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Infradian rhythmicity in milk leukocyte activity together with plasma cortisol and prolaction levels throughout the lactation period in high-yielding crossbred cows has been studied in 10 high-yielding (milk production: 5000 l per lactation) Karan Fries crossbred (Holstein Friesian × Tharparkar) cows. Milk and blood samples were collected from all the experimental animals. Isolation of milk phagocytes (neutrophils and macrophages) and lymphocytes were done by density gradient centrifugation. *In vitro* phagocytic index of milk neutrophils and macrophages was performed by colorimetric NBT reductive assay. Mitogen-induced milk lymphocyte blastogenic response was estimated by colorimetric MTT (tetrazolium) assay. Total plasma cortisol and prolactin were estimated by enzyme immune assay. Highest value of plasma cortisol and prolactin was observed at calving which decreased significantly ($p < 0.01$) on 15th day postpartum for both prolactin and cortisol. Immune activity of milk leukocytes was highest on day 0 colostrum and decreased significantly ($p < 0.01$) on 7th day postpartum. A significant ($p < 0.01$) rise of plasma prolactin was observed around 135th and 225th days postpartum, whereas a peak level of plasma cortisol was observed at 105th, 180th, and 270th days postpartum. Phagocytic index of milk neutrophils and macrophages remains almost in a steady state during mid-lactation period (between 100 and 200 days postpartum). A decline in increasing trend of milk phagocytic activity was observed during late lactation. Mitogen-induced milk lymphocyte blastogenic response was highest on day 0 colostrum which decreased significantly ($p < 0.01$) on 15th day postpartum. Con A-induced milk lymphocyte blastogenic response showed an increasing trend from 120th to 210th days postpartum. Upon correlation study, it showed that the plasma cortisol has a negative effect on milk leukocyte activity, while prolactin has a positive effect, though the effect is lactation stage specific.

Keywords: infradian rhythm; milk leukocytes; cortisol; prolactin; crossbred cows; lactation cycle

1. Introduction

An infradian rhythm refers to the rhythm with a period longer than the period of a circadian rhythm (Lewy et al. 1999). Throughout the production cycle, mammary glands

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of dairy cows undergo marked biochemical, cellular, and immunomodulatory changes to withstand the stress occurred due to partitioning of nutrients between the fetus and the mammary gland during pregnancy, colostrum production, attainment of peak yield, and maintenance of lactation persistency which makes the mammary gland more prone to intra-mammary infections. Circadian rhythmicity in immune system has been reviewed by many workers as lymphocyte proliferation (Ana et al. 1996), activity of natural killer cells (Alvaro & Sarkar 2005) and humoral immune response (Fernandes et al. 1976). Circadian rhythm in mammary tissue carcinogenesis has also been reported (Zhang & Zarbl 2008). Mukherjee et al. (2015) reported seasonal variation in milk leukocyte activity. But, the literature is very scanty on the rhythmic alteration of mammary leukocyte activity throughout the lactation cycle. Therefore, the present investigation has been formulated to find out the infradian rhythmicity in milk leukocyte activity throughout the lactation cycle in high-yielding crossbred cows. An attempt has also been made to correlate the change in milk leukocyte activity with plasma cortisol and prolactin levels throughout the lactation cycle with the assumption of their role in mammary immunity.

2. Materials and methods

2.1. *Selection of experimental animals and sampling*

Ten high-yielding (milk production: 5000 l per lactation) Karan Fries (KF) crossbred (Holstein Fresian × Tharparkar) cows were selected from the herd of National Dairy Research Institute, Karnal, Haryana, India. All the cows selected were kept in a loose housing system with brick flooring and managed as per the practices followed in the institute. They were offered ad lib green fodder and calculated amount of concentrate mixture based on milk production only at the time of milking. Fresh tap water was available ad lib at all time of the day.

Composite milk samples (250 ml/cow) from all four quarters were collected into sterile tubes fortnightly from the day of calving throughout the lactation cycle (up to 300 days postpartum). Blood samples (10 ml/animal) were drawn in sterile heparinized vacutainer tubes by the jugular vein puncture, posing minimum disturbance to the animal during collection on the same day of milk sampling. Immediately after collecting, the samples were transported to the laboratory in ice for further processing.

Blood samples were subjected to centrifugation (3000 rpm for 30 min) to separate plasma and stored at −20 °C for further use.

2.2. *Isolation of milk leukocytes*

Isolation of milk phagocytes (neutrophils and macrophages) and lymphocytes was done by density gradient centrifugation (Mukherjee et al. 2013).

2.3. In vitro *activity of milk leukocytes*

In vitro phagocytic index of milk neutrophils and macrophages was performed by colorimetric NBT reductive assay (Choi et al. 2005). Mitogen-induced milk lymphocyte blastogenic response was estimated by colorimetric MTT (tetrazolium) assay (Mosmann 1983).

2.4. *Estimation of plasma cortisol and prolactin*

Total plasma cortisol was estimated by enzyme immune assay reported by Sarkar et al. (2007). Plasma prolactin was estimated by the methods of Roy and Prakash (2007).

2.5. Statistical analysis

All analyses were done using SYSTAT software package. Data from different experiments are presented as mean ± SE. Significance was tested by employing one-way ANOVA, considering days as a factor. The correlation was tested by the Spearman rank-order correlation. To avoid the confounding effects of stage of lactation and yield, correlation was calculated during individual stages of lactation cycle i.e. early, mid, and late lactation.

3. Results

Phagocytic activity of milk neutrophils and macrophages with plasma cortisol and prolactin concentration in high-yielding crossbred cows has been presented in Figure 1. Highest value of plasma cortisol and prolactin was observed at calving which decreased significantly ($p < 0.01$) on 15th day postpartum for both prolactin and cortisol. Phagocytic index of milk neutrophils and macrophages was also highest on day 0 colostrum and decreased significantly ($p < 0.01$) on the 7th day postpartum. A significant ($p < 0.01$) rise of plasma prolactin was observed around 135th to 225th days postpartum, whereas a peak level of plasma cortisol was observed on 105th, 180th, and 270th days postpartum. Phagocytic index of milk neutrophils and macrophages remains almost in a steady state during mid-lactation period (between 100 and 200 days postpartum). A decline in the increasing trend of milk phagocytic activity was observed during late lactation.

Mitogen-induced milk lymphocyte proliferation response with plasma cortisol and prolactin concentration in high-yielding crossbred cows has been presented in Figure 2. Plasma cortisol and prolactin levels during different days postpartum have been discussed earlier. Mitogen-induced milk lymphocyte blastogenic response was highest on day 0 colostrum which decreased significantly ($p < 0.01$) on 15th day postpartum. During the rest of the peripartum period, mitogen-induced milk lymphocyte blastogenic response remained unaltered; however Con A-induced milk lymphocyte blastogenic response showed an increasing trend from 120th to 210th days postpartum.

Correlation between immune activity of milk leukocytes and plasma concentration of cortisol and prolactin in high-yielding crossbred cows during different stages of lactation cycle has been presented in Tables 1 and 2, respectively. Phagocytic index of milk

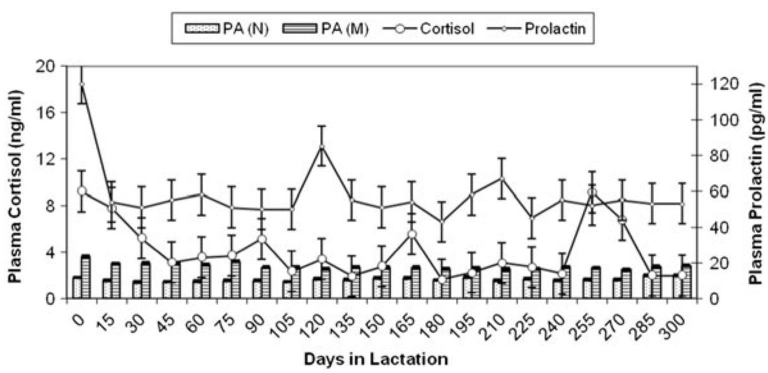

Figure 1. Phagocytic activity of milk neutrophils and macrophages with plasma cortisol and prolactin concentration in high-yielding crossbred cows.
Note: Values are expressed as mean ± SE.

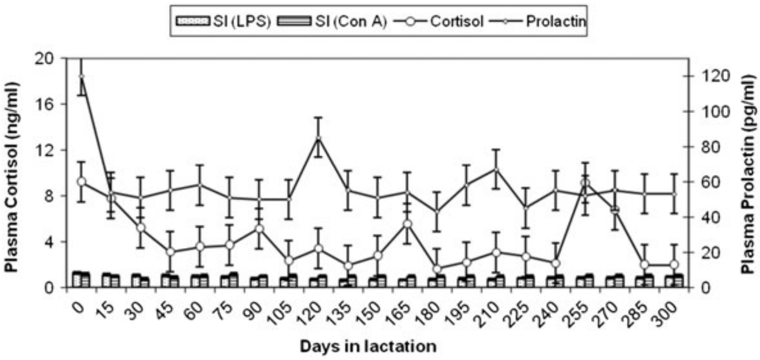

Figure 2. Mitogen-induced milk lymphocyte proliferation response with plasma cortisol and prolactin concentration in high-yielding crossbred cows.
Note: Values are expressed as mean ± SE.

Table 1. Correlation between immune activity of milk leukocytes and plasma concentration of cortisol in elite crossbred cows during different stages of lactation cycle.

	Stages of lactation		
Immune activity of milk leukocytes	Early	Mid	Late
PI (N)	0.40*	−0.57*	−0.60*
PI (M)	0.31	0.31	−0.46*
SI (Con A)	−0.14	0.38	−0.92*
SI (LPS)	0.35	0.21	−0.52*

*Indicates level of significance ($p < 0.05$).

Table 2. Correlation between immune activity of milk leukocytes and plasma concentration of prolactin in elite crossbred cows during different stages of lactation cycle.

	Stages of lactation		
Immune activity of milk leukocytes	Early (0–100 days)	Mid (100–200 days)	Late (200–300 days)
PI (N)	0.12	−0.57*	0.60*
PI (M)	0.23	0.07	−0.03
SI (Con A)	0.61*	0.09	0.75*
SI (LPS)	−0.14	0.05	0.28

*Indicates level of significance ($p < 0.05$).

phagocytes (neutrophils and macrophages) and lipopolysaccharide (LPS)-induced milk lymphocyte blastogenic response were positively correlated with plasma cortisol concentration during the early lactation period. During mid-lactation, PI of milk neutrophils was negatively correlated ($p < 0.05$) with plasma cortisol concentration. But, PI of milk macrophages and mitogen-induced milk lymphocyte blastogenesis was positively correlated with plasma cortisol concentration. Immune activity of all milk leukocytes was negatively correlated ($p < 0.05$) with plasma cortisol concentration during the late lactation stage.

Phagocytic index of milk neutrophils and macrophages was positively correlated with plasma prolactin during early lactation. However, PI of milk neutrophils was negatively correlated with prolactin concentration, but PI of milk macrophages and mitogen-induced milk lymphocyte blastogenesis was positively correlated during mid-lactation. During late lactation, only PI of milk macrophages was found to be negatively correlated with prolactin concentration, whereas PI of milk neutrophils and mitogen-induced milk lymphocyte blastogenesis was positively correlated with prolactin concentration.

4. Discussion

Neutrophils are the first line of defense of the mammary gland which migrate from blood to the mammary gland in response to inflammatory mediators, namely cytokines, complements, and prostaglandins (Zecconi & Smith 2000; Janeway et al. 2001). In the present investigation, highest phagocytic index of milk neutrophils and macrophages was observed in all the cows, which is in accordance with the earlier reports in buffaloes (Dang et al. 2010). Increased activity of colostral phagocytes is due to the presence of a low molecular weight substance of less than 10 kDa (Sugisawa et al. 2003). Altered in functional capabilities of mammary gland, phagocytes were observed during the periparturient period which makes the udder vulnerable to infections (Paape et al. 2002) may be due to the impaired functioning of major defense-related activities, such as phagocytosis, respiratory burst activity, superoxide anion production, random cellular migration, and chemotaxis around this period (Kehrli et al. 1989). Further, there was also a decreased expression of adhesion receptor CD62L (L-selectin), which is necessary for penetration through the endothelium to the sites of infection (Lee and Kehrli 1998). Mehrzad et al. (2001) also reported a decreased chemiluminescence response and viability of milk neutrophils between 3 and 11 d postpartum. An increasing trend of milk phagocytic activity was observed during late lactation in our investigation, possibly due to the lower opsonic activity with decrease in IgM concentrations (Waller 2000). Mitogen-induced lymphocyte blastogenic response is used to measure the immune competence of mammary lymphocytes (Mukherjee et al. 2015). In the present investigation, Concanavalin A (Con A) and LPS were used for stimulating T and B lymphocytes, respectively. Lymphocyte proliferation response was higher in colostrum in accordance with the reports of Ogra et al. (1978) in humans. In the present study, Con A-induced milk lymphocyte blastogenic response showed an increasing trend around mid-lactation. Mukherjee et al. (2013) also reported similar findings in crossbred cows. However, Kehrli et al. (1989) reported diminished leukocyte functions during lactogenesis. Decreased milk lymphocyte function during late lactation was also reported by Mukherjee et al. 2013. Glucocorticoids play an important role in initiation and maintenance of lactation (Akers 1990). At the time of parturition, maternal glucocorticoids are significantly elevated in the blood and milk. During this time, cortisol secretion rate and plasma cortisol concentrations are significantly greater when compared with non-lactating cows or cows in established lactation (Paterson & Linzell 1974). Glucocorticoids are necessary for the formation of milk components (Ray et al. 1996) and to induce differentiation of the lobule-alveolar system (Tucker 1981). The value of plasma cortisol concentration observed in our study was similar to that reported by Pal (1996) in crossbred KF cows. In our study, higher level of plasma cortisol was found at calving which decreased significantly on 15th day postpartum. Similar pattern in the shift of cortisol levels immediately after parturition has been reported in exotic (Goff & Horst 1997) and crossbred cows (Prakash & Madan 1985). Khan and Ludri (2002) observed that the plasma cortisol concentration declined from day 20 to day 2 prepartum, after which it abruptly increased on day 1 prepartum to reach a peak on the day of kidding in goats. The concentration of plasma prolactin in our study was lower when compared to the earlier report of Pal (1996) in crossbred KF cows. We observed large increases in prolactin in peripheral circulation during the day of parturition. In another study where cows were bled twice daily (Ingalls et al. 1971), the peak in prolactin preceded parturition by about 1 day and prolactin returned to prepartum values by 60 h postpartum. In the present study, prolactin peaked at parturition and returned to basal levels within 2 weeks. Whether this parturient peak in prolactin is related to the induction of lactation and/or to parturition as well should be investigated. Possibly, altered prolactin metabolism during lactation, as suggested for pigs (Anderson et al. 1972), masks any active role in reproductive events of the postpartum lactating cow; in heifers, it was observed that prolactin varied with the stages of the estrous cycle (Swanson & Hafs 1971).

Kodagali et al. (1980) reported that the prolactin concentration varied with each stage of lactation, being high, middle, and low in early, mid, and late lactations in Gir cows. Talvelkar and Raikar (1982) reported that prolactin concentrations fell sharply 19 weeks after parturition in Gir cows and 21 weeks after parturition in Gir X HF crossbreeds. They also reported that the stage of lactation significantly correlated with the prolactin concentration. Aliev et al. (1983) reported that the concentration of prolactin was significantly correlated with milk yield in the first three months of lactation in Ala-Tau cows. According to Akasha et al. (1987), serum prolactin concentration declined as lactation progressed. In this investigation, phagocytic index of milk phagocytes and LPS-induced milk blastogenic response in high-yielding cows was negatively correlated with plasma cortisol concentration which is in accordance with earlier reports, which indicated that the glucocorticoids have an inhibitory effect on lymphocyte (Clemens et al. 1979; Pavia et al. 1979; Kincl & Ciaccio 1980) and neutrophil (Nonnecke et al. 1997) functions *in vitro* when administered at pharmacological levels. Roth et al. (1982) reported that the primary anti-inflammatory mechanism of action of glucocorticoids is to prevent neutrophil margination by knocking L-selectin off of neutrophil surfaces (thus reducing neutrophil surveillance for infected tissues) and to potentially reduce the ability of neutrophils to leave the blood stream via decreased expression of CD18 molecules. Increased cortisol during prepartum period suppresses the genes involved in producing inflammatory, antibody, and cytotoxic responses in cattle (Nonnecke et al. 1997). Segei et al. (1980) found inhibition of human lymphocyte blastogenesis by cortisol only when suboptimal mitogen levels were used. The role of prolactin in the immune system has contradictory findings. A series of *in vitro* and *in vivo* studies showed the immunostimulatory effects of prolactin (Gala 1991; Kooijman et al. 1996; Yu-Lee 1997), whereas animals with a targeted disruption of either the prolactin (Horseman et al. 1997) or prolactin receptor (Bouchard et al. 1999) gene suggest that PRL is not essential for normal immune system development or function. In our study, we found lactation stage-specific effect of prolactin over the mammary immunity; this may be due to the fact that prolactin and other pituitary hormones are suggested to act as stress-adaptation molecules important in maintaining steady-state immune system homeostasis (Dorshkind & Horseman 2001). This interpretation is supported by *in vitro* studies showing PRL's protective effect in preventing glucocorticoid-induced lymphocyte cell death (apoptosis) (LaVoie & Witorsch 1995; Buckley 2001) and by *in vivo* studies showing that PRL improves macrophage and splenocyte functions following trauma–hemorrhage and infections (Zellweger et al. 1996).

5. Conclusions

In conclusion, the present study depicts the infradian rhythm of mammary gland immunity together with plasma cortisol and prolactin. Highest activity of milk leukocytes was observed in colostrum together with highest concentration of plasma prolactin and cortisol. A steady-state immune response of mammary gland leukocytes was observed during mid-lactation period which further declined around lactation. Lactation stage-specific effect of plasma cortisol and prolactin was also established.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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