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Abstract: The objective of this study was to investigate the effects of puerperal uterine disease on uterine blood flow using trans-rectal Doppler sonography. Lactating Holstein Friesian cows ($n = 44$) were divided into two groups based on whether they were healthy (UD−; $n = 23$) or had uterine disease (UD+; $n = 21$) defined as retained fetal membranes and/or metritis. General clinical examination, vaginoscopy, trans-rectal palpation, and trans-rectal B-Mode sonography were conducted on Days 8, 11, 18, 25 and then every 10 days until Day 65 after calving. Doppler sonography of the uterine arteries was conducted on Day 8, during diestrus after the second ovulation (Days 40–60 after calving) and during diestrus before breeding (Days 63–75 after calving). Cows with uterine disease had greater ($P < 0.05$) uterine size as assessed trans-rectally compared with cows of the UD group. Sonographic measurements on Day 11 after parturition revealed a greater ($P < 0.05$) horn diameter in cows of the UD+ than in the UD− group. Both uterine size and uterine horn diameter decreased more earlier following parturition ($P < 0.05$) in cows of the UD− group. Blood flow volume (BFV) was greater and pulsatility index was less on Day 8 after calving in cows of UD+ than UD− group ($P < 0.05$). In cows of the UD−, but not in those of the UD+ group, there was a further reduction in BFV subsequent to Day 45 after calving ($P < 0.05$). The results of this study show that uterine blood flow measures by trans-rectal Doppler sonography are affected by puerperal uterine disease.

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The effect of puerperal uterine disease on uterine involution in cows assessed by Doppler sonography of the uterine arteries

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ABSTRACT

The objective of this study was to investigate the effects of puerperal uterine disease on uterine blood flow using tran-srectal Doppler sonography. Lactating Holstein Friesian cows (*n* = 44) were divided into two groups based on whether they were healthy (UD–; *n* = 23) or had uterine disease (UD+; *n* = 21) defined as retained fetal membranes and/or metritis. General clinical examination, vaginoscopy, trans-rectal palpation, and trans-rectal B-Mode sonography were conducted on Days 8, 11, 18, 25 and then every 10 days until Day 65 after calving. Doppler sonography of the uterine arteries was conducted on Day 8, during diestrus after the second ovulation (Day 40 to 60 after calving) and during diestrus before breeding (Day 63 to 75 after calving). Cows with uterine disease had greater ($P < 0.05$) uterine size as assessed trans-rectally compared with cows of the UD group. Sonographic measurements on Day 11 after parturition revealed a greater ($P < 0.05$) horn diameter in cows of the UD+ than in the UD– group. Both uterine size and uterine horn diameter decreased more earlier following parturition ($P < 0.05$) in cows of the UD- group. Blood flow volume (BFV) was greater and pulsatility index was less on Day 8 after calving in cows of UD+ than UD– group ($P < 0.05$). In cows of the UD–, but not in those of the UD+group, there was a further reduction in BFV subsequent to Day 45 after calving ($P < 0.05$). The results of this study show that uterine blood flow measures by trans-rectal Doppler sonography are affected by puerperal uterine disease.

Keywords: Uterus; Blood flow; Metritis; Retained fetal membranes; Bovine

1. Introduction

Many factors affect fertility in dairy cows. Puerperal uterine diseases including retained fetal membranes (RFM) and metritis are associated with an increase in days from calving to first service and a decrease in conception rate at first service (Fourichon et al., 2000; Elkjaer et al., 2013). Results from studies on the effect of uterine disease on uterine involution are ambiguous. According to some authors, the degree of uterine involution in cows is not affected by RFM and abnormal uterine discharge post-partum (Bosu et al., 1984; Holt et al., 1989), whereas others observed delayed uterine involution in cows with puerperal uterine disease (Fonseca et al., 1983; Mateus et al., 2002). Conventional methods for evaluation of uterine involution such as trans-rectal palpation have limitations because of subjective results (Bekana et al., 1994; Okano and Tomizuka, 1987). B-mode ultrasonography allows objective evaluation of the uterus but not of the entire uterus during the early post-partum period (Aslan et al., 2002; Kamimura et al., 1993).

Doppler sonographic evaluation of uterine blood flow has been used for decades to assess uterine involution in women (Jaffa et al., 1996; Nakai et al., 1997; Tekay and Jouppila, 1993). Previous studies in cows evaluated the uterine arteries via Doppler sonography in estrous cycling (Bollwein et al., 2000) and pregnant cows (Bollwein et al., 2002; Panarace et al., 2006) and during the post-partum period (Heppelmann et al., 2012; Krueger et al., 2009). Frequent examinations during the first 2 weeks post-partum revealed a characteristic decrease in uterine blood flow in healthy primiparous cows (Heppelmann et al., 2012). Changes in uterine perfusion until 12 weeks post-partum were recorded in another study with healthy cows (Krueger et al., 2009). The pulsatility index, which measures the resistance in the vascular bed distal to the point of examination underwent distinct changes during the late post-partum period. This led to the conclusion that Doppler sonographic examination of uterine blood flow can serve as an objective method for the description of uterine involution in cows (Krueger et al., 2009). The effects of post-partum uterine disease on uterine blood flow have been described in only one study that was limited to 13 cows examined 2 days after calving (Magata et al., 2013).

During the puerperal period, massive amounts of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were released from the uterus as evidenced in an increase in the metabolite 13, 14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) in peripheral plasma (Lindell et al., 1982; Kindahl et al., 1999). Cows with a disturbed puerperium attributable to RFM and dystocia had greater PGFM plasma concentrations than healthy cows, (Bosu et al., 1984; Nakao et al., 1997).

The main objective of the present study was, therefore, to investigate the effects of post-partum uterine disease on uterine blood flow in dairy cows using trans-rectal Doppler sonography. A secondary goal was to examine the relationship between changes in uterine blood flow and PGFM concentrations.

2. Materials and methods

2.1. Animals

The study was conducted in a herd of 90 lactating Holstein Friesian and Brown Swiss x Holstein Friesian cows at the Research Farm of the University of Veterinary Medicine Hannover, Germany, between February 2009 and July 2010. The cows were housed in a freestall barn and fed a total mixed ration (corn and grass silage with concentrate). Fresh water was available *ad libitum*. The average 305-day milk yield was 10,100 kg. The experimental protocol was approved and conducted in accordance with German legislation on animal rights and welfare (33.9-42502-04-08/1592).

2.2. Study design

Cows were divided into a group of healthy cows without uterine disease (UD–) and a group of cows with RFM and/or metritis (UD+). Fetal membranes were defined as retained when these were not passed within 24 hours after calving. Metritis was diagnosed from Days 4 to 21 (Day 0 = day of calving) by vaginal examination according to a recent study (Sheldon et al., 2009).

On Days 8, 11, 18, 25 and then every 10 days until Day 65 after calving, a general clinical examination, body condition scoring (using a 5-point scale), trans-rectal palpation, vaginoscopy and trans-rectal B-Mode sonography of the internal reproductive organs were conducted. Blood samples were collected weekly. Cows with diseases of other organ systems were excluded from the study. Doppler sonography of the uterine arteries was conducted on Day 8, during diestrus after the second ovulation (Day 40 to 60 after calving) and during diestrus (Day 63 to 75 after calving) before breeding.

For synchronization of time of ovulation, all cows received 0.5 mg of cloprostenol i.m. (Estrumate®, Intervet, Unterschleißheim, Germany) between Days 55 and 60 after calving and 2 days later 0.01 mg of buserelin i.m. (Receptal®, Intervet, Unterschleißheim, Germany). Artificial insemination (AI) was conducted during the subsequent spontaneous estrus. Pregnancy diagnosis was conducted by trans-rectal palpation and sonography 35 to 50 days after AI.

2.3. Trans-rectal palpation and vaginoscopy

During trans-rectal palpation the size of the uterus was assessed. Uterine size was graded according to (Grunert, 1979): uterus retractable and horn diameter <2 cm (score 1), 2 to 5 cm (score 2) or >5 cm (score 3), uterus not retractable but greater curvature palpable (score 4), uterus not retractable and greater curvature incompletely palpable (score 5), and uterus not retractable and greater curvature poorly outlined (score 6).

The vulva was cleaned and vaginoscopy was conducted using a vaginal speculum and a flashlight. During the early post-partum period (< 21 days after parturition), the vaginal examination was conducted to diagnose metritis according to Sheldon et al. (2009). After Day 21, the appearance of vaginal discharge was categorized as absent (Score 0), clear mucus (Score 1), containing flecks of pus (Score 2), consisting of < 50% pus (Score 3) and consisting of > 50% pus (Score 4; from Sheldon et al. (2009), modified).

2.4. Sonography

All sonographic investigations were conducted using a Powervision SSA-370 ultrasonic machine (Toshiba Co., Tokyo, Japan) equipped with a 7.5 MHz microconvex transducer. Cross-sectional images of the uterine horns were obtained by placing the transducer in a transverse direction. The position of the probe for the examination of the uterine horns was approximately 2 cm cranial to the bifurcation. Three images per position were frozen and stored on a Magneto Optical Disc (Sony, Tokyo, Japan). Analysis was conducted off-line on a personal computer using the software FixFoto® (Version 2.74, Joachim Koopmann Software, Wrestedt-Stederdorf, Germany). For further evaluation, the image of the uterus with the greatest contrast and the most circular cross-section was selected from each probe position. The diameter of both uterine horns was estimated. Because the cross-section of the uterine horns was often oval, the diameter was calculated as the mean of the maximum length and width of the endometrium. The ovaries were examined for follicles and corpora lutea.

The pulsed-wave mode was used for Doppler sonography of the uterine arteries. Both uterine arteries were identified as described earlier (Bollwein et al., 2000). The uterine artery ipsilateral to the horn where the previous pregnancy occurred is referred to as ipsilateral artery and the uterine artery contralateral to this horn is referred to as contralateral artery. Blood flow waveforms were

obtained at an insonation angle of 20 to 60 degrees between the Doppler ultrasonic beam and flow direction. The observations were recorded digitally. After each Doppler sonographic examination, the diameter of both uterine arteries was determined using B-mode sonography. For further evaluations, the means of three measurements of the vessel diameter made during one examination were used. Doppler calculations were completed off-line using the software Pixelflux (Chameleon-Software, Leipzig, Germany). Three figures with two similar consecutive flow velocity waveforms with maximum frequency shifts were selected for each investigation. The uterine blood flow was assessed using the blood flow volume (BFV) and the pulsatility index (PI). The PI represents the ratio of the difference between peak systolic frequency shift (PSF) and minimum diastolic frequency shift (MF) to time-averaged maximum frequency shift over the cardiac cycle (TAMF): PI = (PSF–MF)/TAMF. Time-averaged maximum velocity was calculated using the following formula: TAMV [cm/s] = TAMF [Hz] x c [cm/s]/(2F [Hz] x cosα), with c = ultrasonic propagation speed, F = transmitted wave frequency and α = angle between the ultrasound beam and the direction of blood flow. Blood flow volume was computed using the following formula:
BFV [mL/min] = TAMV [cm/s] x π x (D [cm] x 0.5)^2 x 60 with BFV = blood flow volume [mL/min], D = diameter of the uterine artery [cm] and TAMV = time-averaged maximum velocity [cm/sec]. The PI- and BFV-values of the six waveforms were averaged.

2.5. Blood samples and determination of 13, 14-dihydro-15-keto-PGF2α (PGFM)

Blood samples were collected weekly (week 1 to 4) from the jugular vein into tubes containing EDTA, and the tubes were immediately placed on ice. After centrifugation (2000 x *g*, 20 min at 4 °C), plasma was harvested and stored at -20 °C until analysis. PGFM was determined using a competitive enzyme immunoassay (Mishra et al., 2003). The PGFM-horseradish peroxidase conjugate and antiserum were supplied by Prof. Meyer (Physiology Unit, Research Center for

Nutrition and Food Sciences, Technische Universität München, Freising-Weihenstephan, Germany) and PGFM used for the standard curve was purchased from Sigma, Germany. The antiserum used had minimal cross reactions with any of the related prostaglandins, PGE2, PGEM, PGA2, PGAM and PGF2 (<0.01%, (Mishra and Prakash, 2005)). The minimal PGFM detection limit that significantly differed from 0 was 0.5 pg/20 µl plasma/well, which corresponded to 25 pg/ml plasma. The intra-assay coefficient of variation (CV) was 3.5% and the inter-assay CV was 11.4%.

2.6. Statistical analysis

Statistical analyses were conducted using the Statistical Analysis System V9.3 (SAS Institute, Cary, North Carolina). The Shapiro-Wilk test was used to test for normality of the distribution of all variables. Because all variables had non-normal distributions, the median and the median absolute deviation (MAD) values were given. Differences between the UD+ and UD- groups were analyzed using the non-parametric Wilcoxon´s rank sum test (PROC NPAR1WAY). The influence of Day post-partum on variables was determined using the Friedman two-way ANOVA (PROC FREQ). Differences between days within groups were calculated using the Wilcoxon´s signed rank test (PROC UNIVARIATE). The χ^2-test of homogeneity was used to compare categorical data between groups (PROC FREQ). Differences were considered significant at $P < 0.05$.

3. Results

In total, 54 primi- and pluriparous Holstein Friesian cows were used in the study. Five cows were excluded because of diseases of other organ systems (displaced abomasum [$n = 2$], mastitis [$n = 2$] and reticuloperitonitis [$n = 1$]) and another five cows because of technical problems with the ultrasonic machine. This resulted in group sizes of $n = 23$ for UD– and $n = 21$ for UD+. In the UD+ group, four cows (19.1%) had RFM, ten (47.6%) had metritis and seven (33.3%) had RFM and metritis. The cows were 3.7 ± 1.4 years (UD–) and 3.3 ± 1.2 years (UD+) old, parity number was 2.0 ± 1.0 and the body condition score (BCS) was 2.75 ± 0.25 for cows of both groups. These variables did not differ (P > 0.05) between groups.

3.1. Trans-rectal palpation and vaginoscopy

Uterine size was affected by time in both groups (P < 0.0001; Fig. 1). Uterine size decreased (P < 0.0001) by 80% (UD–) and by 70% (UD+) during the first 45 days. After Day 45, there was no further decrease in the UD– group whereas uterine size decreased by another 10% (P < 0.05) in the UD+ group until Day 65. There was a trend for a group effect on uterine size (P = 0.06) for the entire examination period. On Days 8, 11 and 18, the cows of the UD+ group had a greater (P < 0.05) uterine score.

There was no group effect on occurrence of purulent vaginal discharge (Score 2 to 4) for the entire examination period (Day 25 to 65 post-partum; P > 0.05). On Days 35 and 45, more (P < 0.05) cows of the UD+ group had purulent vaginal discharge than cows of the UD– group (Table 1).

3.2. B-mode sonography

The diameter of both uterine horns was affected by time in both groups (P < 0.0001, Fig. 2). Between Days 8 and 45, the diameters decreased by 57% (ipsilateral) / 39% (contralateral; UD–; P<0.0001) and 63% / 52% (UD+; P<0.05). After Day 45, there was no further decrease in cows of the UD– group, whereas uterine diameter decreased by another 2% / 5% in cows of the UD+ group until Day 65. There was no group effect on diameter for the entire examination period (P > 0.05). On Day 11, uterine diameter was larger in cows of the UD+ than those in the UD– group (P<0.05).

3.3. Doppler sonography

The BFV was affected by time in both groups (*P*<0.0001; Fig 3). During the first 45 days after calving, the BFV decreased (*P*<0.05) by 86% (ipsilateral)/ 68% (contralateral; UD–) and by 92% / 95% (UD+). After Day 45, there was no further decrease in BFV in cows of the UD+group, whereas BFV decreased by another 2% / 9% (P < 0.05) in cows of UD– group. There was a group effect on BFV in the contralateral artery for the entire examination period (*P*<0.05); on Day 8, cows in the UD+ group had a greater (*P*<0.05) BFV than cows of the UD–group and on Day 65, BFV in the contralateral artery tended to be greater (*P* = 0.07) in cows of the UD+ than in those of the UD– group.

With the exception of the contralateral artery in the UD– group, there was a time effect on PI (*P*<0.05; Fig. 4). Between Days 8 and 45, the PI of the ipsilateral artery increased (*P*<0.05) by 54% in cows of the UD– group. During the same time period, the PI of the ipsilateral and contralateral arteries increased (*P*<0.05) by 101% and 70%, respectively, in cows of UD+ group. The PI did not change between Days 45 and 65. There was no group effect on PI for the entire examination period (*P*>0.05). On Day 8, the PI of both arteries was less in cows of UD+ than in those of the UD– group (*P*<0.05).

3.4. 13, 14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM)

There was a time effect on PGFM concentration in both groups (*P*<0.0001; Fig. 5). Between Days 7 and 14, PGFM concentrations decreased (*P*<0.05) by 93% and 86% in cows of the UD– and UD+groups, respectively. Afterwards the PGFM-values decreased (*P*<0.05) by a further 1% (UD–) and 6% (UD+) until Day 21. The concentration did not change after Day 21 (*P*>0.05). There was no group effect on PGFM concentrations for the entire study period (*P*>0.05). On Day 21, the PGFM concentration was greater in cows of UD+ than in those of the UD– group (*P*<0.05).

4. Discussion

The results of this study revealed that uterine blood flow was affected by puerperal uterine disease. This was evident by an increased BFV and a decreased PI in the uterine arteries of affected cows 8 days post-partum compared with healthy cows. Blood flow volume in the contralateral artery of affected cows was greater than in healthy cows during the entire examination period. This was similar to women with abnormal puerperium because of retained placenta or endometritis, in which uterine vascular resistance was decreased (Kirkinen et al., 1988; Mulic-Lutvica et al., 2009). Uterine involution in cattle is characterized by a reduction in uterine size and vasoconstriction in uterine vessels (Gier and Marion, 1968; Van Camp, 1991). In healthy cows this translates into a decrease in uterine blood flow and increase in vascular resistance within the first 28 days post-partum (Heppelmann et al., 2012; Krueger et al., 2009). The increased BFV in both arteries of cows with uterine disease in the present study can be interpreted to reflect delayed involution, but the causal relationship between the two phenomena is not clear. Some authors suggested that an increased uterine perfusion is responsible for a delayed uterine involution in women (Kirkinen et al., 1988; Sohn et al., 1988). The vasoconstrictive effect of $PGF_{2\alpha}$ (Slama et al., 1991) did not appear to affect blood flow because the PGFM concentration on Day 8 did not differ between the two groups. PGE_2 may be involved in the increased uterine blood flow in cows with delayed uterine involution; cows with severe post-partum endometritis had greater PGE_2 concentrations in uterine fluid during the first week post-partum than cows with mild endometritis (Mateus et al., 2003). In addition to an immuno-suppressive effect, PGE_2 has potent vasodilating and myorelaxant action (Slama et al., 1991; Still and Greiss, 1978).

Although clinical and histological uterine involution was completed by 40 to 47 days after calving in previous studies (Okano and Tomizuka, 1987; Van Camp, 1991), in the present study it was observed that a further decrease occurred in BFV between Day 45 and 65 in healthy cows. A similar pattern of decreasing uterine perfusion was described in healthy cows studied until Day 86 post-partum (Krueger et al., 2009), which indicated that after completion of uterine involution further vascular changes occur. These late vascular changes do not seem to occur in cows with uterine disease because uterine perfusion remained unchanged between Days 45 and 65 in the present study. It appears unlikely that PGE_2 has an effect at this stage of lactation because the PGE_2 concentration in mares with persistent endometritis was not increased compared with healthy mares (Watson et al., 1987). It is possible that inflammatory vascular changes are responsible for incomplete involution of the vascular bed in cows with uterine disease. Histologically, the angiopathies in cows can be divided into angiosclerosis and pervasculitis (Merbach, 2012). Post-partum endometritis in mares is believed to cause vasculitis with endothelial degeneration resulting in severe damage to the vascular wall (Gruninger et al., 1998). Histomorphological investigations of endometrium in infertile and subfertile cows revealed angiopathies in 77% (Rodenbusch et al., 2007).

However, in contrast to the results of others (Bosu et al., 1984; Holt et al., 1989), we observed an adverse effect of puerperal uterine disease on uterine involution. While there were distinct differences between the two groups of cows in uterine size during trans-rectal palpation, the sonographic examinations revealed only moderate differences. A possible reason for this is that the sonographic examination was limited to a uterine cross-section near the bifurcation, whereas a much larger part of the uterus was assessed during trans-rectal palpation. As expected and in agreement with another study (Holt et al., 1989), more cows of UD+ group had purulent vaginal discharge during the late puerperal period. This confirmed observations by others that metritis and RFM increased the risk of clinical endometritis (Gautam et al., 2009; LeBlanc et al., 2002).

The most pronounced differences between the two groups with respect to uterine size and uterine blood flow variables occurred during the early puerperal period. Interestingly, there was further reduction in uterine size after Day 45 in cows with uterine disease but there were no further changes in uterine blood flow. This suggests that in case of an inflamed uterus, the reduction in uterine size is not necessarily accompanied by vascular involution. It is hypothesized that the inflammatory process affects the vascular system more severely than the connective tissue portion of the uterus, which leads to delayed vascular involution relative to connective tissue involution.

Plasma PGFM concentrations were greater on Day 21 and numerically greater on Day 14 post-partum in cows with uterine disease compared with healthy cows. This was in agreement with other studies, in which cows with uterine infection had greater PGFM concentrations (Del Vecchio et al., 1994) and cows with uterine discharge had longer periods of PGFM release after calving (Lindell et al., 1982) than healthy cows. Another study revealed markedly greater PGFM concentrations 1 to 4 days post-partum in cows with dystocia and/or RFM (Nakao et al., 1997), but comparison of those findings with the present study is difficult.

In conclusion, uterine blood flow was affected by post-partum uterine disease. Diseased cows had a greater BFV and lesser PI particularly in the early post-partum period. In healthy cows, uterine blood flow further decreased after completion of uterine involution whereas in cows with uterine disease the decrease in uterine size between Days 45 and 65 was not accompanied by further changes in uterine blood flow. These results indicate an association between delayed uterine involution and incomplete regeneration of the uterine vascular bed in cows with puerperal uterine disease.

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Table

Table 1

Absolute and relative incidence of purulent vaginal discharge (Score 2 to 4) from Days 25 to 65 post partum in dairy cows without (UD–; *n* = 23) and with uterine disease (UD+; *n* = 21).

	Days post-partum				
Group	25	35	45	55	65
UD-	13.6% (3/22)	4.3%[a] (1/23)	9.1%[a] (2/22)	4.8% (1/21)	26.1% (6/23)
UD+	38.1% (8/21)	33.3%[b] (7/21)	38.1%[b] (8/21)	10% (2/20)	9.5% (2/21)

Within days after calving, values with different letters are different (P<0.05).

Figure legends

Fig. 1. Changes in uterine size (Scores 1-6) determined by trans-rectal palpation in the first 65 days after parturition in dairy cows without (UD–; n = 23) and with uterine disease (UD+; n = 21). Values are medians ± median absolute deviation. Within days after calving, scores with different letters are different between groups UD– and UD+ ($P < 0.05$).

Fig. 2. Changes in diameter of the formerly pregnant uterine horn determined by ultrasonography in the first 65 days after parturition in dairy cows without (UD–; n = 23) and with uterine disease (UD+; n = 21). Values are medians ± median absolute deviation. Within days after calving, values with different letters are different between groups UD– and UD+ ($P < 0.05$).

Fig. 3. Changes in blood flow volume (BFV) in the ipsilateral and contralateral arteries in the first 65 days after parturition in dairy cows without (UD–; n = 23) and with uterine disease (UD+; n = 21). Values are medians ± median absolute deviation. For both arteries within days after calving, values with different letters are different between groups UD– and UD+ ($P < 0.05$). Values with an asterisk differ from corresponding previous values ($P < 0.05$).

Fig. 4. Pulsatility index (PI) of the ipsilateral and contralateral arteries in the first 65 days after parturition in dairy cows without (UD–; n = 23) and with uterine disease (UD+; n = 21). Values are medians ± median absolute deviation. For both arteries within days after calving, values with different letters are different between groups UD– and UD+ ($P < 0.05$). Values with an asterisk differ from corresponding previous values ($P < 0.05$).

Fig. 5. Plasma PGFM concentrations in the first 28 days after parturition in dairy cows without (UD–; $n = 23$) and with uterine disease (UD+; $n = 21$). Values are medians ± median absolute deviation. Within days after calving, values with different letters are different ($P < 0.05$). Values with an asterisk differ from corresponding previous values ($P < 0.05$).