly at 48 h after aglepristone administration (from 99.5 to 162.8 nmol/l, first abortion, p < 0.05), and decreased in group II (from 74.4 to 64.5 nmol/l, first abortion, p > 0.05; Fig. 3a and b).

4. Discussion

In the present study, we induced abortions with two different medications repeatedly. Starting at mid gestation, one group of bitches was treated with the progesterone antagonist aglepristone, another with a combination of aglepristone and the antiprolactin cabergoline. As far as the course of abortion is concerned, there were no significant differences between groups, except a significant earlier start of abortions with the combined treatment after first medication. During an earlier study [18], dogs were mated 1–2 days after ovulations, medication with aglepristone alone started 30 days after ovulations and termination of abortions occurred 4–7 days later, which is comparable to our results with the combined treatment. However, in the present study, medication with aglepristone alone started between days 25 and 37 after mating and in some cases it took more time than
the expected 3–7 days, when abortion was induced after completion of placentation.

Fertility after subsequent abortions has not been investigated yet. However, it was not influenced by up to two abortions in the aglepristone group. Unfortunately, in the group with combined treatment, only two bitches came into heat after the second abortion, which is not enough for a reliable conclusion concerning fertility. However, after the first abortion results are comparable in both groups. All whelpings were completely normal with average to high puppy numbers. During a recent study [27], induction of abortion with a long-acting prostaglandin F(2α) analogue, fenprostalene, starting on day 25 after ovulation, led to low conception rates during the subsequent estrus (50%). In our study, conception rates were higher in both groups.

During the present study, relaxin concentrations varied considerably between individuals, however, they agreed with those reported in the literature [28,29]. According to other researchers, relaxin concentrations are not related to the number of embryos inside the uterus [28,30]; however, concentrations are known to increase between days 28 and 33 of gestation due to

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical data from both groups, before and during abortions 1 and 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Weight kg ± S.D.</td>
</tr>
<tr>
<td>1</td>
<td>21.6 ± 4.7 (17–28)</td>
</tr>
<tr>
<td>2</td>
<td>20.4 ± 3.0 (17–25)</td>
</tr>
</tbody>
</table>

Different letters in the same column indicate statistically significant differences (p < 0.05).

Fig. 1. Average relaxin values after induction of abortion with Alizine (a) and Alizine + cabergoline (b), before and during abortions. Abort. = Abortion. 1, 2 Alizine = 1st and 2nd application of Alizine Values in Fig. 2a were calculated without the bitch Aslik.
ripening of the placenta. In the present study, some abortions were induced before this increase, which might have caused the high individual variations at the beginning of medications.

Only one bitch had outstandingly high values of relaxin before and throughout her first abortion, probably resulting from her endometrial cystic hyperplasia. In all other bitches, no clinical problems occurred during the period of the study, even though comparably high concentrations were measured in individual bitches. We therefore assume, that the relaxin concentrations measured here were within the bound of normal, however, in some cases high values might indicate a uterine pathology. Interestingly, in rats with a pathological uterus during pseudopregnancy, relaxin levels were also elevated more than 20-fold compared to normal cycling rats [31]. Relaxin increases intracellular cAMP in the endometrium of different species (human, monkey, rat), which is an important factor for uterine differentiation, such as decidualization of endometrial stromal cells or pathological situations. The trend towards higher relaxin values in group II was not statistically significant. However, an interaction between glucocorticoids and relaxin, as described for cardiovascular cases [32,33], might explain this phenomenon. The H2 gene relaxin contains a glucocorticoid-responsive element [34], therefore, relaxin expression seems to be influenced by glucocorticoids. As an abortifacient, aglepristone acts like a progestosterone antagonist inside the uterus and does not have direct and immediate luteolytic properties. Aglepristone does not modify plasma concentrations of P4, prostaglandins, oxytocin or cortisol within 24 h after administration. In the present study, P4 concentrations increased 48 h after first medication in the Alizine group, which is comparable to the results of Fieni et al. [35,36]. Values decreased thereafter in accordance with the results of Galac et al. [18] and Fieni et al. [20]. After abortion, P4 concentration usually declines within 8–34 days to basal concentrations. However, plasma P4 concentrations have been observed to remain high for some time after the abortion [18,37,38]. In the present study, at the time of death of the last embryo, P4 concentrations were not basal in all animals and P4 concentrations as high as 84.9 nmol/l were measured 45 days after the beginning of abortion.

After induction of abortion with a combination of aglepristone and cabergoline, P4 concentrations

![Graph](image1)

**Fig. 2.** Average E2 values after induction of abortion with Alizine (a) and Alizine + cabergoline (b), before and during abortions. 1, 2 Alizine = 1st and 2nd application of Alizine.

![Graph](image2)

**Fig. 3.** Average P4 values after induction of abortion with Alizine (a) and Alizine + cabergoline (b), before and during abortions. 1, 2 Alizine = 1st and 2nd application of Alizine.
decreased within 48 h after first medication. In a previous study [1], P4 concentrations decreased in at least 66.7% of cases, when cabergoline was given at day 30 after mating. The combined medication used in the present study accelerated the beginning of abortion significantly, a feat that could be explained by the synergy of the anti-progesterone action and the indirect luteolytic effect of cabergoline.

From the present results, no influence of any medication on the ovarian secretion of E2 could be detected. On average, the course of E2 in both groups was similar to that observed in pregnant dogs [39,40] and cats after induction of abortion with aglepristone [35,36].

In conclusion, with both medication schemes, in the majority of cases the subsequent cycles were normal and fertile. We therefore suggest that the here measured relaxin, E2 and P4 concentrations until day 45 after the end of abortions, were within the bound of normal. In addition, both schemes were found to be useful for repeated induction of abortion in bitches. Nevertheless, after the first application, the combined medication lead to a significant quicker termination of abortion, rendering the combination of aglepristone and cabergoline an interesting alternative to the sole application of aglepristone.

Considering in addition the different course of P4 serum concentrations in both groups, we suppose that the interplay of these drugs should be investigated more closely.

References


Decrease of body temperature after aglepristone treatment in bitches

Y. Corrada a, P. García b, P.E. de la Sota b, M. Huzman a, M.F. Landoni a, C. Gobello a, *

a Clinical Department, Faculty of Veterinary Sciences, National University of La Plata, La Plata CC 296, Argentina
b Basic Sciences Department, Faculty of Veterinary Sciences, National University of La Plata, La Plata CC 296, Argentina

Received 31 July 2004; received in revised form 18 October 2004; accepted 8 November 2004

Abstract

Body temperature responses and the timing of abortions were evaluated in pregnant bitches with the anti-progestin aglepristone. Fifteen purebred and crossbred, 25–45 days pregnant, were included in this study and seven untreated bitches at the same stage of pregnancy served as controls. Treated bitches were administered two applications of aglepristone (10 mg/kg SC) 24 h apart for pregnancy termination. Pregnancy termination was confirmed by ultrasonographic assessment. Body temperature was rectally measured three times a day for 6 days beginning 24 h before treatment or pregnancy diagnosis in the treated and control bitches, respectively. Additionally, serum progesterone concentrations were assessed at time points during the study in the treated bitches. Pregnancy was terminated in 14 treated bitches in a mean ± S.E.M. of 4.3 ± 0.7 days after treatment. Control bitches remained pregnant. In the treated bitches, but not in the controls, body temperature significantly decreased 24 h after the beginning of the treatments (P < 0.01) and then gradually returned to pre-treatment values. Correlation between the day of mean minimum body temperature and the day of pregnancy termination was low (0.07; >0.05). Progesterone did not show significant change throughout the study. Body temperature does not seem to be a suitable variable to clinically monitor the aborting effect of aglepristone. Decrease of body temperature after aglepristone treatment could represent further evidence of its hypothalamic effects.

Keywords: Bitch; Pregnancy termination; Aglepristone; Body temperature

© 2005 Elsevier B.V. All rights reserved.

0378-4320/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
1. Introduction

Anti-progestins are synthetic steroids which bind with great affinity to progesterone (P₄) receptors preventing native P₄ from exerting its biological effects (Hoffmann and Schuler, 2000). In dogs, two anti-progestins have been studied mifepristone (RU 486; Concannon et al., 1990; Linde-Forsberg et al., 1992) and aglepristone (RU 534), although only aglepristone is available in the veterinary market of some European and American countries. Aglepristone functions as a true P₄ antagonist at the uterus without initially decreasing P₄ serum concentrations. It is, therefore, utilized in P₄ dependent uterine conditions and diseases. The main clinical indication for anti-progestins is termination of unwanted pregnancy.

Aglepristone has shown to efficiently terminate gestation in bitches within 7 days (Fieni et al., 1996, 2001; Galac et al., 2000), being one of the safest options for this purpose. Elevated plasma prolactin concentrations were described within 24 h aglepristone treatment without changing P₄ blood concentrations (Fieni et al., 1996; Galac et al., 2000). Prolactin returned to basal concentrations in 2–4 days. Progesterone concentrations declined within 8 and 34 days to basal concentrations due to premature luteolysis (Galac et al., 2000). A decrease of the interestrus intervals by shortening not only of diestrus but also of anestrus was noted in the three studies after aglepristone treatment (Fieni et al., 1996, 2001; Galac et al., 2000).

These effects (i.e. prolactin release, premature luteolysis and shortening of the anestrus) of aglepristone may be due to a direct or indirect action of aglepristone on hypothalamic-pituitary axis. Detailed studies in dogs on the effect of P₄ receptor antagonist at the hypothalamic-pituitary level are lacking.

In a first study in which mifepristone was used for pregnancy termination, body temperature declined 24–48 h after treatment and then returned to normal (Linde-Forsberg et al., 1992). More recently, in aglepristone-treated pregnant bitches, rectal temperature was described to decrease within 48 h after abortion and then to return to normal values (Fieni et al., 1996). Therefore, body temperature monitoring would potentially be a practical tool to follow up anti-progestin effects and to clinically diagnose pregnancy termination. It was, therefore, of interest to describe the body temperature variations in aglepristone-treated pregnant bitches in relation to time of administration and pregnancy termination.

2. Materials and methods

Fifteen 1–13-year-old crossbred and purebred pregnant bitches, 6–28 kg, that were privately owned were treated with two applications of aglepristone (Alizine®, Virbac, France) SC, 10 mg/kg 24 h apart for pregnancy termination. Treatments occurred during the morning hours. The bitches had been bred 25–45 days before and pregnancy confirmed by ultrasonography (Toshiba Core Vision Pro, Japan; Mattoon and Nyland, 1995). During the period of the study, seven bitches with the same characteristics were used as controls. All the bitches were hospitalized during the study, fed commercial dog food and given water ad libitum.

The bitches were clinically examined and body temperature was rectally measured three times a day (8 a.m., 4 a.m. and 12 p.m.) for six consecutive days beginning 24 h before
treatment or pregnancy diagnosis in the treated and control bitches, respectively. Pregnancy termination was clinically diagnosed by the appearance of vaginal discharges, expulsion of fetuses, or typical behavioral changes and immediately confirmed by ultrasonography (Mattoon and Nyland, 1995).

Additionally, blood samples by peripheral venepuncture were collected for serum P4 concentration determination before treatment, at the time of ultrasonographic confirmation of pregnancy interruption and 72 h later in the treated bitches. Serum P4 was measured by radioimmunoassay, using a solid phase kit (Coat-A-Count, DPC®, Los Angeles, USA). For this kit, the sensitivity at 95% binding was 0.1 ng/ml and intra-assay CV was 5.7%.

Descriptive statistics was performed for days to achieve pregnancy termination and expressed as mean ± S.E.M. Body temperature on the treated and control groups were analyzed by ANOVA for repeated measures and Student–Newman was performed as post-test. A correlation analysis was also performed between the day of mean minimum body temperature and the day of pregnancy termination. The level of significance was set at 0.05.

3. Results

Pregnancy was terminated in 14 of 15 treated bitches either by resorption (n = 8) or abortion (n = 6) in a mean ± S.E.M. of 4.3 ± 0.7 days (range 2–8) after initiation of treatments. As expected, earlier pregnancies terminated mainly by resorption while older ones by abortion. The non-responding bitch was treated with another pharmacological aborting protocol on day 9. Control bitches remained pregnant and whelped normal litters.

![Fig. 1. Mean body temperature of 15 pregnant bitches treated with two applications of aglepristone SC (10 mg/kg 24 h apart) for pregnancy termination. Bars on the symbols represent SEM. Asterisks point out significantly different (<0.01) values among the days of the treated bitches. (Inset) Mean body temperature of seven control bitches which did not receive any treatment.](image-url)
Body temperature significantly differed between treated and control bitches (Fig. 1, inset) and varied throughout the study in the treated bitches, consistently decreasing 24 h after the beginning of the treatments ($P < 0.01$) and returning gradually to pre-treatment values (Fig. 1). Correlation between the day of averaged minimum body temperature and the day of pregnancy termination was low (0.07) and non-significant. Progesterone did not show significant change in the treated bitches, although a tendency for a decrease was evident as expected in this phase of pregnancy (Fig. 2).

4. Discussion and conclusion

Aglepristone successfully terminated pregnancy in most of the treated bitches. In line with a previous report, in the present trial efficacy was slightly less than 100% (Fieni et al., 1996). Gestation interruption occurred in the presence of $P_4$ concentrations that were typical of those during pregnancy confirming the local uterine effect of the receptor blocker. Progesterone is a thermogenic hormone. Its action at central nervous system is responsible for decreases in body temperature (Guyton and Hall, 1997). Therefore, body temperature variations in the present study could not be attributed to a peripheral decrease of serum $P_4$.

In contrast to a former study (Fieni et al., 1996), in the present study, no chronological relationship was found between pregnancy termination and variation on body temperature. Temperature consistently decreased 24 h after treatment in all the animals, independently of the time at which pregnancy termination occurred. The decline in body temperature, even in the bitch in which the treatment failed, further confirmed this observation. A similar
temperature pattern was described in three bitches after mifepristone administration in an earlier report (Linde-Forsberg et al., 1992).

Thus, decreased body temperature after treatment could represent additional evidence of anti-progestin effects on the hypothalamus and other central nervous system centers. The abrupt decrease in body temperature may be explained by occupation of central P₄ receptors by the antagonist, mimicking a sudden decline in P₄ concentrations. Interestingly, plasma prolactin has shown an opposite pattern of variation after aglepristone administration (Fieni et al., 2001; Galac et al., 2000). Prolactin is known to increase in response to P₄ deprivation in this species (Gobello et al., 2001). Thus, prolactin may promote similar, although opposite, actions of anti-progestins at hypothalamus.

It is concluded that body temperature does not seem to be a reliable variable to clinically monitor aglepristone aborting effect because no relationship between this variable and pregnancy termination time was determined in the present study. Additionally, decrease of body temperature after aglepristone treatment could represent further evidence of its hypothalamic and other central nervous system center effects.

Acknowledgement

The authors would like to thank Virbac, France for Alizine® supply.

References