The luteal blood flow, area and pixel intensity of corpus luteum, levels of progesterone in pregnant and nonpregnant mares in the period of 16 days after ovulation

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ABSTRACT: The objective of the present study was to examine if luteal blood flow (LBF) monitoring could be used as an additional prognostic tool for early pregnancy diagnosis, and we particularly focused on the differences in LBF between pregnant and nonpregnant mares. Furthermore, other possible developmental differences of corpus luteum (CL) between pregnant and nonpregnant mares were evaluated. The CL (n = 119) of 27 mares were monitored once daily in B- and Power-Doppler Mode on days 1, 2, 9, 12, and 16 after ovulation (day 0 = ovulation). The data were evaluated using the MIXED Linear Model with repeated measures, and parameters were estimated by the REML method. The course of LBF, area of CL, and pixel intensity differed in nonpregnant mares on a day-to-day basis in contrast to more stable values in pregnant mares. Further, the profiles of the courses were identical until day 9, but since day 12 the differences between pregnant and nonpregnant mares started to be prominent. The LBF, pixel intensity, and level of progesterone (P4) were similar in all mares until day 16, when smaller LBF, lower pixel intensity, and lower levels of P4 were found in nonpregnant mares (P = 0.04, P = 0.02, P < 0.05, respectively). In pregnant and nonpregnant mares the LBF was weakly (r = 0.29 in both) and pixel intensity strongly (r = 0.48 and 0.59, respectively) correlated to the levels of P4. LBF was strongly correlated to the area of CL in pregnant as well as nonpregnant mares (r = 0.72 and 0.64, respectively). In accordance with the results presented in our study we can state that LBF monitoring is not a suitable tool for early pregnancy diagnosis or prognosis as the differences between pregnant and nonpregnant mares are notable – similarly to other indicators of CL status – just after the onset of luteolysis (day 16) when embryo itself is detectable.

Keywords: horse; pregnancy; ultrasonic imaging; Doppler ultrasonography; image analysis

Early pregnancy diagnosis is an important management tool which greatly enhances the efficiency of a breeding program in mares. Breeders want to check their mares for pregnancy as early as possible mostly for economic purposes. There are several more or less reliable methods for early pregnancy diagnosis (reviewed in Ginther, 1992).

In the early pregnancy period the CL plays a crucial role (Ginther et al., 1985). Luteal tissue produces progesterone (P4) and generally the plasma values > 10 ng/ml are sufficient for successful embryo development and maintenance of pregnancy in the early period (Squires et al., 1974; Ginther, 1992). Pregnancy at such an early period could therefore

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be diagnosed by measuring P4 levels (Willmann et al., 2011). Unfortunately, the system is not 100% reliable, as huge variations among measurements performed within one day (Van Niekerk et al., 1973), in individual days following the ovulation (Ginther et al., 2007b), even among individual mares (Perkins et al., 1993) have been published.

The levels of P4 are dependent upon the secretory activity of CL, which is a tributary to blood circulation in the vessels surrounding the entire ovary and in CL (Miyazaki et al., 1998). Currently more widely used colour-Doppler ultrasonography is a suitable method for noninvasive luteal blood flow (LBF) monitoring (Ginther and Utt, 2004) in veterinary practice. The relationship between LBF and P4 production was demonstrated by Herzog et al. (2010) and Garcia-Ispierto and Lopez-Gatius (2012) in cows and Ginther et al. (2007a) and Bollwein et al. (2002) in mares during the estrous cycle. Both the latter studies identically mention that LBF reliably reflects the levels of P4. Obviously by LBF monitoring it is not possible to detect the absolute levels of P4 but it raises the question if inadequate luteal vascularization could be the cause of infertility in mares (Bollwein et al., 2002).

The only study in mares dealing with differences in blood flow between pregnant and nonpregnant (cycling) mares was published by Bollwein et al. (2003) and they were monitoring blood flow in the uterine arteries. They concluded that in comparison with the estrous cycle, the blood flow in the uterus increases since the second week of pregnancy and there are individual variances in uterine blood flow among mares. In this study the pulsed Doppler ultrasonography was used, which is a time-consuming technique requiring well-trained and skilled veterinarians and is hardly practicable in practice.

In contrast, Power-Doppler ultrasonography is not as time consuming and does not require as much experience as pulsed Doppler. Power Doppler ultrasonography easily adds blood flow information to the B-mode image about anatomy and function, which improves the diagnostic and prognostic skill of veterinarians (Ginther and Utt, 2004). Since there are differences in uterine blood flow in early pregnant and nonpregnant mares (Bollwein et al., 2003), the question arises if there are also differences in LBF. So far it has not been stated if it would be possible to use LBF monitoring as an additional relevant diagnostic tool for early pregnancy diagnosis in mares.

Therefore, firstly we intended to examine if LBF monitoring could be used as an additional prognostic tool for early pregnancy diagnosis, while particularly the differences in LBF between pregnant and nonpregnant mares were evaluated. Secondly, other possible developmental differences of CL between pregnant and nonpregnant mares were assessed.

MATERIAL AND METHODS

Animals

The study proceeded in accordance with Decree No. 207/2004 on the Protection, Breeding and Utilization of Experimental Animals and was carried out between March and June 2011 in the Equine Reproduction Center Pardubice-Mnětice. Twenty-seven gynecologically healthy Czech warm-blood mares with a mean age of 10 years were chosen for this study. The mares were artificially inseminated at the time of ovulation (day 0). The ovulation was determined by ultrasonographic examination of ovaries in 6-hour intervals as disappearance of the ovulatory follicle.

Ultrasonography

All examinations were performed by one operator and carried out at the same time (7:00–9:00 h). Luteal dynamics were monitored with a real-time linear array ultrasound scanner equipped with a 7.5 MHz linear rectal probe MyLab™30Vet (Esaote, Maastricht, the Netherlands) on days 1, 2, 9, 12, and 16. The mares were checked for pregnancy on days 9, 12, and 16 after insemination and were retrospectively divided into groups of pregnant (n = 9) and nonpregnant (n = 18). The B-mode was used to identify the CL and then the digital video record of CL was taken. Afterwards, the Colour Doppler Mode (Power-Mode) was activated to display the blood flow in CL. When the image on the screen was free of artifacts, the entire area (with careful slow continuous motion of the probe) of CL was recorded (Bollwein et al., 2002; Ginther et al., 2007a) and all the records were saved on the hard disk of the ultrasound machine. All the examinations were performed at a constant B-mode and Power-Doppler settings: gain 82%, PRC 6-3-H, PRS 2 and gain 70%, PRF 2.8 KHz, PRC 3-L-H, and PRS 3, respectively.

Image analysis

Digital video records were exported from the hard disk of the ultrasound machine to a PC equipped with a specialized software (MyLab™Desk, Version 7, 2011) developed directly for processing of images from MyLab™Vet30 (Esaote) (Rajmon et al., 2012). Three optimal pictures of CL (both in B- and Power-Mode) were chosen here. The optimal picture in Power-Mode means a picture of CL with maximum number of colour pixels in the CL and for B-mode it was the picture with the largest diameter of CL. Afterwards, the pictures were exported to NIS-Elements AR software (Version 3.10, 2009) for further detailed digital image analysis. The region of each CL was manually selected and the following parameters were analyzed: LBF (cm^2), area of CL (cm^2), and pixel intensity of CL (scale 0–255; 0 = black and 255 = white). The pixel intensity of CL was evaluated only on the luteal tissue of CL in accordance with Singh et al. (1997). The mean values of the three selected images were calculated and one mean value was used as referential.

Progesterone assay

The blood samples were obtained from the jugular vein right after ultrasound examination on days 9, 12, and 16. The serum was separated within 1 h, centrifuged (at 3000 *g* for 10 min), and frozen to –20°C until analysis of serum level P4. Levels of P4 were estimated by the chemiluminiscence immunoanalysis (ADVIA Centaur® progesterone kit; Siemens Solution Diagnostics, Erlangen, Germany) at the Institute of Clinical Biochemistry and Laboratory Diagnostics of the 1st Medical Faculty (Charles University, Prague, Czech Republic), and intra- and interassay CVs were 5.3 and 3.6% respectively on a scale of 1.2 to 48.7 ng/ml.

Statistical analysis

All data were subjected to the exploration analysis aimed at testing dependent variables and at identifying outliers or incomplete data. Finally a total of 119 CL were evaluated. LBF, area of CL, and pixel intensity were analyzed using the mixed linear model with repeated measurements. Parameters were estimated using the REML method of the MIXED Procedure of SAS (Statistical Analysis System, Version 9.1, 2006). In the model, random (co)variances were summarized by residual **R** matrix, which was assumed to be a block diagonal with identical submatrices, each corresponding to an individual mare. As alternatives, the compound symmetry, unstructured, and spatial power covariance structures were compared. In accordance with Akaike's Information Criterion and Bayesian Information Criterion (Littell et al., 2000), the spatial power covariance structure was found to be the most appropriate for LBF, whereas the unstructured was used for the area of CL and pixel intensity. The Least Squares Means were calculated and multiple comparisons were made with *P*-values adjusted using Tukey's procedure.

RESULTS

In nonpregnant animals there was a strong variability ($P < 0.05$) in each of the indicators among the monitored days. Nevertheless, the indicators reflected the development of CL, and as a representative example the differences between the monitored days for LBF in nonpregnant mares are shown in Figure 1. For greater clarity the authors agreed not to show these statistically significant results in figures, as Bollwein et al. (2002) and Ginther et al. (2007a) had already published similar day-to-day differences in mares.

The blood flow signals were detectable since day 1 in all CL, and during the 16 days after ovulation the LBF varied (Figure 2a). The mean area of colour pixels in the CL was higher in pregnant ($n = 40$) compared to nonpregnant ($n = 79$) mares (0.88 ± 0.2 and 0.74 ± 0.14 cm^2, respectively; $P = 0.18$). In pregnant mares it was possible to visualize the LBF on all monitored days, in contrast to the 7 (36.8%) nonpregnant mares where the CL was visible but without blood flow signals on day 16, which caused less blood flow on day 16 in nonpregnant mares (lower by 0.5 ± 0.16 cm^2 compared to day 16 in pregnant mares, $P = 0.04$). An approximately two-fold increase in LBF between days 2–9 ($P < 0.05$) was detected in both groups of animals, and CL of pregnant and nonpregnant mares on day 12 tended ($P = 0.1$) to be more engorged, respectively. In pregnant mares the area of colour pixels reached its peak on day 12 in contrast to day 9 in nonpregnant mares. In contrast to the

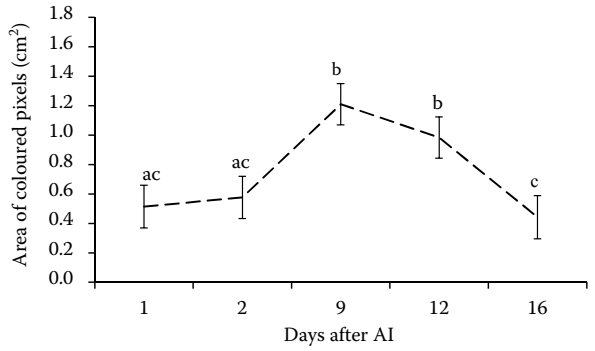

Figure 1. Day-to-day differences in luteal blood flow in nonpregnant mares on days 1, 2, 9, 12, and 16 post ovulation

values are LSM ± SEM, [a–c] results differ on $P < 0.05$ level

other parameters, only the blood flow differed between the monitored days in pregnant mares ($P < 0.05$). Specifically, the mean area of colour pixels on day 1 (0.5 ± 0.2 cm^2) differed from that on day 9 (1.1 ± 0.2 cm^2) and day 12 (1.4 ± 0.2 cm^2) ($P = 0.04$ and $P = 0.003$, respectively), and on day 2 (0.48 ± 0.2 cm^2) and day 12 ($P = 0.008$).

The mean pixel intensity of luteal tissue (mean intensity of grey pixels on a scale of 0–255) did not differ ($P > 0.05$) between pregnant and nonpregnant mares on days 1, 2, 9, and 12 (Figure 2b). A lower intensity of grey pixels was found on day 16 in nonpregnant compared to pregnant mares (60 ± 10 vs. 86 ± 12, respectively; $P = 0.02$). In pregnant mares the pixel intensity values did not vary during the experimental period. On the other hand, in nonpregnant mares the lowest values were on day 1 and the highest on day 12 (79 ± 9 and 100 ± 4, $P = 0.02$). There was a steep decrease between days 12–16 (from 100 ± 4 to 61 ± 9, respectively; $P < 0.0001$).

Throughout the monitored period the mean maximum area of CL in pregnant and nonpregnant mares was 5.6 ± 0.8 and 5.9 ± 0.6 cm^2 ($P > 0.05$), respectively. From Figure 2c it is obvious that CL was the largest on day 2 in both groups of animals. The area of CL in pregnant mares did not vary during the entire experimental period, while in nonpregnant mares there were differences in size between days 1 and 2 (5.9 ± 0.9 and 8.2 ± 0.8 cm^2, respectively; $P < 0.05$) and days 2 and 12 (8.2 ± 0.8 and 5.0 ± 0.4 cm^2, respectively; $P = 0.002$).

The mean levels of P4 (ng/ml) are shown in Table 1, and except day 16 there was no difference between pregnant and nonpregnant mares. The level of P4 was similar during the monitored period (days 9, 12, and 16) in pregnant mares, but in nonpregnant mares the level decreased by

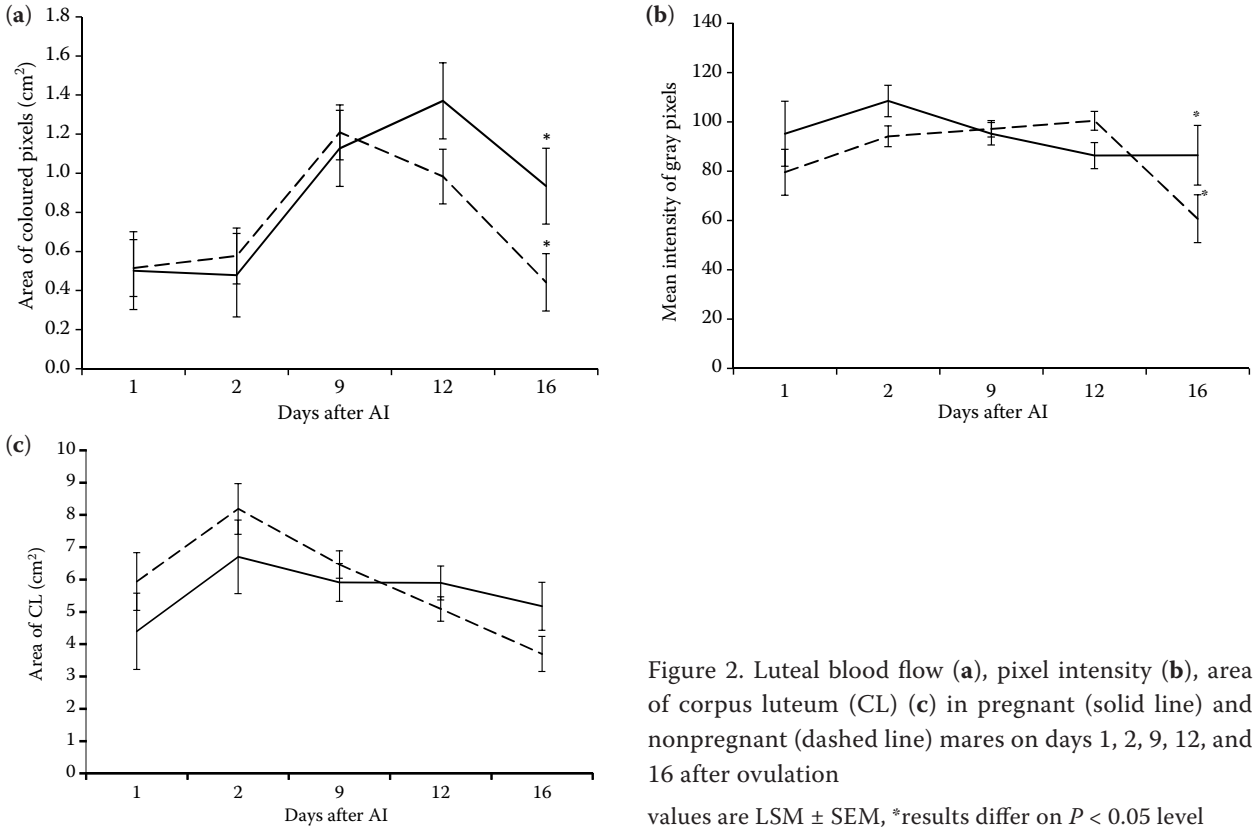

Figure 2. Luteal blood flow (**a**), pixel intensity (**b**), area of corpus luteum (CL) (**c**) in pregnant (solid line) and nonpregnant (dashed line) mares on days 1, 2, 9, 12, and 16 after ovulation

values are LSM ± SEM, *results differ on $P < 0.05$ level

Table 1. Mean progesterone concentrations (ng/ml) in pregnant and nonpregnant mares on days 9, 12, and 16 after ovulation

Status	Progesterone (ng/ml)		
	day 9	day 12	day 16
Pregnant (n = 27)	15.0 ± 1.9	13.4 ± 1.9	14.0 ± 1.9*
Nonpregnant (n = 54)	17.7 ± 1.4[a]	13.2 ± 1.4[b]	5.2 ± 1.4*[c]

*values with asterisks in a column differ ($P < 0.05$)

[a–c]values with different superscripts in a row differ ($P < 0.05$)

4.5 ± 2.1 ng/ml from day 9 to day 12 ($P = 0.03$) and by 8.1 ± 2.0 ng/ml from day 12 compared to day 16 ($P = 0.0002$).

In pregnant mares the correlations were strong for LBF and the area of CL, the area of CL and the level of P4, and for pixel intensity and the level of P4 ($r = 0.72$, $P < 0.0001$; $r = 0.56$, $P < 0.0001$; and $r = 0.48$, $P < 0.05$, respectively). Weak and non-significant correlations were found for LBF and pixel intensity, LBF and the level of P4, and the area of CL and pixel intensity in the pregnant group ($r = 0.14$, $r = 0.29$, $r = 0.29$; respectively, all $P > 0.05$). When we evaluated these correlations with reference to days (days 9, 12, and 16), we found strong but non-significant correlation for LBF and P4 production on day 12 only ($r = 0.49$, $P > 0.05$) and for the area of CL and pixel intensity on day 16 only ($r = 0.45$, $P > 0.05$). In nonpregnant mares all the correlations were significant ($P < 0.05$), probably due to higher number of mares involved (approximately twice the number of pregnant mares). As in pregnant mares, we found no relationship of LBF and P4 production in nonpregnant mares ($r = 0.29$, $P < 0.05$). Strong correlations were found for LBF and the area of CL, the area of CL and pixel intensity, the area of CL and level of P4, pixel intensity and the level of P4 ($r = 0.64$, $r = 0.61$, $r = 0.49$, $r = 0.59$; respectively, all $P < 0.05$). Regarding to the individual days (9, 12, 16) in the monitored period, the relationships varied from very weak and sometimes negative to very strong and significant (e.g. area of CL and pixel intensity on days 12 and 16, from $r = -0.18$ to 0.85, respectively).

DISCUSSION

In the present study we investigated several CL developmental indicators (LBF, pixel intensity, area of CL, and level of P4) in terms of determination of a possible LBF prognostic/diagnostic validity and the differences in CL quality between pregnant and nonpregnant mares. We did not observe any differences in the area of CL between the two monitored groups of mares. The differences connected with pregnancy were manifested in a different P4 production, pixel intensity, and also in LBF compared to nonpregnant mares.

In nonpregnant mares the LBF after ovulation corresponded with the previously reported pattern during the estrous cycle (Bollwein et al., 2002). In the first two days after artificial insemination (AI) the LBF was similar and the peak of LBF was reached on day 9, which is consistent with the work of Ginther et al. (2007a), who published a similar development and peak on day 10. On the other hand, Bollwein et al. (2002) reported LBF peak on day 5. The reason for such different results could be in methodological approach. Besides different numbers of mares, frequency of ultrasound examination, Bollwein et al. (2002) used Power-Mode (PW) in their study and Ginther et al. (2007a) the Colour Doppler Mode (CFM). Also, there could be other factors such as breed or age involved. Until day 9 the LBF was nearly the same in pregnant and nonpregnant mares. Starting with day 9 there was a systematic decrease of LBF continuing through day 12, and the lowest LBF was reached on day 16 in nonpregnant mares. The LBF in pregnant mares continued to increase until day 12 and then it slightly decreased to day 16. Bollwein et al. (2003) showed a similar trend in resistance index (RI) and time-averaged maximum velocity (TAMV) of uterine arteries, where day 9 was also the starting point of different courses of blood flow in the arteries of pregnant and nonpregnant mares. Moreover, in this work (Bollwein et al., 2003) the significant differences between pregnant and nonpregnant mares started to be manifested in both parameters on day 11. In the present study the starting point of different courses of LBF was obvious on day 9 (Figure 2a)

and LBF in pregnant and nonpregnant mares differed on days 12 and 16 (P = 0.1 and P = 0.04, respectively). Since there were 37% of CL visible in nonpregnant mares (in B-mode), but without blood flow signals on day 16, the Power-Doppler monitoring is a very useful tool for evaluation of CL functionality. The values of LBF in nonpregnant mares on day 16 are comparable with published results during the estrous cycle – in this study the LBF decreased to day 2 levels similarly to results given in other studies (Bollwein et al., 2002; Ginther et al., 2007a).

It has been shown in cows that the digital image analysis of CL echotexture is an effective tool for prediction of the functional status of the luteal tissue (Herzog et al., 2008). It would be practical to find out if this phenomenon also exists in mares. Therefore in our study we evaluated pixel intensity of CL through the use of the digital image analysis. The results of the pixels intensity evaluation in CL (scale 0–255; 0 = black and 255 = white) were almost the same in pregnant and nonpregnant mares during the experiment. The values differed only on day 16 (P = 0.02), which is most likely connected with the luteolytic process. It is interesting that mean pixel intensity values significantly differed in nonpregnant mares between individual days (significances not shown) in the experimental period, while in the pregnant we did not find this trend. We think this difference in nonpregnant mares reflects different functional status of CL as it can be seen in the levels of P4 where the values in pregnant mares are stable while in nonpregnant mares they significantly vary (Table 1). Townson and Ginther (1989) analyzed the pixels in CL during 132 h after the onset of luteal development during the estrous cycle, and it is clear that pixel values (scale 0–255) increased between 0–48 h (approximately from 105 to 140) and in the period of 48–132 h they slowly decreased (from 140 to 120). More recently, similar results have been reported by Checura et al. (2002), when mean pixel values decreased from 0 to 6 h and increased from 6 to 8 h and remained stable from 8 to 24 h after ovulation. Herzog et al. (2008) and Siqueira et al. (2009) investigated the relationship between pixel intensity and function of bovine CL. Siqueira et al. (2009) did not find any changes of mean pixel values (0–255) during the estrous cycle in contrast to Herzog et al. (2008) and Gallienne et al. (2012), who reported significant differences in bovine and ovine CL, respectively. The above mentioned results of different studies performed on cows are ambiguous, and the authors of the present manuscript did not find any similar study in cyclic or pregnant mares.

The mean maximum area of CL was similar on all monitored days in pregnant and nonpregnant mares, and the development of this indicator (a curve) was similar until day 9 in both groups (Figure 2c). In nonpregnant mares there was a continuous decrease from day 2, and the minimum area of CL was reached on day 16, while in pregnant mares the decrease was more gradual. Townson and Ginther (1989) published similar absolute values (cm^2) 24 h after ovulation as we did on day 1. CL in that study (Townson and Ginther, 1989) reached smaller sizes 48 h after the onset of development than found by our as well as Bollwein's laboratory, but such differences are not unusual, since Bollwein et al. (2002) published high variations of CL area among individual mares. This could be partially because of normal occurrence of clots in some CL (Pierson and Ginther, 1985). In this study we were not able to determine if the mare was pregnant or not according to the area of CL in contrast to Vecchio et al. (2012), who described differences of area of CL in pregnant and nonpregnant buffaloes.

On the basis of our results, we were not able to prove that the levels of P4 differ with the pregnancy status of the mare during days 1–12. The authors are aware that the results of P4 levels in serum are higher than in some other publications, e.g. Townson et al. (1989), Sevinga et al. (1999), but in this study we focused particularly on the comparison of differences between pregnant and nonpregnant animals. Sevinga et al. (1999) published significant differences in P4 production in pregnant and nonpregnant mares on days 8–9. Ginther et al. (1985) showed different P4 levels on day 11 but not on day 7. Obviously the results between these two studies, even among individual mares (Perkins et al., 1993), vary. To prove that even little differences in P4 levels (although the levels of P4 are > 10 ng/ml) in pregnant and nonpregnant animals are the cause of conception failure, we probably need specifically designed studies. The luteolytic process in a regular cycle of nonpregnant mares takes place around day 16 post ovulation (Nagy et al., 2004), which was, according to the decrease in size, also apparent in our study.

We also evaluated if there are dependent changes among LBF, area of CL, pixel intensity, and P4 production in pregnant and nonpregnant mares.

In pregnant and nonpregnant mares the LBF was weakly correlated (r = 0.29 and 0.29, respectively) to the changes of serum P4 concentrations, although levels of P4 were dependent on the area of CL (r = 0.56 and 0.49, respectively). Also, the pixel intensity of luteal tissue was reflected better (r = 0.48) in P4 production than LBF in pregnant mares as well as in nonpregnant (r = 0.59). Generally we can conclude, that relationships among evaluated indicators were more constant in pregnant than in nonpregnant mares, although there were only nine mares involved in the pregnant group. Bollwein et al. (2002) published stronger (r = 0.58) correlations between LBF and the level of P4 in mares during the estrous cycle and also in the blood flow in uterine arteries during early pregnancy (Bollwein et al., 2003). Strong correlations of LBF are obvious over the estrous cycle in cows (Herzog et al., 2010), but in the mid-luteal phase Lutgenau et al. (2011) concluded that the level of P4 is independent on LBF. The relationship of LBF and P4 levels in our study is similar to published results in mid-luteal phase in cows (Luttgenau et al., 2011), and the reason could be that we calculated the correlations during days 9, 12, and 16, which corresponds to the mid-luteal and late-luteal phase. The relationship of pixel intensity and P4 levels was studied for example in cows (Tom et al., 1998; Herzog et al., 2008), and ewes (Davies et al., 2006; Gallienne et al., 2012) and they found clear positive correlations between these indicators. There are studies in mares (Townson and Ginther, 1989; Checura et al., 2002) of the pixel intensity a few hours after ovulation during the estrous cycle, but the results of pixel intensity were not correlated to the levels of P4. If compared to studies published on cows in the estrous cycle (Tom et al., 1998; Herzog et al., 2008), our results in pregnant and nonpregnant mares correspond with the published data.

CONCLUSION

The present findings revealed different LBF dynamics in pregnant and nonpregnant mares only during the luteolytic period (day 16). In nonpregnant mares there was a huge day-to-day variability (significant difference) in all monitored indicators of luteal development in contrast to pregnant mares, where this phenomenon was very limited. Further, based on our results, we can say that monitoring of LBF is not a suitable prognostic method for early pregnancy diagnosis, but can be used as a very useful tool for identification of vanishing CL in mares.

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