



Association of peripartum progesterone, estradiol, cortisol, PGFM and relaxin concentrations with retention of fetal membranes in crossbred dairy cows

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ABSTRACT

Retention of fetal membranes (RFM) in bovines not only lowers fertility and productivity but also leads to significant economic loss to the dairy farms. The present study compared peripartum progesterone, estradiol, cortisol, Prostaglandin F₂ metabolite (PGFM) and relaxin concentrations in crossbred cows with normal parturition (NP) and with RFM. Blood samples were collected from 33 dairy cows at weekly interval from 30 days prepartum till 7th day before calving, followed by every alternate days till calving and 1st and 2nd day postpartum. Significantly higher plasma cortisol concentrations was observed between day 3 prepartum and day 2 postpartum in RFM cows (n=6) compared to NP cows (n=6). Plasma estradiol level was significantly lower in RFM group than in NP group on the day of calving while progesterone concentrations did not differ between the groups. In RFM cows, PGFM level was significantly lower on day 1 and day 2 postpartum compared to NP cows. On the day of calving and on day 1 postpartum, relaxin concentration was significantly ($P<0.05$) higher in NP cows compared to RFM cows. The ROC analysis, commonly used for development of diagnostic threshold value, revealed that cows with cortisol concentration above 7.35 ng/ml and PGFM concentration below 1,072 pg/ml on day-1 were associated with 5.99 times higher risk of RFM. It may be inferred that peripartum plasma cortisol and PGFM concentrations may be useful for identification of crossbred cows at the risk of developing RFM.

Keywords: Dairy cows, Hormones, RFM

Retention of fetal membranes (RFM), a condition in which fetal membranes are not expelled within 12 hours after parturition, leads to huge economic loss to the dairy industry (Beagley *et al.* 2010). In addition to compromised reproduction, the milk loss due to RFM in taurine cattle was observed to be about 753 kg per lactation in temperate climate (Dubuc *et al.* 2011). In subtropical climate, RFM-affected crossbred cows produced significantly lower milk than normal cows (Kumari *et al.* 2015). Although consequences of RFM on postpartum production and reproduction performance have been well documented, the etiology and mechanism of RFM is yet to be understood (Attupuram *et al.* 2016).

Multiple etiologies have been associated with occurrence of RFM in dairy cattle including altered endocrine profile (Wischral *et al.* 2001, Gupta *et al.* 2005), impaired inflammatory process (McNaughton and Murray 2009, Boro *et al.* 2014, 2015), altered neutrophil function (Kimura *et al.* 2002, Pathak *et al.* 2015), metabolic disorder (Beagley *et al.* 2010, Kumari *et al.* 2016), altered MHC expression (Davies *et al.* 2004) and reduced expression of short chain fatty acids (Boro *et al.* 2014). A complex endocrinological process is involved in normal parturition and expulsion of

fetal membranes; any deviation from normal endocrine changes can lead to RFM (Beagley *et al.* 2010). Initiation of normal parturition includes elevation of fetal cortisol level leading to an increase in circulating estrogen level and decrease in progesterone level (Wischral *et al.* 2001). Circulating fetal cortisol helps in the conversion of placental progesterone to estrogen by enhancing the activity of 17 α -hydroxylase enzyme (McNaughton and Murray 2009). Elevated estrogen enhances the secretion of maternal PGF₂ α which causes lyses of pregnancy corpus luteum (Beagley *et al.* 2010). Although an increased level of relaxin at parturition upregulates collagenase activity and favours breakdown of feto-maternal interface (Beagley *et al.* 2010), relaxin concentration in RFM cows has not been studied. Similarly, the predictive value of reproductive hormones for RFM in cows has not been studied in detail. If hormone concentrations can be used to identify the cows at the risk of developing RFM, suitable management measures can be implemented to prevent the losses associated with the condition. Keeping these in view, the present study estimated peripheral plasma concentrations of cortisol, estradiol, progesterone, PGFM and relaxin in normal cows and compared with the cows that developed RFM. Receiver operating characteristic analysis was carried out to find out the threshold values of hormones in relation to RFM so

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that it can be used as a tool to identify the cows at the risk of developing RFM.

MATERIALS AND METHODS

The study was conducted on Karan Fries crossbred cows at Livestock Research Centre, ICAR-National Dairy Research Institute, Karnal. Forty days prior to expected date of calving, 33 cows (3rd–5th parity, 420–500 kg body weight and previous lactation yield of 4,000–4,500 kg). Body condition of cows ranged between 3.5 and 4.0 in 6 point scale (Prasad 1994). Cows were maintained under similar management conditions. About 1 week before calving, cows were shifted to calving pen and retained till 4–5 days after calving. The animals were fed as per farm standard (National Research Council, 2001) and closely observed for calving and expulsion of fetal membranes following birth. Cows with abnormal parturition (dystocia, still birth and premature birth) or cows calved 3 days prior to the expected date of calving were excluded. Finally, blood samples from 12 cows (6 animals each from normal parturition (NP) and RFM groups) were used for estimation of hormones. Cows that failed to expel fetal membranes within 12 hours after calving were considered as RFM. Experimental procedures were approved by the Institutional Animal Ethics Committee (IRC project code B-25).

Blood samples were collected at weekly interval from 30 days prepartum till 7th day before calving, followed by every alternate day till calving and 1st and 2nd day after calving between 7:00 and 8:00 AM. Samples were centrifuged at 4°C (1,000 g for 20 minutes) to separate and store plasma in cryovials at –20°C until further analysis. Cortisol, progesterone, estradiol (Endocrine Technologies Inc., USA), PGFM (B-Bridge Technologies Inc., USA) and relaxin (USCN Life Science, USA) concentrations were estimated using ELISA kits as per the instructions given by the manufacturers. Inter assay variations for cortisol, progesterone, estradiol, PGFM and relaxin were 8.6, 12.3, 9.2, 8.9 and 9.1%, respectively while intra-assay variations were 5.7, 6.1, 4.2, 6.4 and 5.4%, respectively. Standard curves of hormone were calculated using GraphPad PRISMS 3.0 software (GraphPad software, USA). Sensitivity of cortisol, progesterone, estradiol, PGFM and relaxin estimation were 1.0 ng/ml, 0.5 ng/ml, 5 pg/ml, 46.2 pg/ml and 8.65 pg/ml, respectively.

Statistical analyses were carried out using SigPlot 11® Software package (Systat software Inc., USA). The effect of RFM, time of parturition and their interaction with various hormones was analyzed by two-way analysis of variance (ANOVA) using General linear model. Tukey post hoc test was used to compare pairwise difference and was considered as statistically significant when $P \leq 0.05$. Hormones that showed significant difference between cows with and without RFM were further analyzed by Receiver operating characteristic (ROC) analysis (Patbandha *et al.* 2013) to develop accuracy as indicated by area under curve (AUC), threshold value (value having maximum combined sensitivity and

specificity) and likelihood ratio [sensitivity/(1-specificity)].

RESULTS AND DISCUSSION

Periparturient hormonal profiles: Cortisol concentration remained almost constant up to day-5 of calving and then increased and reached significantly (Table 1, $P < 0.05$) higher level on the day of calving, then decreased significantly on day 1 and 2 post-partum both in RFM and NP groups which is supported by Dang *et al.* (2013). Cortisol remained comparatively higher in RFM cows as compared to normal cows during the entire sampling period, but the differences were statistically significant ($P < 0.05$) on day -3, day -1, day 0, day 1 and day 2 of calving which is in agreement with previous reports (Gupta *et al.* 2005, Wischral *et al.* 2001). In consonance with our results, a significantly higher level of cortisol just after calving in RFM cows than in NP cows was reported earlier (Kaczmarowski *et al.* 2006). Hypoxia condition of fetus before and at the time of parturition may increase fetal cortisol, which might be the reason for elevated maternal cortisol concentration (Beagley *et al.* 2010). Thus, elevated cortisol on the day of calving, up to certain level, is a physiological alteration rather than pathological, but beyond that levels it may alter the expulsion of fetal membranes. Higher cortisol concentrations in RFM cows compared to NP cows indicate stress in cows that developed RFM. Elevated concentrations of cortisol during parturition have been shown to have an immunosuppressive effect and impair the neutrophil functions by affecting the activity and life pattern of neutrophils (Burton *et al.* 2005). Further, circulating cortisol concentrations were reported to be negatively correlated to expression of Fas, CD 62L, CD 18/11b and Caspase genes in neutrophils (Pathak *et al.* 2015). Thus elevated cortisol concentrations and associated alterations in neutrophil functions might be a reason for development of RFM in these cows.

Although plasma progesterone did not differ statistically between RFM and NP cows (Table 1), declined significantly ($P < 0.05$) from day 14 before calving till the day of calving in both the groups. Plasma estradiol level was significantly ($P < 0.05$) higher on the day of parturition in NP cows compared to pre- and postpartum period (Table 1). However, in RFM cows, the estradiol level was higher on day-1 prepartum. There was a significant difference in estradiol concentrations between NP cows and RFM cows on the day of parturition ($P < 0.05$) which is in agreement with previous reports (Thomas *et al.* 1992, Kankofer *et al.* 1996, Wischral *et al.* 2001). Other study also reported that RFM cows had significantly lower prepartum (from day 10 to day 3) estradiol concentrations as compared with the normal cows (Shah *et al.* 2007). It is evident from our results that progesterone concentration was higher, although non-significant, in RFM cows compared to NP cows. It is possible that conversion of progesterone into estradiol was altered in RFM cows leading to less estradiol concentrations (Thomas *et al.* 1992). Further, it has been reported that decreased progesterone and increased estrogen immediately prior to parturition indicates conversation of progesterone

Table 1. Peripheral blood plasma concentrations of cortisol, progesterone, estradiol, PGFM and relaxin during peripartum period in normal (n=6) and RFM (n=6) cows

Cow	Days related to calving										
	-30 (±2)	-21 (±2)	-14 (±2)	-7	-5	-3	-1	0	+1	+2	
Cortisol (ng/mL)											
Normal Parturition	2.91 ^a ±0.53	3.44 ^{ab} ±0.60	4.28 ^{abc} ±0.58	5.00 ^{abc} ±0.48	5.18 ^{bc} ±0.54	6.04 ^{Ac} ±0.97	6.36 ^{Ac} ±0.42	8.96 ^{Ad} ±0.99	6.32 ^{Ac} ±1.06	5.48 ^{Abc} ±0.68	
RFM	4.33 ^a ±0.60	5.59 ^a ±0.94	5.55 ^a ±0.34	6.30 ^{ab} ±0.58	6.53 ^{ab} ±0.57	8.54 ^{Bbc} ±0.81	9.31 ^{Bc} ±0.48	14.73 ^{Bd} ±1.00	10.79 ^{Bc} ±0.86	8.61 ^{Bbc} ±1.38	
Progesterone (ng/mL)											
Normal Parturition	7.23 ^c ±1.61	6.51 ^{de} ±1.39	5.69 ^{cde} ±1.32	4.06 ^{bcd} ±1.40	3.07 ^{abc} ±0.70	1.82 ^{ab} ±0.35	1.48 ^{ab} ±0.20	0.49 ^a ±0.17	0.82 ^a ±0.35	0.93 ^a ±0.44	
RFM	7.65 ^c ±0.76	6.63 ^{de} ±0.88	6.69 ^{cde} ±1.42	5.47 ^{bcd} ±1.10	4.33 ^{abc} ±1.30	3.34 ^{ab} ±0.75	2.14 ^{ab} ±0.62	1.42 ^a ±0.54	1.08 ^a ±0.57	0.64 ^a ±0.28	
Estradiol (pg/mL)											
Normal Parturition	44.22 ^b ±5.92	48.7 ^{bc} ±6.13	50.28 ^{bc} ±2.88	56.97 ^{bcd} ±3.38	58.21 ^{bcd} ±2.37	60.87 ^{cd} ±2.65	62.8 ^{cd} ±3.9	69.86 ^{Bd} ±1.85	9.97 ^a ±0.91	6.91 ^a ±0.91	
RFM	41.58 ^b ±2.37	48.38 ^{bc} ±3.34	45.69 ^{bc} ±3.92	52.09 ^{bc} ±4.07	54.88 ^{bc} ±4.22	59.93 ^c ±3.04	60.45 ^c ±3.50	57.25 ^{Abc} ±4.85	7.32 ^a ±0.71	5.69 ^a ±0.44	
PGFM (pg/mL)											
Normal Parturition	111.2 ^a ±35.8	185.2 ^a ±82.8	136.8 ^a ±84.9	117.8 ^a ±49.1	59.0 ^a ±8.1	67.3 ^a ±4.4	1714.7 ^{Bb} ±358.7	2724.4 ^{Bc} ±117.4	2172.8 ^{Bb} ±212.3	1842.7 ^{Bb} ±340.3	
RFM	98.5 ^a ±33.8	118.4 ^a ±25.8	149.9 ^a ±25.4	133.44 ^a ±47.5	247.78 ^a ±48.9	424.3 ^a ±111	840.01 ^{Ab} ±176.7	1966.14 ^{Ad} ±100.4	1495.16 ^{Ac} ±150.6	1240.95 ^{Ac} ±149.58	
Relaxin (pg/mL)											
Normal Parturition	111.3 ^{ab} ±9.9	146.7 ^{abc} ±28.8	107.7 ^{ab} ±13.9	94.5 ^a ±8.3	87.7 ^a ±7.8	136.5 ^{abc} ±31.5	99.0 ^a ±8.7	186.1 ^{Bc} ±33.1	166.1 ^{Bbc} ±23.6	153.7 ^{abc} ±8.8	
RFM	106.9 ^a ±16.5	150.9 ^a ±35.9	117.8 ^a ±14.6	139.4 ^a ±12.7	149.3 ^a ±21.3	128.8 ^a ±14.9	145.4 ^a ±26.1	107.9 ^{Ab} ±12.8	84.1 ^{Aa} ±9.2	121.6 ^a ±25.1	

Means with different superscripts in a row (a, b, c, d, e) and in a column (A, B) differ significantly (P≤0.05).

to estrogen under the influence of foetal cortisol to clear any residue of fetal membranes after parturition (Frazer 2001). Moreover, in our study, RFM cows had higher level of circulating cortisol before and on the day of parturition, which might have altered the normal conversion of progesterone to estrogen.

Present study showed significantly higher concentrations of PGFM in NP cows from a day before calving to day 2 after calving (P<0.05), which is in agreement with Wischral *et al.* (2001). They observed higher PGFM levels at parturition in normal cows compared to RFM cows. Prepartum elevation of plasma estrogen enhances secretion of prostaglandin that causes lysis of CL, so circulating PGFM level gradually increases around one week before calving and reached peak on the day of parturition (Frazer 2001). We also observed a significant increase in plasma PGFM in both groups from 3 days preceding calving and peak level on the day of parturition and then declined markedly which is similar to the findings of Mallick and Prakash (2011), who reported peak level of PGFM on the day of calving (1043.4 pg/ml) than one day before (359.8 pg/ml) and after calving (150 pg/ml) in crossbred cows. Estrogen induces prostaglandin synthase (PTGS2) leading to synthesis of endometrial PGF2α (Whittle *et al.* 2000, Streyl *et al.* 2012), which abolishes the progesterone block during the immediate peripartum period to facilitate myometrial contractions (Shenavai *et al.* 2012). These findings are further supported by our results that PGFM concentrations were higher in NP cows compared to RFM cows from day 3 prepartum till days 2 postpartum.

Plasma relaxin level was significantly lower (P<0.05) on the day of calving and on day 1 after calving in RFM cows compared to NP cows. Relaxin level was significantly (P<0.05) higher on the day of calving than during the prepartum period in NP group, while in RFM group no difference was observed during peripartum period. The role of relaxin on relaxation of pelvic ligament and cervix during prepartum and around parturition is well studied in rat, mice and guinea pig (Sherwood 2004). In pregnant beef cattle, administration of exogenous relaxin 5 days before expected date of normal calving resulted premature normal delivery by enhancing pelvic canal expansion and cervical relaxation without retention of fetal membranes though there is premature induction of parturition (Musah *et al.* 1986). Similarly, calving process is advanced in Holstein dairy heifers due to exogenous relaxin administration on 266 or 267 days of gestation which caused pelvic expansion and cervical dilatation (Bagna *et al.* 1991). Relaxin may have role in modification of connective tissue, particularly collagenase degradation during late pregnancy and around parturition in cattle and helps in expulsion of fetal membranes (Musah *et al.* 1987, Beagley *et al.* 2010). To our knowledge circulating relaxin concentrations in RFM cows has not been studied earlier. Though the role of relaxin in the cervical ripening through degradation of collagenous matrix is known (Engelen *et al.* 2009), similar function in the placentome maturation is yet to be explored. However

Table 2. ROC analysis of blood hormones in RFM condition

Hormones	AUC	Optimum threshold value	Se (%)	95% CI for Se	Sp (%)	95% CI for Sp	LR+	P value
<i>Cortisol (ng/ml)</i>								
-3 d	0.80	6.01	100.00	54.07–100.00	66.67	22.28–95.67	3.00	0.078
-1 d	0.97	7.35	100.00	54.07–100.00	83.33	35.88–99.58	5.99	0.006
0 d	0.97	11.44	100.00	54.07–100.00	83.33	35.88–99.58	5.99	0.006
+1 d	0.92	8.73	83.33	35.88–99.58	83.33	35.88–99.58	4.99	0.016
+2 d	0.80	6.00	83.33	35.88–99.58	66.67	22.28–95.67	2.50	0.078
<i>Estradiol (pg/ml)</i>								
0 d	0.87	67.65	85.71	42.13–99.64	85.71	42.13–99.64	5.99	0.018
<i>PGFM (pg/ml)</i>								
-1 d	0.88	1072.00	100.00	54.07–100.00	83.33	35.88–99.58	5.99	0.024
0 d	1.00	2335.00	100.00	54.07–100.00	100.00	54.07–100.00	infinite	0.003
+1 d	0.88	1681.00	83.33	35.88–99.58	83.33	35.88–99.58	4.99	0.024
+2 d	0.72	1397.00	83.33	35.88–99.58	66.67	22.28–95.67	2.50	0.200
<i>Relaxin (pg/ml)</i>								
0 d	0.77	171.40	100.00	54.07–100.00	66.67	22.28–95.67	3.00	0.109
+1 d	1.00	113.70	100.00	54.07–100.00	100.00	54.07–100	infinite	0.003

AUC, Area under curve; Se, Sensitivity; Sp, Specificity; CI, Confidence interval; LR+, Likelihood ratio positive.

it has been reported that circulating relaxin level increases around parturition to enhance uterine collagenase activity and also other tissues like cervix, pelvic symphysis and ligaments for easy separation of fetal membranes (Frazer 2001) and the results of the present study support these findings. We observed 88% increase in relaxin level on the day of calving in NP cows, which might have enhanced the separation of fetal membranes in these cows, however in RFM cows such increase was not observed.

Receiver operating characteristic (ROC) analysis: Area under the ROC curve (AUC) and optimum threshold value along with their corresponding sensitivity (Se) and specificity (Sp) of different hormones at different times in relation to calving are given in Table 2. The AUC for cortisol on the day before calving (day -1) was 0.97 indicating 97% accuracy to differentiate RFM cows from NP cows ($P<0.05$). Similarly, AUC of plasma PGFM on day -1 differentiated RFM cows from NP cows with 88% accuracy ($AUC=0.88$, $P<0.05$). The AUC showed that cortisol had 8% higher accuracy compared to PGFM concentrations in differentiating RFM from NP cows. Optimum threshold values of cortisol on day -1 was 7.35 ng/ml and cows with elevated cortisol concentrations above this threshold level were 5.99 times more likely to develop RFM, but cows with PGFM level below the optimum threshold values (1072 pg/ml) were 5.99 times more likely to develop RFM. Huzzey *et al.* (2011) observed higher association of plasma cortisol >12.35 ng/ml (>34.1 nmol/L) during 2 weeks before calving with RFM in Holstein cows and similar association observed in crossbred cows. Though sample size is small, we observed that monitoring cortisol and PGFM concentrations on a day prior to calving could predict RFM in dairy cows.

Taken together, the concentrations of cortisol were significantly higher in RFM cows compared to NP cows,

while the concentrations of PGFM were significantly higher in NP cows compared to RFM cows. Among the hormones estimated, based on ROC analysis, it was observed that cortisol and PGFM concentrations had significant predictive values for RFM in dairy cows.

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