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Use of the progesterone (P_4) receptor antagonist aglepristone to characterize the role of P_4 withdrawal for parturition and placental release in cows

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Abstract

In late pregnant cows, progesterone (P_4) is mainly of luteal origin. However, the trophoblast may provide high local P_4 concentrations in the uterus. To test for the importance of a complete P_4 withdrawal for parturition-related processes and placental release, the P_4 receptor (PGR) blocker aglepristone (Ap) was administered to three cows on days 270 and 271 of pregnancy. A complete opening of the cervix was observed 46.5±7.3 h after the start of treatment. However, expulsion of the calves was impaired obviously because of insufficient myometrial activity, and placental membranes were retained for at least 10 days. Measurement of P_4 concentrations indicated that PGR blockage induced luteolysis. To investigate the role of P_4 withdrawal for the prepartal tissue remodeling of the placentomes, the caruncular epithelium was evaluated by morphometry, and the percentage of trophoblast giant cells (TGCs) relative to the total number of trophoblast cells were assessed. Caruncular epithelium in Ap-treated cows (D272+Ap) was immature (30.5±3.3%) and not different from untreated controls (elected cesarean section (CS) on day 272; D272-CS; 31.5±1.4%), whereas it was significantly reduced at normal term (D280.5±1.3; 21.0±6.1%; $P=0.011$). Correspondingly, the percentage of TGCs were 20.1±1.4 in D272+Ap, 22.1±4.8 in D272-CS, and 9.8±3.9 at term ($P=0.001$). No effect was detected on placental estrogen synthesis. The results showed that in late pregnant cows, P_4 withdrawal only induces a limited spectrum of the processes related to normal parturition and is not a crucial factor for the prepartal tissue remodeling in placentomes and the timely release of the placenta.

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Introduction

In dairy cows, release of the placenta usually takes <6–8 h after expulsion of the fetus. The retention of the fetal membranes for more than 12–24 h is generally considered as a pathological situation named retained fetal membranes (RFMs). RFMs are a common abnormality in dairy cows, occurring in 5–10% of otherwise normal calvings. It is regarded as a major cause of reproductive disorders in the puerperal and postpuerperal period and may lead to significant economic losses at the herd level. A considerable number of factors have been implicated to be associated with RFM such as breed, dystocia, twin pregnancy, gestation length, season, herd, environment, parity, nutrition, and hormonal imbalances. However, irrespective of the many investigations performed, the underlying pathogenetic mechanisms are still largely unknown, and consequently any preventive measures are of uncertain nature (Wetherill 1965, van Werven *et al*. 1992, Laven & Peters 1996, Han & Kim 2005, McNaughton & Murray 2009).

The profound understanding of the etiology and pathogenesis of RFM is also hampered by the lack of definite information on the regulatory mechanisms allowing for a physiological placental release. However, it is obvious that a tightly controlled reduction of feto-maternal adherence is a prerequisite for the birth of a vital fetus and the timely release of the placenta. Accordingly, in late gestation and in the immediate prepartal period, distinct remodeling processes have been observed in the microarchitecture of the bovine feto-maternal interface, named (morphological) placental maturation, including a significant reduction of the caruncular epithelium (Björckman 1954, Grunert 1985, Woicke *et al*. 1986) and a marked decrease in the number of trophoblast giant cells (TGCs; Williams *et al*. 1987, Gross *et al*. 1991). In cows with RFM, it has been shown that this reduction in caruncular epithelium

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(Grunert 1985) and the numbers of TGCs are less pronounced (Williams *et al*. 1987, Gross *et al*. 1991). However, the actual significance of these processes for placental separation and the underlying regulatory mechanisms are still not understood.

Although the essential role of progesterone (P_4) in the maintenance of pregnancy is generally accepted, the mechanisms reducing the availability of P_4 or bringing its functions to an end near term to allow labor and the delivery of the offspring – which exhibit significant differences among mammalian species – are still not fully clear. In most domestic animal species, a prepartal withdrawal of P_4 is considered as a prerequisite for normal parturition resulting from either luteolysis or a switch in placental steroidogenesis from P_4 precursors to other steroids, depending on the source of P_4 during late gestation (Zakar & Hertelendy 2007). In contrast to many other species with either the ovary or the placenta as the sole significant source of P_4 during late gestation (Hoffmann 1994, Meyer 1994), the situation in the late pregnant cow is more complex because of the coexistence of the two sources. Throughout bovine gestation, the corpus luteum is the major source of P_4, and the prepartal decline of peripheral P_4, which is considered as a prerequisite for physiological parturition in cattle, is clearly a result of luteolysis (Hoffmann *et al*. 1979). The bovine placenta exhibits a significant steroidogenic activity (Hoffmann *et al*. 1976, Hoffmann & Schuler 2002, Schuler *et al*. 2008) but contributes only marginally to peripheral maternal plasma P_4 levels, and its capacity to maintain pregnancy in the absence of luteal P_4 production is restricted to a short phase approximately on days 180–250 (Estergreen *et al*. 1967, Day 1977*a*, Chew *et al*. 1979, Johnson *et al*. 1981, Pimentel *et al*. 1986, Conley & Ford 1987). However, despite its negligible contribution to peripheral maternal plasma levels, also during late gestation, the bovine placenta has the capacity to produce high P_4 concentrations at the feto-maternal interface (Tsumagari *et al*. 1994), where P_4 receptors (PGRs) are highly expressed in the caruncular stroma (Schuler *et al*. 1999). This observation suggests that the caruncle is under the control of placental rather than luteal P_4.

Among the various essential functions exerted by P_4 for the establishment and maintenance of pregnancy is the generation of a pregnancy-specific state of differentiation of the endometrium allowing fetal attachment and subsequent intrauterine fetal development (Bagchi *et al*. 2005, Forde *et al*. 2009). Thus, at the end of pregnancy, the prepartal withdrawal of P_4 may not only allow for the expulsion of the fetus but may also play an important role in the induction of processes leading to the reduction of feto-maternal adherence with placental P_4 being an essential factor in respect to the latter process. In the prepartal phase, a switch in bovine placental steroidogenesis – considered as functional placental maturation – occurs from the predominant production of P_4 and estrone sulfate to the synthesis of free estrogens induced by fetal cortisol (Comline *et al*. 1974, Hunter *et al*. 1977, Wagner *et al*. 1992, Schuler *et al*. 1994). The dynamics of the prepartal collapse in placental P_4 production and its role in the network of signals controlling parturition in cattle are still unclear. However, for the reasons mentioned earlier, an incomplete or delayed decline of placental P_4 production, which would not become obvious from P_4 measurements in peripheral maternal blood, may be an important cause for placental retention in cattle by impairing the remodeling of placentomal tissue and other putative mechanisms for the reduction of feto-maternal adherence such as the downregulation of adhesion molecules, extracellular matrix degradation (Walter & Boos 2001), or immune mechanisms related to allograft rejection (Davies *et al*. 2006). This idea is supported by a high incidence of RFM after induction of abortion or parturition by ovariectomy (Venable & McDonald 1958, Estergreen *et al*. 1967, Chew *et al*. 1979) or the administration of prostaglandins (Day 1977*b*, Königsson *et al*. 2002), which induce luteolysis but have not been considered to significantly affect placental steroidogenesis.

To obtain information on the dependency of individual parturition-related processes including placental maturation and release on P_4 withdrawal, the antiprogestin aglepristone (Ap), which blocks receptor-mediated effects of P_4 irrespective of its origin (Teutsch & Philibert 1994), was administered to pregnant cows on days 270 and 271 of gestation. Subsequently, the process of parturition and placental release were monitored clinically. To study the effect of treatment on placental morphological maturation, the integrity of the caruncular epithelium and the percentage of TGCs were assessed, and placental estrogens were monitored in maternal blood as indicators of functional placental maturation. P_4 concentrations were measured to confirm that spontaneous prepartal decline has not occurred before the time of treatment, and to monitor the impact of treatment on luteal function throughout the time of the experiment. The effects of treatment were investigated in comparison to untreated controls matched for gestational age (day 272) and cows at normal term.

Results

Clinical observations after Ap administration

After administration of the antiprogestin on days 270 and 271, a rapid enlargement of the udder and milk filling of the teats was observed in two of the three Ap-treated cows (D272+Ap) during the last 24 h before parturition. These changes were less obvious in the third animal that exhibited a significant udder edema already at the beginning of housing 2 weeks before the start of the experiment. In untreated control animals (D272-cesarean section (CS)), corresponding changes only became

obvious on the second day after the surgical removal of the fetus performed on day 272 and about 1 day after cloprostenol treatment.

In all D272+Ap cows, a slight raise of the tail was observed as the first sign of impending parturition. At this time, which was 46.5±7.3 h after the first Ap administration, vaginal exploration yielded a fully open cervix. In the following 1–2 h, a substantial activity of the abdominal press started, which, however, did not result in any noticeable progress of fetal expulsion. Occasionally, the animals showed signs of discomfort such as restlessness, kicking with the hind legs against the lower abdomen, and vocalization. As 2 h after the detection of a fully open cervix, still no progress in the expulsion of the calves was noticeable, they were manually extracted, which took place 48.5±7.3 h after the first Ap administration. In all the three cases, extraction of the calves was apparently hindered by insufficient myometrial activity and a rigid consistency of the caudal vagina and vulva. During extraction of the calves, a spontaneous release of milk was observed. At the first milking immediately after parturition, visually normal colostrum was obtained in one animal, whereas colostral character was less pronounced in the remaining two cows. Clearly different from antiprogestin-treated cows, in D272-CS cows, only small volumes of precolostrum could be milked on the day of surgery. Significant amounts of milk from these cows could only be obtained starting from the third day after surgery.

All three D272+Ap cows developed pronounced and persistent RFM, characterized by a tear-resistant consistency of the fetal membranes and a high number of placentomes involved. During the first 48 h after extraction of the calves, no attempts were made to manually remove the fetal membranes, and no medication for RFM treatment was administered. During the following days, treatment consisted of attempts to remove the fetal membranes manually and intrauterine antibiotic treatment every other day, in case of fever supplemented with systemic antibiotic treatment. However, only minor placental components could be removed from the uterus. No significant release of fetal membranes took place until day 10 *post partum*, when a discharge of major autolytic placental components started. RFM of similar type was also observed in all three cows of the untreated control group (D272-CS), in which a significant discharge of autolytic placental components did not occur before day 8 after CS. The four cows of the normal term group released the fetal membranes within 12 h after spontaneous expulsions of a vital calf on day 280.5±1.3 after insemination.

All calves from the D272+Ap and the D272-CS groups were fully developed but exhibited moderate degrees of immaturity (delayed first stand and first suck, recurrent negative base excess in blood gas analysis indicating immaturity of the respiratory system), which made significant clinical care necessary to ensure successful adaption to extrauterine life.

Temperature profiles in Ap-treated cows

From day 268 until 20–30 h after the first Ap administration (beginning of day 271), body temperatures (Fig. 1) were variable within the physiological range of late pregnant cows (38.0–39.2 °C). Thereafter, a period of rather constant values of 38.4±0.15 °C was observed until the beginning of day 272, when the body temperature became more variable again and rose further up reaching subfebrile and febrile temperatures during the following days in two of three animals.

Steroid hormone profiles

In two of the three D272+Ap cows, a considerable decrease in P_4 concentrations started about 33 h after the first Ap administration, reaching levels around 1.5 ng/ml at the time when the calf was extracted (Fig. 2A). In the third animal, P_4 was still high during the extraction of the calf. It started to decrease immediately thereafter, i.e. 49 h after the first Ap administration. In D272-CS cows, P_4 concentrations did not change in days 269–272, and they were at 5.9±1.0 ng/ml immediately before CS (Fig. 2B). In two cows of this group, a significant drop of P_4 levels was only observed associated with the start of prostaglandin treatment 14 and 26 h after surgery respectively, which was administered to D272-CS cows to induce luteolysis in order to support uterine involution. In the third animal, P_4 concentrations >4 ng/ml were maintained for a period of at least 9 h after surgery, after which return to basal levels occurred spontaneously.

In D272+Ap cows, estrone and estrone sulfate concentrations fluctuated between samplings but there were no obvious changes during the period between the first Ap administration and the extraction of the calves, whereas estradiol-17β (E_2) concentrations continued to increase (Fig. 3A–C). After extraction of the calves, estrogen concentrations remained virtually constant for another 36 h, followed by a decrease to baseline levels within 2–3 days. In D272-CS animals, no significant

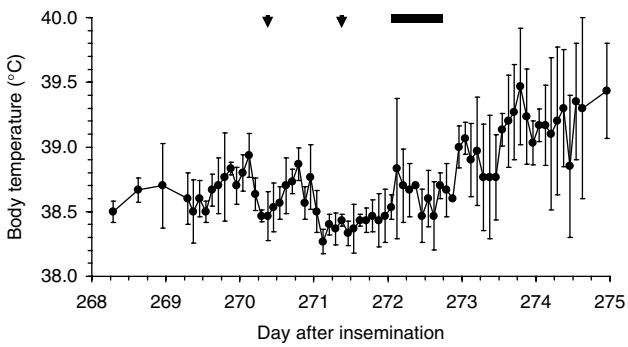

Figure 1 Body temperature (mean±s.d.) in three cows (group D272+aglepristone (Ap)) treated with the progesterone receptor blocker Ap on days 270 and 271 after insemination (arrows). The black bar indicates the period in which the calves were extracted.

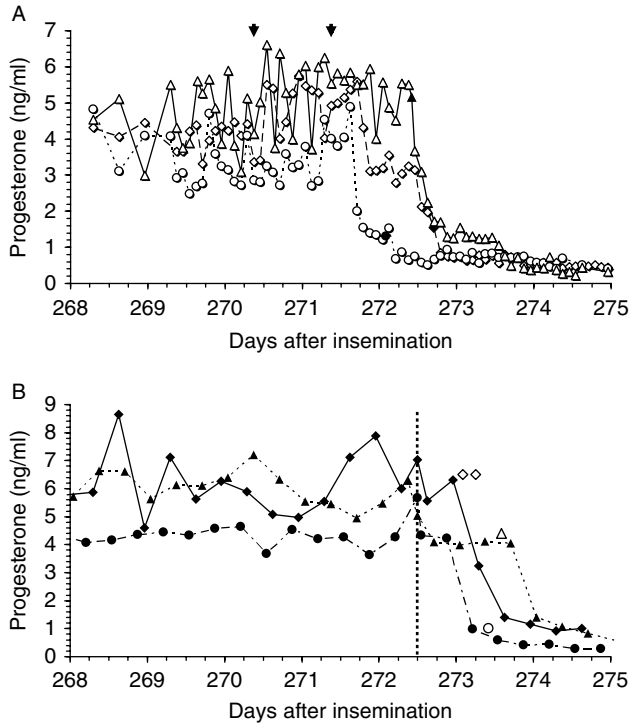

Figure 2 (A) Progesterone concentrations in three pregnant cows treated with the progesterone receptor blocker aglepristone (Ap) (arrows) on days 270 and 271 after insemination (group D272+Ap). The closed symbols indicate the time when the calves were extracted.
(B) Progesterone concentrations in three pregnant control cows, in which elected cesarean sections were performed on day 272 after insemination to collect placentomal tissue (group D272-CS). The open symbols indicate the time of prostaglandin administrations, which were intended to support uterine involution by the induction of luteolysis.

changes of estrogen concentrations were observed during the last 5 days before surgery (Fig. 3D–F). The values determined before surgery were 1.2±1.0 ng/ml for estrone, 13.0±3.6 ng/ml for estrone sulfate, and 89.2±67.1 pg/ml for E_2. After surgery in two D272-CS cows, estrogen concentrations were maintained until the end of the observational period, which was 51–61 h after the removal of the calf. In the third animal, a return to basal levels within 24 h after surgery occurred for estrone and estrone sulfate, but not for E_2.

Cortisol concentrations in D272+Ap cows were not affected by treatment with Ap but showed a transient increase starting with fetal extraction and lasting for about 2 days (data not shown).

In term animals ($n=4$; Fig. 4), the prepartal decline of P_4 occurred 12–36 h before the onset of labor. Concentrations of free estrogens fluctuated in the last week prepartum with an increase toward parturition in two animals, whereas this was not the case in the other two animals. Concentrations of estrone sulfate were fairly constant during late gestation and, as those of the free estrogens, returned to basal level within 24 h after expulsion of the fetus.

Histomorphological observations in placentomes

Placentomes of D272+Ap cows were clearly immature with mostly cuboidal caruncular epithelial cells and a high frequency of TGCs (Fig. 5A), as was seen in D272-CS animals (Fig. 5B). Placentomes from term animals showed the outcome of the normal prepartal tissue remodeling consisting of a flattening of the caruncular epithelium and a reduction in the number of TGCs (Fig. 5C). However, a considerable variability of these findings was observed depending on the individual animal and individual crypts or villi respectively within the placentomes.

With regard to TGC density (Fig. 5D), a one-factorial ANOVA yielded a significant influence on the experimental group ($P=0.0012$). However, in a pairwise comparison of experimental groups, no difference was found between D272+Ap (20.1±1.4%) and D272-CS (22.1±4.8%) cows, whereas density in the term group (9.8±3.9%) was significantly lower than in the D272+Ap group and in the D272-CS group ($P<0.01$ each). Evaluation of data from the morphometric assessment of the caruncular epithelium (Fig. 5E) by a one-factorial ANOVA also showed a significant influence on the experimental group ($P=0.0113$). Again, no difference was found between D272+Ap (30.5±3.3%) and D272-CS cows (31.5±1.4%), whereas they were significantly different from the term group ($P<0.05$ each), in which the caruncular epithelium was clearly reduced (21.0±6.1%).

Discussion

Treatment of pregnant cows with the antiprogestin Ap starting on day 270 induced onset of parturition and significantly reduced gestational length (272.4±0.3 vs 280.5±1.3 days in untreated controls; $P<0.001$). The first noticeable effect of Ap treatment was a trend to lower body temperature starting 20–30 h after the first antiprogestin treatment. The prepartal course of body temperature in D272+Ap cows clearly resembles features described for the phase preceding normal term characterized by a transient decrease of 0.4 °C (48–22 h; Birgel *et al.* 1994) and the absence of diurnal variation (48–8 h; Lammoglia *et al.* 1997). The time between the onset of antiprogestin treatment and birth (48.7±7.5 h) is in the same range as reported after the initiation of parturition using the similar type antiprogestin, mifepristone, on days 277 and 278 in beef cows (55±3 h; Li *et al.* 1991) or beef heifers (43±7 h; Dlamini *et al.* 1995).

In order to characterize the role of P_4 withdrawal for parturition and placental release widely unbiased by other parturition-related processes, in this study, day 270 was chosen to start antiprogestin treatment clearly before the main period of morphological and functional maturation of the placenta. Histomorphological investigations in control animals on day 272 confirmed that at this time placentomes were still profoundly immature.

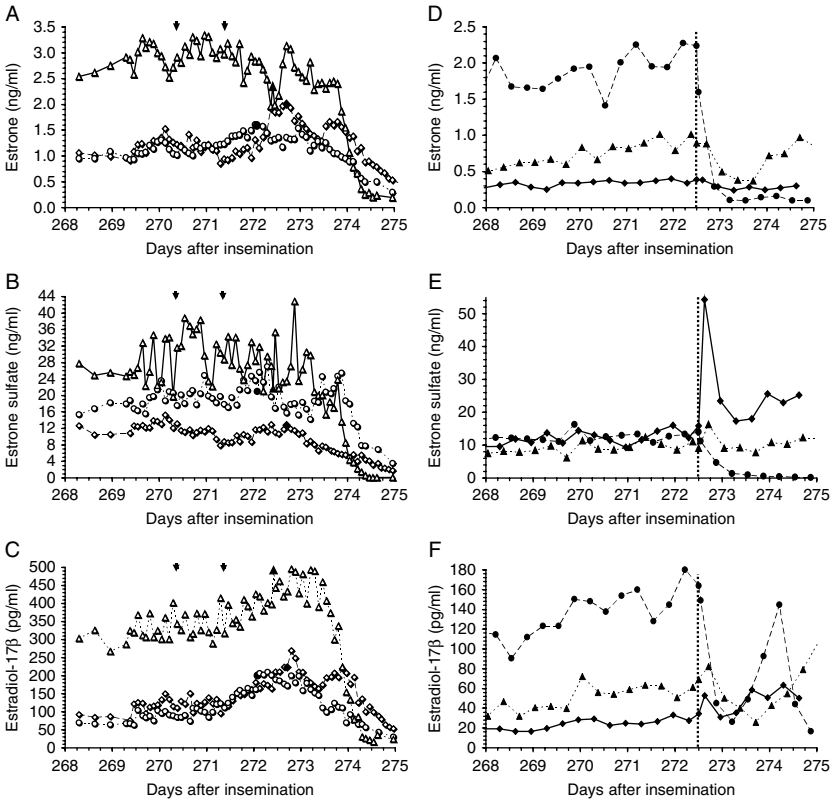

Figure 3 Concentrations of (A) estrone, (B) estrone sulfate, and (C) estradiol-17β in three pregnant cows treated with the progesterone receptor blocker aglepristone (Ap) (arrows) on days 270 and 271 after insemination (group D272+Ap). The closed symbols indicate the time when the calves were extracted respectively. (D) Estrone, (E) estrone sulfate, and (F) estradiol-17β concentrations in three pregnant control cows, in which elected cesarean sections (CS) were performed on day 272 after insemination to collect placentomal tissue (group D272-CS). The dotted line indicates the time of surgery.

The administration of Ap ∼10 days before the end of mean gestational length induced a complete opening of the cervix within 46.5±7.3 h after the start of treatment. However, in all three cases, a considerable use of traction was necessary to extract the calves. Clinical observations indicated that dystocia was due to an insufficient myometrial activity and a poor widening of the posterior birth canal. In contrast to our study, no dystocia was observed after the induction of parturition in multiparous beef cows using mifepristone starting on day 277 (Li *et al*. 1991), and the incidence was low in beef heifers (Dlamini *et al*. 1995). The reason for this significant difference between these results is not clear but may be related to the time when antiprogestin treatment was started. Although the mean gestational length in beef cattle used by Li *et al*. (1991) and Dlamini *et al*. (1995) was slightly longer than in Holstein cows used in our study (beef cows: 286±2.3 days; beef heifers: 284±1.5 days; Holstein: 280.5±1.3 days), uterine activation by upregulating a cassette of contraction-associated proteins and/or the conditioning of the systems providing uterotonins such as oxytocin and $PGF_{2\alpha}$ (Whittle *et al*. 2001) – presumably by signals from the fetal side – may have been substantially different at the onset of antiprogestin treatment between the two experimental settings. A similar situation has been postulated for the dog, where, depending on the time of antiprogestin treatment, parturition with impaired (Hoffmann *et al*. 1999) or efficient (Baan *et al*. 2005) expulsion of the puppies had been observed. The assumption that in our study antiprogestin treatment was started at an earlier stage of readiness for parturition compared with the study by Li *et al*. (1991) and Dlamini *et al*. (1995) is also substantiated by the observation that in our experiment calves from Ap-treated animals and from day 272 controls were viable but needed considerable clinical care during the first week after

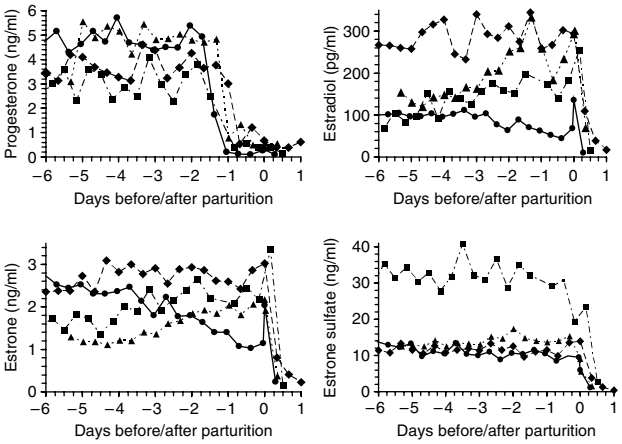

Figure 4 Concentrations of progesterone, estradiol 17β, estrone, and estrone sulfate in four untreated control cows during late gestation and around normal term. Gestational length was 280.5±1.3 days, and the placenta was released within 12 h after spontaneous expulsion of the calves.

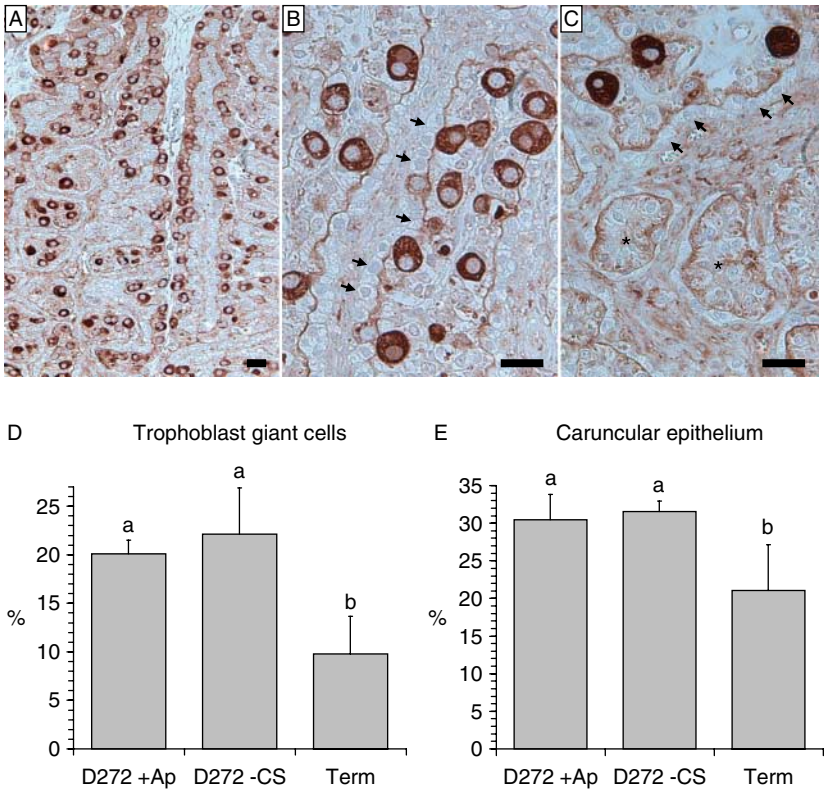

Figure 5 Effect of progesterone receptor blockage on placentomal morphology. (A) Placentome from an aglepristone (Ap)-treated cow. The high density of trophoblast giant cells (TGCs) stained with PHA lectin histochemistry is indicative of immaturity. (B) Placentome from a D272 control animal. The high density of trophoblast cells and the cuboid caruncular epithelial cells (arrows) shows that the prepartal remodeling at the feto-maternal interface has not yet started to a significant extent. (C) Placentome from a cow at normal term. The density of TGCs has considerably reduced. The asterisks mark cross-sectioned chorionic villi surrounded by a significantly reduced caruncular epithelium, whereas at other sites, cuboid caruncular epithelial cells may still be present (arrows). (D) Percentage of TGCs ($\bar{X}$+S.D.) related to the total number of trophoblast cells. Placentomes were collected from Ap-treated animals after extraction of the calves on day 272 (D272+Ap; $n=3$), from pregnant cows during elected cesarean section (CS) on day 272 (D272-CS; $n=3$) and at normal term ($n=4$). Columns with different superscripts differ with $P<0.01$. (E) Results from the morphometrical quantification of the caruncular epithelium. Values represent the percentage ($\bar{X}$+S.D.) of the area corresponding to the caruncular epithelium relative to the total area encircled by the basal membrane of the caruncular epithelium in cross-sectioned maternal crypts. Columns with different superscripts differ with $P<0.05$. Bar=25 μm.

parturition to survive, whereas Li *et al.* (1991) and Dlamini *et al.* (1995) reported that calves from their experiments born around day 279 were vigorous.

When investigating the role of P_4 withdrawal for parturition and placental release in cows by the administration of Ap during late gestation, all relevant P_4-responsive cells in the maternal compartment should have been reached by the antiprogestin administered including the caruncular stroma, the only part of bovine placentomes expressing the PGR (Schuler *et al.* 1999). As no PGRs were detected in the fetal part of the placentome (Schuler *et al.* 1999) and as P_4 concentrations in bovine fetuses are below 0.2 ng/ml during late gestation (Schuler 2000), it is rather unlikely that antiprogestins have significant direct effects on the fetal compartment. Moreover, the fact that no effects of Ap treatment were observed on trophoblast steroidogenesis and TGC density also questions significant indirect effects of P_4 on the fetal compartment mediated by PGRs expressed in the caruncle.

The interesting new aspect on the endocrine control of parturition in cattle relates to the observation that among the complex events of bovine parturition, only a very limited number of processes such as the opening of the cervix and the second phase of lactogenesis could be clearly identified to depend directly on P_4 withdrawal, whereas other important parturition-related events such as induction of labor and placental maturation apparently need significant signaling from the fetal side instead or in addition. The essential role of P_4 withdrawal for the opening of the cervix in cattle has been demonstrated earlier by Fairclough *et al.* (1984) using a different experimental approach. The pronounced and persistent RFM after antiprogestin-induced parturition clearly falsifies our working hypothesis of a complete withdrawal of placental P_4 at the caruncular level as a

prerequisite for the timely release of the placenta in cattle. Moreover, the lack of detectable effects on placentomal histomorphology questions a role of placental P_4 in cows as a discrete paracrine regulator of caruncular differentiation. Thus, the role of P_4 production in the bovine placenta remains fully unclear. In groups D272+Ap and D272-CS, no shedding of placental tissue resembling the normal process of placental detachment did occur, and the release of the fetal membranes from the caruncles was more by autolysis of cotyledons as obvious from vulval discharge starting on days 8–10 *post partum*. This observation suggests that the mechanisms leading to the physiological release of the placenta normally take place to a significant extent only after day 272 and are dependent on the presence of a viable fetus. Once the fetus has been separated from the placenta, no effectual after ripening of a profoundly immature placenta will occur. This statement must be seen in light of the observation that in the Ap-treated cows after removal of the calves, estrogen levels remained elevated for another 36–48 h pointing out that the cotyledonary trophoblast cells were at least in part still viable over a considerable time after removal of the fetus. Different from our study, in the preceding studies using mifepristone on days 277 and 278 in multiparous beef cows (Li *et al*. 1991), no RFM occurred, a trend to an increased incidence of RFM was only observed in heifers (Dlamini *et al*. 1995). However, as suggested earlier concerning the divergent observations on the incidence of dystocia, the different outcome is obviously related to the difference in time when antiprogestin treatment was started.

Interestingly, and as was observed when late pregnant cows were treated with mifepristone (Li *et al*. 1991, Dlamini *et al*. 1995), all Ap-treated cows showed luteal regression starting 33–50 h after the first Ap treatment, with the extraction of the calves being performed at the onset of luteal regression in one case and at its end in the two remaining cases. Luteal regression in D272+Ap cows was accomplished within about 24 h, matching the situation of a normal parturition (Hoffmann *et al*. 1979). Thus, luteolysis was clearly induced by antiprogestin treatment, an observation also made in the dog (Kowalewski *et al*. 2009). Luteolysis may result from a direct effect on luteal PGRs (Rekawiecki *et al*. 2008) or an indirect one by stimulating the synthesis of luteolytic prostaglandins. To allow for this effect of an antiprogestin treatment, the endocrine environment of late gestation seems to be a prerequisite, as Ap when administered to early pregnant cows using the same dosages led to a significant increase in P_4 levels during the observational period of 7 days after the start of treatment (Breukelman *et al*. 2005).

The pioneer substance of antiprogestins, mifepristone (RU38486), was originally recognized as a potent antiglucocorticoid in humans (Teutsch & Philibert 1994). Thus, after administration of mifepristone in primates, a rapid increase in cortisol resulting from a disruption of the negative feedback loop of the hypothalamo-pituitary–adrenal axis has been observed (Healy *et al*. 1985, Lamberts *et al*. 1991, Koper *et al*. 1997). For the structurally closely related Ap (RU46534; Teutsch & Philibert 1994), no information on antiglucocorticoid activity in cattle is available. The fact that maternal cortisol levels remain unchanged until labor does not point to a significant antiglucocorticoid activity of Ap in cattle.

In conclusion, the results show that PGR blockage in late pregnant cows only induces an incomplete subset of events associated with normal parturition. The fact that PGR blockage not even rudimentarily triggered the prepartal remodeling of placentomal microarchitecture normally occurring in late pregnancy and that in placentomes no noteworthy reduction in feto-maternal adhesion could be observed for more than 1 week after antiprogestin-induced parturition gives strong evidence that the timely release of the bovine placenta predominantly depends on signaling from the fetus proper.

Materials and Methods

Animals, treatments, and sample collection

The Ap treatment group (group D272+Ap) consisted of three pregnant Holstein–Friesian cows with registered dates of artificial insemination. On day 270 of gestation, they received 3 g of the PGR blocker Ap (kindly provided by Virbac Tierarzneimittel GmbH, Bad Oldesloe, Germany) in 100 ml solvent s.c. at four different sites of the lower thoracic wall (~5 mg/kg body weight) after shaving and disinfecting the injection site. This procedure was repeated 24 h later, using the contralateral thoracic wall. The abortifacient efficiency of this dosage regime has been previously shown in early pregnant heifers (Breukelman *et al*. 2005). After the first Ap injection, the animals were visually controlled in 2 h intervals for signs of impending parturition, completed by vaginoscopy or manual exploration to confirm the onset of active labor. The calves were extracted 2 h after noticing the first signs of active labor such as a completely open cervix and frequent use of the abdominal press. Immediately after the extraction of the calves, three to five placentomes were removed per vaginam. To obtain placentomes from untreated control animals matched for gestational age, elected CSs were performed in three Holstein–Friesian cows on day 272 after artificial insemination (group D272-CS). All three cows were treated once or twice with 150 μg cloprostenol between 14 and 26 h after surgery to induce luteolysis in order to support uterine involution. Placentomes from four cows at normal term (group term) were collected per vaginam immediately after spontaneous delivery of healthy calves. In these cows, placentae were released within 12 h after expulsion of the calf.

From the animals of the group D272+Ap, blood samples were taken from the jugular vein in 2 h intervals on days 269–274 and in 8 h intervals on days 268 and 275. In group D272-CS animals, blood sampling was in 8 h intervals from day 268 until day 275. Animals of the term group were sampled in 8 h intervals from day 271 after insemination until the day

after parturition. Additional blood samples were taken from the cows immediately before calving (group D272+Ap, term group) or before CS (group D272-CS). In the D272+Ap group, body temperature was measured concomitantly with blood sampling.

The animal experiment was approved by the committee on the use of animals for research purposes at the regional council (Regierungspraesidium Giessen, no. V54-19c-20-15(I) Gi 18/14-Nr.41/2007) according to the German animal protection law.

Steroid hormone measurements

P_4 was measured following the method by Hoffmann *et al.* (1973). Plasma (0.1 ml) was extracted with hexane, and the antiserum used was directed against 4-pregnene-11a-ol-3,20-dione-hemisuccinate-BSA. Intra- and inter-assay coefficients of variation (CV) were 8.8 and 8.9% respectively. The measurement of E_2 concentrations was performed by a sequential assay (Strecker *et al.* 1979) as previously described (Hoffmann *et al.* 1992). Plasma (0.25 ml) was extracted with toluene; the antiserum used was directed against E_2-6-carboximethyloxim (CMO)–BSA. The minimum detectable concentration was at 1 pg/ml. Intra- and inter-assay CV were 7.1 and 17.6% respectively. Free and conjugated estrone were measured as outlined by Hoffmann *et al.* (1996). Free estrone was measured after extraction of plasma samples with toluene. The antiserum was raised against estrone-6-CMO-rat serum albumin. Estrone sulfate was measured by the same method after removal of free estrone and cleavage of conjugated estrogens with β-glucuronidase/arylsulfatase from helix pomatia (Serva GmbH, Heidelberg, Germany). Intra- and inter-assay CV were between 9.4 and 12.5%, and the lower limit of detection was at 0.1 ng/ml. For the measurement of cortisol, plasma was extracted with ethyl acetate. The antiserum was raised against cortisol-3-CMO–BSA. Intra- and inter-assay CV were 9 and 14% respectively. The detection limit was at 0.5 ng/ml (Hoffmann *et al.* 1994).

Assessment of TGC numbers

In order to identify trophoblast cells at advanced stages of differentiation, the ability of *Phaseolus vulgaris* leucoagglutinin (PHA-L; Vector Laboratories, Burlingame, CA, USA) to specifically bind to sugar moieties of glycoproteins produced by differentiating and mature TGCs was used. Lectin histochemistry was performed as previously described (Klisch *et al.* 2006) with slight modifications. Sections of 4 μm prepared from formalin-fixed paraffin-embedded tissue were dewaxed in xylene, rinsed in three changes of ethanol, rehydrated in descending concentrations of ethanol, and rinsed in running tap water. The slides were then rinsed in ICC buffer (PBS/0.3% Triton X-100), and incubated for 45 min in a humid chamber at 37 °C with 10 μg/ml biotinylated PHA-L (Vector Laboratories). After washing in ICC buffer (5 min), the slides were incubated with a preformed streptavidin/biotin–peroxidase complex (from the Vectastain Elite ABC kit 6101, Vector Laboratories) for 30 min at room temperature. After washing with ICC buffer (5 min), the sections were incubated with the substrate (NovaRED substrate kit, Vector Laboratories) for 4.5 min. As control, the lectin was replaced by ICC buffer. In order to assess the percentage of mature trophoblast cells, the total number of trophoblast cells and the number of distinctly PHA-L-positive trophoblast cells were registered in one section from each animal by viewing 20 chorionic villi randomly distributed over the complete height of the interdigitation zone. A one-factorial ANOVA was used to test for an influence of the experimental group, followed by a pairwise comparison of experimental groups by Tukey–Kramer multiple comparison test (GraphPad Software, Inc., San Diego, CA, USA).

Evaluation of the reduction in the caruncular epithelium

For the quantification of caruncular epithelium reduction, sections were prepared from formalin-fixed tissue as described earlier and stained with hematoxylin–eosin. Maternal crypts surrounding nine cross-sectioned chorionic villi of a predefined size range randomly distributed over the complete height of the interdigitation zone were evaluated in one section from each animal. The area corresponding to the carunclar epithelium surrounding each of the villi was measured using the IM1000 software (Leica, Bensheim, Germany) and expressed as percentage of the area encircled by the basal membrane of the caruncular epithelium. Finally, for each of the animals, the mean percentage was calculated. Data were subjected to a one-factorial ANOVA for an influence on the experimental group, followed by a pairwise comparison of experimental groups by Tukey–Kramer multiple comparison test (GraphPad Software, Inc). The level of significance was set at $P<0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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