

Low progesterone concentration in early pregnancy is detrimental to conceptus development and pregnancy outcome in horses

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ABSTRACT

High progesterone concentrations in the early luteal phase support pregnancy, whereas subphysiological progesterone concentrations delay embryonic development at least until placentation. In this study, fetal growth and development of pregnancy was investigated in pregnancies with prostaglandin $F_{2\alpha}$ -induced low progesterone concentrations (PGF) in the early luteal phase and control pregnancies (CON) in the same mares ($n = 12$). Mares were inseminated and in PGF pregnancies received the prostaglandin $F_{2\alpha}$ analogue cloprostenol ($62.5 \mu\text{g}$) on days 0–3 after ovulation to induce subphysiological progesterone concentrations; CON pregnancies remained untreated. Mares were assigned to PGF or CON treatments in alternating order and received the opposite treatment in the following year. Blood was collected and conceptus size determined repeatedly by transrectal ($\leq$ day 101) and transabdominal ($>$ day 101) ultrasonography. After birth, foals were weighed, measured and submitted to a clinical examination. Treatment PGF resulted in fewer pregnancies than CON treatment. All foals born from CON pregnancies were healthy and mature, whereas 4/7 PGF pregnancies were either lost (one embryonic death, one abortion) or resulted in the birth of compromised foals ($P = 0.018$). Size of the conceptus (e.g., diameter day 49: PGF 6.6 ± 0.7 , CON 7.7 ± 0.7 cm, $P = 0.006$) and embryo proper (e.g., crown rump length day 54; PGF 4.4 ± 0.8 , CON 5.8 ± 0.6 cm, $P = 0.015$) differed between treatments. These size differences decreased over time and at birth PGF foals did not differ significantly from CON foals. In conclusion, reduced progesterone concentration in the early luteal phase leads to delayed conceptus growth beyond placentation and increased pregnancy loss.

1. Introduction

In horses, as in several other domestic animal species (Spencer and Bazer, 2002), physiological progesterone concentration in the early luteal phase supports pregnancy whereas experimentally reduced progesterone concentration delays embryonic development at least until placentation (Okada et al., 2020a). In mares with reduced postovulatory progesterone concentration, the physiological downregulation of endometrial progesterone receptors is retarded and this in turn leads to changes in histotroph composition (Willmann et al., 2011a; b; Beyer et al., 2019). Similar findings have previously been reported for cattle (Okumu et al., 2010; Lonergan, 2011). In cows, early postovulatory progesterone concentration is positively correlated with the endometrial expression of several

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genes related to conceptus development (Lonergan, 2011). Elevated progesterone concentration enhances conceptus elongation and stimulates interferon tau secretion (Garrett et al., 1988; Mann et al., 2006; Satterfield et al., 2006). An association between reduced progesterone concentrations and early pregnancy loss in cattle has been reported (Lonergan, 2011). Also, in mares, endogenous progesterone concentrations five days after ovulation were positively correlated to pregnancy rate at day 14 (Hollinshead et al., 2022). Postovulatory progestin treatment stimulated conceptus growth in aged but not in younger mares (Willmann et al., 2011b). Conceptus fixation in the uterus of mares occurred later when plasma progesterone concentration was reduced after ovulation by low doses of a prostaglandin $F_{2\alpha}$ agonist compared to untreated pregnant control mares (Okada et al., 2020a). When conceptuses were collected by transcervical flushing of the uterus on day 34, i.e., shortly before the beginning of placentation, those from pregnancies with reduced progesterone concentration before day 14 were smaller, lighter, and morphologically less developed than embryos from untreated control pregnancies (Okada et al., 2020a). Histomorphometric analysis showed also a more developed endometrium in control pregnancies compared to pregnancies with reduced progesterone concentration (Okada et al., 2022). Subphysiological progesterone concentrations are thus likely to increase pregnancy failure in mares and at least to some extent contribute to economic losses in horse breeding.

Together, the negative effects of subphysiological plasma progesterone concentration in early equine pregnancy on development of the conceptus and endometrium until placentation (Okada et al., 2020a, 2022) support the idea of progestin supplementation during the early postovulatory period in mares with a history of early pregnancy loss (Willmann et al., 2011a,b). Such mares may benefit from progestin treatment because of transiently low endogenous progesterone concentration in blood. On the other hand, low progesterone concentration in the early postovulatory phase diminishes the negative feedback on the hypothalamic-pituitary axis in early pregnancy and, thus, stimulate a luteal tissue response and formation of secondary corpora lutea (Okada et al., 2020b) with a subsequent increase in progesterone synthesis. This response might occur too late to counteract the effects of low progesterone concentration in the early postovulatory phase (Okada et al., 2020a).

To what extent subphysiological plasma progesterone concentration in the early postovulatory phase affects fetal development beyond the initiation of placentation has not, to the best of our knowledge, been investigated. In this study, therefore, conceptus growth throughout gestation, embryonic and fetal loss, as well as foal characteristics were compared in pregnancies with experimentally reduced progesterone concentration in the early luteal phase and physiological control pregnancies in the same mares. We hypothesized that despite a rapid normalization of plasma progesterone concentration, the alterations induced by transient initial progesterone deprivation persist throughout pregnancy and lead to the birth of smaller and potentially compromised foals.

2. Materials and methods

2.1. Animals

Initially, 12 Shetland mares were included into the study. Mares were between 3 and 19 years old (10.6 ± 4.7 years, $\pm$ SD) and weighed 200 ± 40 kg ($\pm$ SD, range 146–260 kg). Before the study, mares underwent a thorough breeding soundness evaluation that included transrectal ultrasonography of the genital tract, an endometrial biopsy for histopathology and an endometrial swab for bacteriological analysis. The absence of pathologies, endometrial bacterial growth, and a histological classification of the endometrium as I or IIA (Kenney, 1978) were prerequisites for enrolment of mares into the study. Throughout pregnancy, mares were kept in groups in outdoor paddocks with access to a stable. They were fed hay twice daily; water and mineral supplements were available continuously. Mares were transferred to straw-bedded individual foaling stables between days 305 and 310 of pregnancy calculated from the day of ovulation. Size of the foaling boxes was 3.5×3.5 m. Mares were monitored continuously via video cameras in the foaling stables when clinical signs of imminent parturition were evident. All foalings were uneventful and no assistance was required.

2.2. Experimental design

The experiment was approved by the competent authority for animal experimentation in Austria (Federal Ministry for Science and Research, license number 2020–0.547.889).

Estrous mares were inseminated with raw semen from a fertile Shetland stallion at 48-h intervals until detection of ovulation (Day 0; i.e., disappearance of the preovulatory follicle) and randomly assigned to a $PGF_{2\alpha}$ treatment (PGF) or control pregnancy (CON). In the treatment pregnancy group, mares received the $PGF_{2\alpha}$ analogue cloprostenol ($62.5 \mu\text{g}$ once daily i.m.; Estrumate; MSD, Vienna, Austria) for four consecutive days from Day 0 until Day 3 with the aim to reduce luteal progesterone secretion during the early postovulatory phase (Beyer et al., 2019). The cloprostenol dose was adapted from Haflinger to Shetland mares considering that the weight of Shetland mares was approximately 50 %. Mares were left untreated in the control pregnancy group. The order of treatments was changed among mares making sure that half of the mares started with the CON and the other half with the PGF pregnancy. Mares received the opposite treatment in the subsequent year and thus served as their own controls. Mares and their pregnancies were only included in analysis of fetal growth and foaling outcome if both the PGF and the CON pregnancy developed at least until the stage of detection of the embryo proper by transrectal ultrasound. Blood samples were collected throughout pregnancies for progesterone analysis at different intervals (daily until day 15, every five days between days 15 and 60, every two weeks between days 60 and 280, every 10 days between day 280 and foaling). Detection of pregnancy was performed by transrectal ultrasonography (DP-6600Vet; 6LE5Vs rectal transducer, 5–8 MHz, Mindray, Shenzhen, China) and conceptus size was determined by transrectal ($\leq$ day 101 of pregnancy) and transabdominal ultrasonography ($>$ day 101 of pregnancy; C5–1s convex transducer 1.4–5.1 MHz, Mindray) at regular intervals (see below).

Immunoglobulin G (IgG) concentration was determined in colostrum at foaling by sugar refractometer as described previously (Chavatte et al., 1998). Colostrum was collected from both mammary complexes of each mare and the mean of both measurements used for further comparisons. Foals were checked for signs of dysmaturity by clinical examination and white blood count (polymorphonuclear leukocyte/lymphocyte ratio; N/L ratio) immediately after birth. In the foals, time from birth to first standing and to first suckling their dam's udder was recorded. Foals were weighed and measured, and placental weight and size were determined as described previously (Beythien et al., 2017). Transfer of immunoglobulins in foals was measured 18–22 h after birth by densimeter (590a; Animal Reproduction Systems, Ontario, Canada).

2.3. Progesterone analysis

Progesterone in plasma was determined with an enzyme immunoassay for progesterone (Enzo Progesterone ELISA, Cat. No.: ADI-901–011, Enzo Life Sciences, Farmingdale, NY, USA) as described previously and validated for equine plasma in our laboratory (Nagel et al., 2012). The antiserum cross-reacts 100 % with 5 α -pregnane-3,20-dione, thus besides progesterone measuring accurately the most important pregnancy-specific equine progestin. According to the manufacturer, cross-reactivity is 3.5 % with 17-OH-progesterone and <1 % for other steroids tested. When referring to progesterone (in ng/mL) in the results section of this manuscript, this therefore includes progesterone-immunoreactive steroids such as 5 α -pregnane-3,20-dione. The intra-assay coefficient of variation was 6.1 % and the inter-assay coefficient of variation was 8.2 %. The minimal detectable concentration of the assay was 6.1 pg/mL.

2.4. Determination of conceptus development

From day 12 until day 101 after ovulation, pregnancy was evaluated at regular intervals (every second day from day 12–24; every 5 days until day 50, every two weeks until day 101) by transrectal ultrasonography to monitor conceptus growth and development (DP-6600Vet; Mindray). The size of the embryonic vesicle and the embryo proper were measured in two dimensions at a 90-degree angle by electronic calipers of the ultrasound machine. The mean of the two measurements was calculated to be the mean diameter of the vesicle. The size of the embryo proper was determined by measuring its biggest length, i.e., its craniocaudal axis. This measure closely corresponds to the crown-rump length of the embryo proper (Okada et al., 2020a). Diameter of the fetal ocular orbita was measured every two weeks from day 60–101 by transrectal and from days 130–300 by transabdominal ultrasonography.

2.5. Fetomaternal heart rate and heart rate variability

On days 290 and 300 of gestation, fetomaternal electrocardiogram (ECG) recordings were made with the Televet 100 system (version 4.1.3, Kruuse, Marslev, Denmark) as described previously by our group (Nagel et al., 2010). This ECG device uses a filter allowing to display and analyze maternal and fetal cardiac action both combined and separately and to amplify the fetal signal for evaluation. Data were transferred to a computer via Bluetooth. During recordings, mares remained in their normal surroundings. Recordings were always made in the morning between 8:00 and 10:00 am.

For analysis of heart rate and heart rate variability (HRV), Kubios HRV Software (Biomedical Signal Analysis Group, Department of Applied Physics, University of Kuopio, Finland) was used. For determination of heart rate and HRV, three 5-min intervals from the start, middle and end of each recording were selected taking into account also signal quality. For all further calculations, mean values from these three intervals were used. To remove trend components, data were detrended and, in addition, an artifact correction was made as in previous studies on horses following established procedures (Nagel et al., 2010; Schmidt et al., 2010). From the recorded beat-to-beat (RR) intervals, heart rate and the HRV variable root mean square of successive RR differences (RMSSD) was calculated for the mare and her fetus.

2.6. Statistical analysis

Data were analyzed with the SPSS statistics software (version 28, IBM-SPSS, Armonk, NY, USA). Data distribution was normal, and variances were homogenous. Changes over time throughout pregnancy such as size development and progesterone concentration profiles were analyzed by GLM (general linear model) for repeated measures ANOVA with both time and treatment (PGF and CON) as within subject factor, taking into account that the same mares were studied in two consecutive pregnancies. Data obtained at one time point only, such as gestation length or foal size, were compared between treatments by Wilcoxon signed rank test. The number of viable foals per treatment was compared by χ^2 analysis. For all comparisons, a *P*-value <0.05 was considered significant. Data are given as mean $\pm$ standard error (SEM).

3. Results

3.1. Pregnancy results and clinical findings in foals

From the initial number of 12 mares, six were assigned to PGF and CON treatment, respectively in year one, eventually resulting in three PGF and six CON pregnancies. Only these nine mares were inseminated again in the second year and assigned to the opposite treatment, resulting in additional four PGF and three CON pregnancies. Out of the seven PGF pregnancies, three healthy foals and two compromised foals were born, whereas one CON pregnancy was aborted on day 202 and eight CON pregnancies resulted in the birth of

healthy foals. Thus 3/12 PGF inseminations and 8/9 CON inseminations resulted in the birth of healthy foals ($P = 0.016$, χ^2 -test, Table 1). The aborted CON fetus had a laterally deviated rostral maxilla and bacteriological culture yielded moderate growth of *Bacteroides pyogenes*.

Both a CON and a PGF pregnancy in two subsequent years was achieved in seven mares and these 14 pregnancies were included into further analysis. All seven CON pregnancies proceeded uneventful to term and resulted in the birth of healthy and viable foals. In contrast, one PGF pregnancy was lost between days 29 and 34 and another PGF fetus between days 59 and 73. In the fetus, sporadic growth of *Weissella* spp. was detected by bacterial culture. One foal born from an uneventful PGF pregnancy had increased serum amyloid A concentration at birth (250 mg/L) which increased to a peak of 1500 mg/L during the first day of life. The foal suffered from moderate meconium impaction, had a dilated, but intact urinary bladder and increased pulmonary sounds on auscultation. The foal was treated initially with sulfadiazine-trimethoprim and then with penicillin and gentamicin. Urination was stimulated by bethanechol treatment. The foals condition improved, and no clinically apparent health problems were evident from day 3 of life onwards. Another foal born from a PGF pregnancy had respiratory problems from the day of birth onwards, characterized by whistles and increased respiratory effort. Based on clinical findings and on ossification of the carpal and tarsal joints on radiography of the foal was classified as mature. On tracheal endoscopy performed 11 days after birth, the dorsoventral diameter of the trachea was increased and the latero-lateral diameter decreased, resembling a moderate to severe saber-sheath trachea. Despite supportive care and antibiotic treatment, the condition of the foal deteriorated continuously. The foal was therefore euthanized 36 days after birth. On necropsy, the saber-sheath trachea was confirmed. In addition, a purulent bronchopneumonia was diagnosed. The other three foals from PGF pregnancies were mature and healthy at least throughout the first three months of life. The seven PGF pregnancies thus resulted in three non-compromised, viable foals and the seven CON pregnancies in the birth of seven viable and healthy foals ($P = 0.018$, χ^2 -test; Table 1).

3.2. Plasma progesterone concentration

Plasma progesterone concentrations were analyzed separately for the time intervals day 0–34 ($n = 7$ complete pairs for analysis) and day 35 to term ($n = 5$ complete pairs for analysis, two mares with early pregnancy loss excluded). During the first interval, progesterone concentrations increased but the pattern differed between PGF and CON pregnancies with a reduced concentration in the PGF pregnancies (time $P < 0.001$, treatment $P < 0.001$, time x treatment $P < 0.001$; Fig. 1). During the second time interval, progesterone concentration increased and dropped rapidly shortly before foaling (time $P < 0.001$) but did not differ between PGF and CON pregnancies; Fig. 1).

3.3. Conceptus growth

Size of the conceptus determined by transrectal ultrasound until day 50 of pregnancy increased ($P < 0.001$) and was smaller in PGF than in CON pregnancies ($P = 0.006$; Fig. 2a; e.g., day 49: PGF 6.6 ± 0.7 , CON 7.7 ± 0.7 cm). Similarly, size of the embryo proper increased ($P < 0.001$) and this increase was less pronounced in PGF than in CON pregnancies of the same mares (treatment $P = 0.015$, time x treatment $P = 0.006$; Fig. 2b; e.g., day 54: PGF 4.4 ± 0.8 , CON 5.8 ± 0.6 cm). As expected, the diameter of the fetal orbita increased over time ($P < 0.001$), but the diameter of the orbita was smaller in PGF compared to CON pregnancies between days 60 and 101 of gestation ($P = 0.007$; e.g., day 73: PGF 0.73 ± 0.03 , CON 1.05 ± 0.03 cm). From day 129 of gestation onwards, there was no longer a significant difference in diameter of the fetal orbita between PGF and CON pregnancies (Fig. 3).

3.4. Gestation length, fetomaternal heart rate and immunoglobulin G transfer

The four PGF pregnancies resulting in birth of healthy and viable foals did not differ either in gestation length or in colostrum IgG content determined by Brix refractometer from the respective control pregnancies in the same mares. Average gestation length for all foals was 320 ± 2 days and the one non-viable foal resulting from a PGF pregnancy was born on day 323 of gestation. The five PGF foals born at term did not differ from the respective CON foals in average weight, size, time to first standing, suckling, meconium release and urination and the N/L ratio in blood immediately after birth (Table 2). Time from birth to first suckling in the non-viable foal born from a PGF pregnancy, however, was 127 min and thus longer than in any other foal of the study,

As expected, heart rate was higher in the fetus than in its dam ($P < 0.001$) at the same time. A slight increase in maternal heart rate over time but decrease in fetal heart rate was reflected by a significant time x mare and foal interaction ($P = 0.031$). There was no

Table 1

Information on the total number of mares inseminated, their pregnancies and foals born.

Endpoint	Prostaglandin F _{2α} (PGF)	Control (CON)
Mares inseminated (n)	12	9
Mares pregnant (n)	7	9
Healthy foals (n)	4	8
Mares included (CON+PGF pregnancy; n)	7	7
• Embryonic loss (< day 40; n)	1	0
• Fetal loss (> day 40; n)	1	0
• Problem foals (n)	2	0
• Healthy foals (n)	3	7

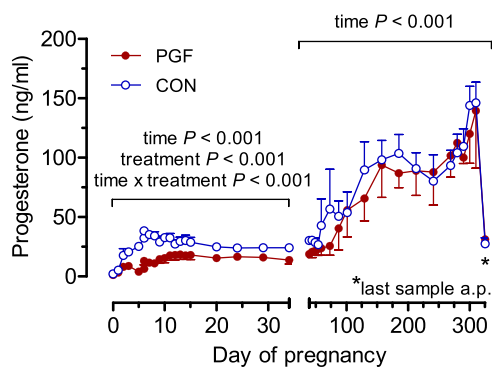


Fig. 1. Progesterone concentration throughout pregnancy in plasma of mares treated with a $\text{PGF}_{2\alpha}$ analogue during the first four days after ovulation (PGF) and in untreated control pregnancies (CON) of the same mares. Values are means and SEM, $n = 7$ complete pairs for day 0–34 and $n = 5$ complete pairs for day 35 to foaling.

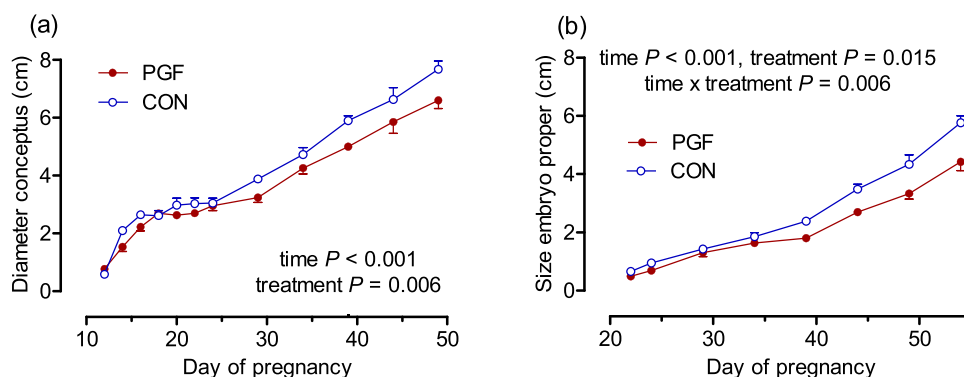


Fig. 2. Maximal diameter of (a) the conceptus from day 12–50 and (b) the embryo proper from day 22–54 of pregnancy in mares treated with a $\text{PGF}_{2\alpha}$ analogue during the first four days after ovulation (PGF) and in untreated control pregnancies (CON) of the same mares, values are means and SEM, $n = 6$ complete pairs.

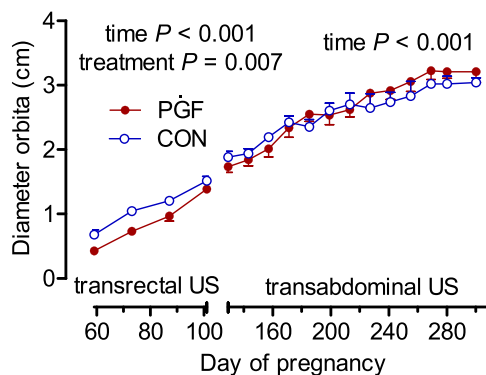


Fig. 3. Maximal diameter of the fetal orbita from day 60–101 of pregnancy determined by transrectal ultrasonography (US; $n = 6$ per group) and from day 129–300 of pregnancy determined by transabdominal US ($n = 5$ per group) in mares treated with a $\text{PGF}_{2\alpha}$ analogue during the first four days after ovulation (PGF) and in untreated control pregnancies (CON) of the same mares, values are means and SEM.

significant difference between PGF and CON pregnancies, neither in the mares nor their fetuses (Fig. 4a,b). The HRV variable RMSSD was lower in the fetus than in the mare ($P = 0.023$) but there was neither a significant difference between PGF and CON pregnancies nor a significant change over time.

Immunoglobulin G concentration in plasma of foals at 18 h after birth was >2000 mg/dl and thus above the measuring range of our analysis system in the four healthy PGF foals and in all seven CON foals. In the non-viable PGF foal, plasma IgG concentration was 942 mg/dl.

Table 2

Foaling and foal data for mares carrying their foals to term either treated with a PGF_{2α} analogue during the first four days after ovulation (PGF) and in untreated control pregnancies (CON) of the same mares (means $\pm$ SEM; Wilcoxon pairwise comparisons for 5 pairs).

	PGF	CON	P-value
Characteristics of mares			
Gestation length (d)	319 $\pm$ 4	321 $\pm$ 2	0.581
Brix in colostrum	29.9 $\pm$ 0.4	26.7 $\pm$ 1.0	0.066
Weight after foaling (kg)	213 $\pm$ 13	215 $\pm$ 15	0.892
Placenta weight (kg)	2.0 $\pm$ 0.1	1.9 $\pm$ 0.1	0.786
Placenta surface (m ²)	0.65 $\pm$ 0.05	0.64 $\pm$ 0.04	0.893
Amnion weight (kg)	0.7 $\pm$ 0.1	0.5 $\pm$ 0.1	0.066
Allantois weight (kg)	1.3 $\pm$ 0.1	1.3 $\pm$ 0.0	0.489
Characteristics of foals			
Weight (kg)	19.2 $\pm$ 0.8	20.5 $\pm$ 1.4	0.500
Weight (% of mare)	9.1 $\pm$ 0.5	9.5 $\pm$ 0.3	0.225
Height at withers (cm)	62 $\pm$ 2	65 $\pm$ 2	0.138
Elbow-carpus (cm)	21.1 $\pm$ 0.5	21.1 $\pm$ 0.9	0.893
Carpus-fetlock (cm)	16.8 $\pm$ 0.8	16.3 $\pm$ 0.5	0.686
Carpus circumference (cm)	10.1 $\pm$ 0.3	10.0 $\pm$ 0.2	0.796
Thorax circumference (cm)	60.9 $\pm$ 1.3	61.4 $\pm$ 0.9	0.893
Time to first standing (min)	30.0 $\pm$ 4.3	29.6 $\pm$ 4.8	0.893
Time to first suckling (min)	89.4 $\pm$ 17.1	73.2 $\pm$ 15.4	0.686
Time to first meconium (min)	147.4 $\pm$ 34.6	121.4 $\pm$ 25.3	0.500
Time to first urine (min)	472 $\pm$ 49	456 $\pm$ 80	0.686
Neutrophil/lymphocyte ratio	4.5 $\pm$ 1.3	3.4 $\pm$ 0.5	0.345

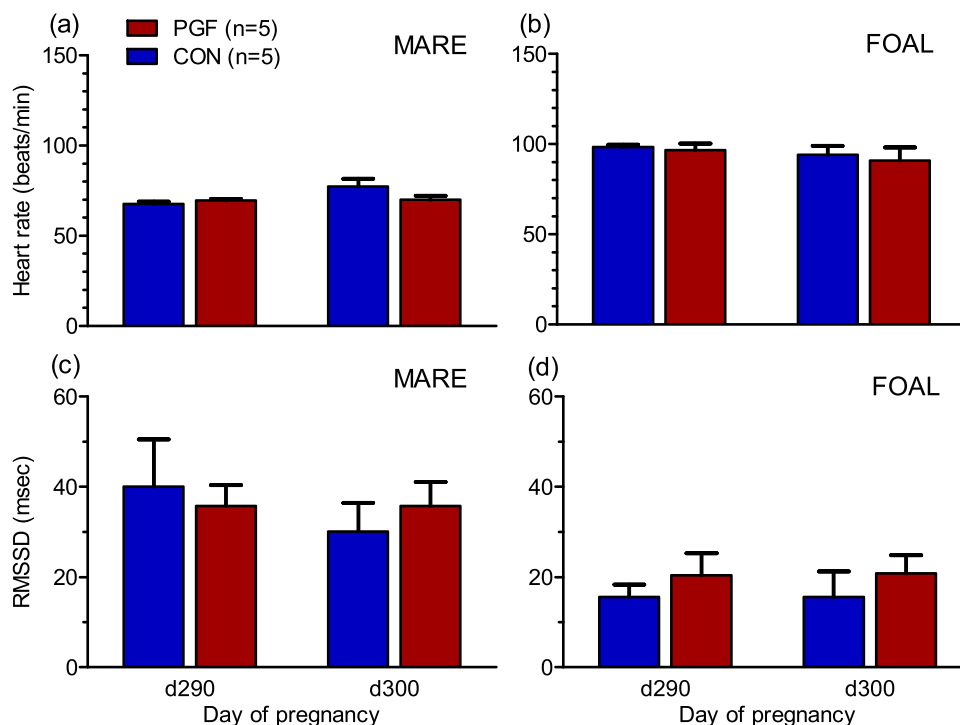


Fig. 4. (a,c) Maternal and (b,d) fetal (a,b) heart rate and (c,d) heart rate variability (RMSSD) on days 290 and 300 of pregnancy in mares treated with a PGF_{2α} analogue during the first four days after ovulation (PGF) and in untreated control pregnancies (CON) of the same mares, values are means and SEM. For heart rate mare vs. foal $P < 0.001$ and time $\times$ mare and foal $P = 0.031$, for RMSSD mare vs. foal $P = 0.023$.

4. Discussion

Our study indicates that subphysiological progesterone concentration in the early luteal phase of pregnancy in mares influences conceptus development well beyond the beginning of placentation. Conceptus size differences between pregnancies with experimentally induced low progesterone concentration and control pregnancies decreased, however, over time and were no longer detectable after the end of the eCG phase. Nevertheless, subphysiological progesterone concentration in the early postovulatory phase

was associated with two pregnancy losses. Furthermore, one compromised foal that had to be euthanized at five weeks of age was born from a mare with subphysiological progesterone concentration. This foal did not suckle before two hours after birth. It is unclear if the delayed neonatal adaption in this foal was anyhow associated with early luteal phase maternal progesterone concentration of the dam or was caused by other unidentified factors.

The present data extend recent findings from our group on pregnancy and conceptus development until day 34 when progesterone concentration was experimentally reduced during the first 10 days of pregnancy (Okada et al., 2020a,b; 2022). These studies showed smaller and lighter embryos in mares with subphysiological progesterone concentration in the early luteal phase compared to controls. The present data indicate that in pregnancies that continue to term and result in the birth of viable and healthy foals, the initially reduced growth can be compensated. Such compensation did, however, not happen in all pregnancies as reflected in the finding that pregnancy losses occurred more often in mares with subphysiological progesterone concentration than in control pregnancies. In a recent study, embryos and fetuses collected from early pregnancy losses were smaller than clinically normal embryos and fetuses (Kahler et al., 2021). Reduced conceptus size thus reflects predisposition for pregnancy loss and subphysiological progesterone concentration very early in pregnancy delays conceptus growth. Further studies should address the effects of progestin treatment in early pregnancy compared to non-progestin-treated mares with subphysiological progesterone concentration.

Furthermore, pregnancy rate on day 12 after insemination was reduced in PGF-treated mares, whereas all CON mares were diagnosed pregnant on day 12. In our experimental model, plasma progesterone was reduced in PGF pregnancies from day 3–14 after ovulation. In horses, conception and early embryonic development can occur in a severely progesterone-deprived environment (Leisinger et al., 2018; Mak et al., 2019), experimentally induced by repeated consecutive injections of PGF_{2α} starting within 12 h from detection of ovulation (Coffman et al., 2014). But already on day 7, embryos obtained from progesterone-deprived mares were markedly smaller than embryos obtained from non-PGF_{2α}-treated controls or PGF_{2α}-treated mares supplemented with the progestin altrenogest and were classified as degenerated when collected on day 8 (Leisinger et al., 2018; Mak et al., 2019). Undisturbed luteal function during early pregnancy is thus critical in eliciting proper embryonic and endometrial gene expression (Leisinger et al., 2019; Pinto, 2020; Okada et al., 2020a, 2022). It is thus highly likely that a proportion of PGF_{2α}-treated mares in our study had initially been pregnant but had already lost their pregnancy because of reduced progesterone concentrations before pregnancy diagnosis on day 12. In agreement with the present study in Shetland mares, smaller embryos were collected from PGF_{2α}-treated Haflinger mares compared to untreated controls, but the initial pregnancy rate assessed on day 10 after ovulation was similar in PGF_{2α}-treated and control Haflinger mares (Okada et al., 2020a). We interpret this difference as an effect of horse body mass with the Haflinger, a near full-size breed, and the Shetland as one of the smallest horse breeds. Although the dose of the PGF_{2α} analogue cloprostenol was reduced from 125 µg in the approximately 450 kg Haflingers (Okada et al., 2020a) to 62.5 µg in the 200 kg Shetland mares of the present study, this may still result in a higher effective dose of cloprostenol in the Shetlands. A higher incidence of early embryonic loss due to treatment is thus feasible in the smaller mares.

With several experimental pregnancies apparently lost before pregnancy diagnosis, observations on further development of confirmed pregnancies potentially until foaling were based on a smaller number of experimental animals than expected from our previous study in Haflinger mares (Okada et al., 2020a). Conclusions on pregnancy development beyond placentation therefore need to be interpreted with some care. Further studies could also include mares with spontaneously reduced progesterone concentration in early pregnancy either left untreated or supplemented with progestins.

In the one foal with a respiratory pathology that finally had to be euthanized, an abnormal morphological appearance of the trachea was diagnosed by endoscopy and confirmed on necropsy. The dorsoventral diameter of the trachea was increased and the latero-lateral diameter decreased, resembling a saber-sheath trachea. Findings in a single foal must be interpreted with caution, but in our previous study on day-34 embryos, the trachea was classified as developed in all control embryos, but only in four out of seven PGF_{2α}-treated embryos (Okada et al., 2020a). These results suggest that abnormalities of the trachea may have originated already from the pre-placentation phase of pregnancy.

Fetal growth retardation and a higher incidence of neonatal illness have been described in aged broodmares and could potentially be caused by less efficient placental development and by reduced fetomaternal nutrient transfer because of endometrial degeneration (Morley and Townsend, 1997; Wilsher and Allen, 2003). In contrast, mare age was not related to foal birth weight and gestation length in other studies (Elliott et al., 2009; Neto da Silva et al., 2022) and it has been suggested that older pluriparous mares develop a heavier, more vascularized placenta which might represent an adaptation of older mares to provide an appropriate nutrition to their fetus (Neto da Silva et al., 2022; reviewed by Derisoud et al., 2022). Growth-retarded foals were not born in our study, further indicating endocrine rather than endometrial factors as the cause for pregnancy loss. Also, no differences in placental weight and surface at foaling existed between PGF_{2α}-treated and control pregnancies although differences in the state of placental development were determined on day 34 of pregnancy in mares from a similar experimental approach (Okada et al., 2022).

Normal term pregnancies in PGF_{2α}-treated mares of the present study are also indicated by fetomaternal heart rate and heart rate variability. Findings agree with a previous study aimed at comparing fetomaternal heart rate and heart rate variability between Shetland ponies and large-size Warmblood horses (Nagel et al., 2011). For the fetus, the only way to react to reductions in oxygen supply is a reduction of cardiac action and decrease in heart rate. Subsequently, decompensation through loss of central regulatory mechanisms leads to persistent tachycardia and finally bradycardia and cardiac arrest (Manning, 2002; Bocking, 2003). Normal fetal heart rate is thus an indicator of fetal well-being. Heart rate variability, i.e., short-term fluctuations in heart rate, reflects the balance of sympathetic and parasympathetic tone. Decreases in heart rate variability reflect a shift towards more sympathetic dominance, while increased values indicate a shift towards parasympathetic dominance (Von Borell et al., 2007). In humans, fetal heart rate variability rises markedly after week 30 of gestation, reflecting maturation of the autonomous nervous system which controls cardiac activity (Wheeler et al., 1979; Van Leeuwen et al., 1999). Reduced heart rate variability is considered a sign of fetal compromise (Dawes et al.,

1992) but heart rate variability also provides information on the developmental stage of the fetal autonomic nervous system (Schneider et al., 2008).

Progesterone profiles in the present study extend recent findings (Okada et al., 2020a; b) beyond placentation and until parturition. Treatment with PGF_{2α} on days 0–3 of pregnancy reduced plasma progesterone concentration until approximately day 14 when compared to control pregnancies. In the present study, the dose of PGF_{2α} was reduced compared to the previous study (Okada et al., 2020a), considering the size difference between Shetland mares and the full-size type Haflinger mares studied previously. This resulted in similar progesterone concentration profiles in both control and experimental mares of the two studies. In the present study, progesterone concentration profiles were continued until foaling and, after approximately day 14 of pregnancy, were close to identical in PGF_{2α}-treated and control pregnancies. Because chorionic girdle height was reduced in PGF_{2α}-treated mares on day 34 of pregnancy (Okada et al., 2022), a delayed onset of eCG secretion, formation of accessory corpora lutea and the associated increase in plasma progesterone concentration in this phase might have been expected but was not evident from our results.

5. Conclusions

Reduced plasma progesterone concentration in the early luteal phase results in increased early embryonic loss and delays fetal growth beyond placentation. Treatment with synthetic progestins early in pregnancy may thus be justified in mares with reduced endogenous progesterone synthesis in the early luteal phase. It is therefore recommended to determine blood progesterone concentration on day 5 after ovulation before such a treatment is initiated.

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CRediT authorship contribution statement

Lisa-Hélène Wagner: Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft. Carolina T.C. Okada: Conceptualization, Investigation, Methodology, Formal analysis. Maria Melchert, Martim Kaps, Svenja Claaßen: Investigation, Methodology. Jörg Aurich: Formal analysis, Methodology, Funding acquisition, Writing – original draft. Camille Gautier: Methodology, Validation, Formal analysis. Christine Aurich: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no financial or personal relationship with other people or organizations that could inappropriately influence or bias this article. As an Editor with Animal Reproduction Science, Dr. Christine Aurich was not privy to any of the review process other than the anonymous reviews that were returned to her as corresponding author.

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References

- Beyer, T., Rink, B.E., Scarlet, D., Walter, I., Kunert, S., Aurich, C., 2019. Early luteal phase progestin concentration influences endometrial function in pregnant mares. *Theriogenology* 125, 236–241.
- Beythien, E., Aurich, C., Wulf, M., Aurich, J., 2017. Effects of season on placental, foetal and neonatal development in horses. *Theriogenology* 97, 98–103.
- Bocking, A.D., 2003. Assessment of fetal heart rate and fetal movements in detecting oxygen deprivation in-utero. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 110 (Suppl. 1), 108–112.
- Chavatte, P., Clément, F., Cash, R., Grongnet, J.-F., 1998. Field determination of colostrum quality by using a novel practical method. *Proc. Am. Assoc. Equine Pract.* 44, 206–209.
- Coffman, E.A., Pinto, C.R.F., Snyder, H.K., Leisinger, C.A., Cole, K., Whisnant, C.S., 2014. Antiluteogenic effects of serial prostaglandin F_{2α} administration in cycling mares. *Theriogenology* 82, 1241–1245.
- Dawes, G.S., Moulden, M., Redman, C.W., 1992. Short-term fetal heart rate variation, decelerations, and umbilical flow velocity waveforms before labor. *Obstet. Gynecol.* 80, 673–678.
- Derisoud, E., Auclair-Ronzaud, J., Palmer, E., Robles, M., Chavatte-Palmer, P., 2022. Female age and parity in horses: how and why does it matter? *Reprod. Fertil. Dev.* 34, 52–116.
- Elliott, C., Morton, J., Chopin, J., 2009. Factors affecting foal birth weight in Thoroughbred horses. *Theriogenology* 71, 683–699.
- Garrett, J.E., Geisert, R.D., Zavy, M.T., Morgan, G.L., 1988. Evidence for maternal regulation of early conceptus growth and development in beef cattle. *J. Reprod. Fertil.* 84, 437–446.
- Hollinshead, F.K., Mehegan, M.K., Gunn, A., Nett, T., Bruemmer, J.E., Hanlon, D.W., 2022. The correlation of endogenous progesterone concentration in diestrus on early pregnancy rate in Thoroughbred mares. *J. Equine Vet. Sci.* 118, 104127.
- Kahler, A., McGonnell, I.M., Smart, H., Kowalski, A.A., Smith, K.C., Wathe, D.C., de Mestre, A.M., 2021. Fetal morphological features and abnormalities associated with equine early pregnancy loss. *Equine Vet. J.* 53, 530–541.
- Kenney, R.M., 1978. Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a note on early embryonic death. *J. Am. Vet. Med. Assoc.* 172, 241–262.

- Leisinger, C.A., Medina, V., Markle, M.L., Paccamonti, D.L., Pinto, C.R.F., 2018. Morphological evaluation of day 8 embryos developed during induced aluteal cycles in the mare. *Theriogenology* 105, 178–183.
- Leisinger, C.A., Klein, C., Markle, M.L., Premanandan, C., Sones, J.L., Pinto, C.R.F., Paccamonti, D.L., 2019. Altered gene expression in embryos and endometrium collected on day 8 of induced aluteal cycles in mares. *Theriogenology* 128, 81–89.
- Loneragan, P., 2011. Influence of progesterone on oocyte quality and embryo development in cows. *Theriogenology* 76, 1594–1601.
- Mak, C.K., Medina, V., Markle, M., Pinto, C.R.F., 2019. 49 collection of day 7 equine embryos in aluteal cycles in mares. *Reprod. Fertil. Dev.* 31, 150.
- Mann, G.E., Fray, M.D., Lammings, G.E., 2006. Effects of time of progesterone supplementation on embryo development and interferon-T production in the cow. *Vet. J.* 171, 500–503.
- Manning, F.A., 2002. Fetal biophysical profile: a critical appraisal. *Clin. Obstet. Gynecol.* 45, 975–985.
- Morley, P.S., Townsend, H.G.G., 1997. A survey of reproductive performance in Thoroughbred mares and morbidity, mortality and athletic potential of their foals. *Equine Vet. J.* 29, 290–297.
- Nagel, C., Aurich, J., Aurich, C., 2010. Determination of heart rate and heart rate variability in the equine fetus by fetomaternal electrocardiography. *Theriogenology* 73, 973–983.
- Nagel, C., Aurich, J., Palm, F., Aurich, C., 2011. Heart rate and heart rate variability in pregnant warmblood and Shetland mares as well as their fetuses. *Anim. Reprod. Sci.* 127, 183–187.
- Nagel, N., Erber, R., Bergmaier, C., Wulf, M., Aurich, J., Möstl, E., Aurich, C., 2012. Cortisol and progesterin release, heart rate and heart rate variability in the pregnant and postpartum mare, fetus and newborn foal. *Theriogenology* 78, 759–767.
- Neto da Silva, A.C., Costa, A.L., Teixeira, A., Alpoim-Moreira, J., Fernandes, C., Fradinho, M.J., Rebordão, M.R., Silva, E., Ferreira da Silva, J., Bliebernicht, M., Alexandre-Pires, G., Ferreira-Dias, G., 2022. Collagen and microvascularization in placentas from young and older mares. *Front. Vet. Sci.* 8, 772658.
- Okada, C.T.C., Kaps, M., Scarlet, D., Handschuh, S., Gautier, C., Melchert, M., Aurich, J., Aurich, C., 2020a. Low plasma progesterin concentration during the early postovulatory phase impairs equine conceptus development in the late preimplantation phase. *Reprod. Fert. Dev.* 32, 1156–1167.
- Okada, C.T.C., Kaps, M., Perez Quesada, J., Gautier, C., Aurich, J., Aurich, C., 2020b. Diestrous ovulations in pregnant mares as a response to low early postovulatory progesterone concentration. *Animals* 10, 2249.
- Okada, C.T.C., Kaps, M., Reichart, U., Walter, I., Gautier, C., Aurich, J., Aurich, C., 2022. Low plasma progesterone concentration during the early luteal phase delays endometrial development and the beginning of placentation in mares. *Anim. Reprod. Sci.* 247, 107149.
- Okumu, L.A., Forde, N., Fahey, A.G., Fitzpatrick, E., Roche, J.F., Crowe, M.A., Loneragan, P., 2010. The effect of elevated progesterone and pregnancy status on mRNA expression and localisation of progesterone and oestrogen receptors in the bovine uterus. *Reproduction* 140, 143–153.
- Pinto, C.R.F., 2020. Impact of the corpus luteum on survival of the developing embryo and early pregnancy in mares. *Theriogenology* 150, 374–381.
- Satterfield, M.C., Bazer, F.W., Spencer, T.E., 2006. Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. *Biol. Reprod.* 75, 289–296.
- Schmidt, A., Möstl, E., Wehnert, C., Aurich, J., Müller, J., Aurich, C., 2010. Cortisol release and heart rate variability in horses during road transport. *Horm. Behav.* 57, 209–215.
- Schneider, U., Frank, B., Fiedler, A., Kaehler, C., Hoyer, D., Liehr, M., Hauelsen, J., Schleussner, E., 2008. Human fetal heart rate variability—characteristics of autonomic regulation in the third trimester of gestation. *J. Perinat. Med.* 36, 433–441.
- Spencer, T.E., Bazer, F.W., 2002. Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. *Front. Biosci.* 7, 1879–1898.
- Van Leeuwen, P., Lange, S., Bettermann, H., Grönemeyer, D., Hatzmann, W., 1999. Fetal heart rate variability and complexity in the course of pregnancy. *Early Hum. Dev.* 54, 259–269.
- Von Borell, E., Langbein, J., Després, G., Hansen, S., Leterrier, C., Marchand-Forde, J., Marchand-Forde, R., Minero, M., Mohr, E., Prunier, A., Valance, D., Veissier, I., 2007. Heart rate variability as a measure of autonomic regulation of cardiac activity for assessing stress and welfare in farm animals—a review. *Physiol. Behav.* 92, 293–316.
- Wheeler, T., Cooke, E., Murrills, A., 1979. Computer analysis of fetal heart rate variation during normal pregnancy. *Br. J. Obstet. Gynaecol.* 86, 186–197.
- Willmann, C., Budik, S., Walter, I., Aurich, C., 2011a. Influences of treatment of early pregnant mares with the progestin altrenogest on embryonic development and gene expression in the endometrium and conceptus. *Theriogenology* 76, 61–73.
- Willmann, C., Schuler, G., Hoffmann, B., Parvizi, N., Aurich, C., 2011b. Effects of age and altrenogest treatment on conceptus development and secretion of LH, progesterone and eCG in early-pregnant mares. *Theriogenology* 75, 421–428.
- Wilsher, S., Allen, W.R., 2003. The effect of maternal age and parity on placental and fetal development in the mare. *Equine Vet. J.* 35, 476–483.