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Phagocytic activity of blood neutrophils and its relationship with plasma concentration of TNF-α, IL-6 and milk SCC in crossbred cows during early lactation

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ABSTRACT

To study the inter-relationship between plasma concentrations of tumour necrosis factor-a (TNF-α) and interleukin-6 (IL-6) with somatic cell count (SCC) and phagocytic activity (PA) of blood neutrophils, blood and milk samples were collected from 16 Karan Fries (KF) cows (8 high producing and 8 low producing) during 75 days of early lactation and from 4 mastitic KF cows during different days of clinical mastitis. PA of blood neutrophils was estimated by nitroblue tetrazolium (NBT) reductive assay. Plasma TNF-α concentration was highest on the day of calving (1.52 ng/ml) and it declined significantly in low yielding cows up to 45th day (0.44 ng/ml), whereas, it declined nonsignificantly in high yielding cows. Peak values of IL-6 were obtained at calving and during peak yield in low yielding cows, whereas, in high yielding cows, peak values were obtained towards peak yield only. PA was lowest at calving in both the group of cows and then increased significantly to normalize with the progression of lactation. Plasma concentration of cytokines TNF-α and IL-6 were significantly higher in mastitic animals as compared to normal animals. Significantly higher concentration of both the cytokines were observed during the first day (TNF-α, 2.09 ng/ml; IL-6, 26.2 ng/ml) of mastitis which later declined by the fifth day (TNF-α, 0.59 ng/ml; IL-6, 13.91 ng/ml) of mastitis. PA of blood neutrophils was lowest (0.15) on day 1 of mastitis and increased significantly by the fifth day (0.31) of mastitis. Plasma concentrations of IL-6, TNF-α and milk SCC during different days of mastitis were negatively correlated with PA of blood neutrophils. This study indicated that the plasma concentration of TNF-α and IL-6 can be used as an indicator of mammary stress around calving in high producing cows.

Key words: IL-6, Neutrophils, Phagocytic activity, SCC, TNF-α

Milk production in dairy cattle is a highly elastic trait as it is easily influenced by many internal and external factors. Due to negative energy balance and physiological stress during the first 100 days of lactation, the high-producing cows are most susceptible to negative external stimuli. Impairment of bovine host defense during this period is also associated with high incidence of diseases like mastitis which may be caused due to endocrine factors associated with metabolic and physical changes (Burton and Kehrli 1995). Use of bovine cytokines in immunotherapy of mastitis or as adjuvants in the recent years in the immuno-potentiation of the mammary gland has revealed their important role in the regulation of the mammary gland defenses. Cytokines such as IL-2, IFN-γ and TNF-α have been used as adjuvant or as innovative therapeutic means in treatment and/or diagnosis of mastitis (Alluwaimi 2004). Detailed knowledge about the immune response and important defense factors which is essential to prevent and treat various infections during lactation cycle is not still properly understood. Therefore, the present study was initiated to evaluate the immune activity of neutrophils (first line of defense) during the early lactation period and its relationship with plasma concentration of IL-6, TNF-α and milk SCC.

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MATERIALS AND METHODS

Multiparous Karan Fries (KF) crossbred (Holstein Friesian × Tharparkar) cows (16) were selected from the institute's herd and divided into 2 groups (high and low yielding) based on their production potential. High yielding animals (8) were having production potential of above 5000 liter/lactation, whereas, low yielding animals (8) were having production potential below 3500 liters per lactation. Samples were also collected from 4 mastitic cows (production potential of above 5000 litre/lactation) of early lactation period, to compare them with uninfected cows. All the cows were kept in loose housing system with brick flooring and managed as per the standard management practices followed in the institute's herd. They were offered *ad lib.* green fodder and calculated

amount of concentrate mixture based on milk production was fed to them only at the time of milking. Fresh tap water was available *ad lib.* at all times of the day.

Both blood and milk samples were collected from normal cows during different fortnights starting from the day of calving to 75th day of early lactation period. From mastitic animals, samples were collected for consecutive 5 days of onset of clinical mastitis to their complete recovery. Isolation of PMN from peripheral blood was performed by hypotonic lysis of RBC and then centrifugation as described by Mehrzad *et al.* (2004). A semi-quantitative microscopic nitroblue tetrazolium (NBT) assay is used to determine *in vitro* procedure for measuring phagocytosis of blood neutrophils as described by Chai *et al.* (2005). Breifly 1 million viable neutrophils were culture with zymosan (650µg/ml) and NBT (250 µg/ml) in 96 well tissue culture plate. The plate was allowed to incubate at 37°C in a humidified CO_2 incubator (95% air and 5% CO_2) for 2h. During the incubation phagocytic cells produce O_2^- anions. These O_2^- reduce the yellow colour water soluble NBT to water insoluble blue or purple colour formazan crystals. The OD of the reduction product was determined at 540 nm.

SCC of milk samples were estimated as per Dang *et al.* (2008). Quantitative estimation of bovine cytokines (IL-6 and TNF-α) was done by using ELISA kit. The results were expressed as mean ± standard error of mean. Significance was tested by employing two ways ANOVA with the help of software SYSTAT.

RESULTS AND DISCUSSION

Phagocytic activity of blood neutrophils along with plasma concentration of TNF-α, IL-6 and milk SCC in high and low yielding KF cows during early lactation period are presented in Figs 1–4. PA was significantly (P<0.01) higher in low yielding cows as compared to high yielding cows during the early lactation period. Mean PA value in high yielding KF cows was 0.201 ± 0.005, whereas, same was 0.243 ± 0.005 for low yielding KF cows during the study period. PA was lowest on the day of calving and increased during the later period of early lactation in both the groups.

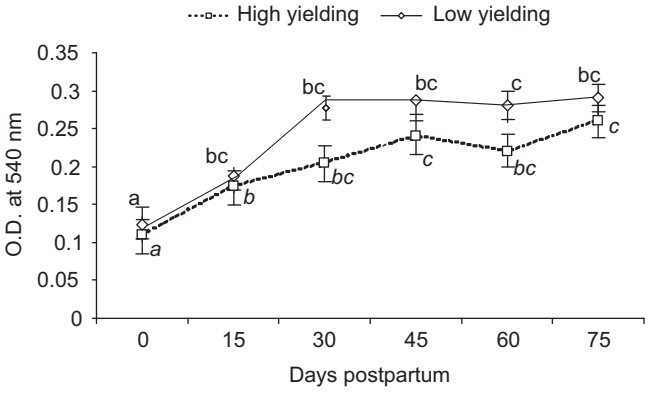

Fig.1. Phagocytic activity of blood neutrophils in high and low yielding Karan Fries cows during early lactation period.

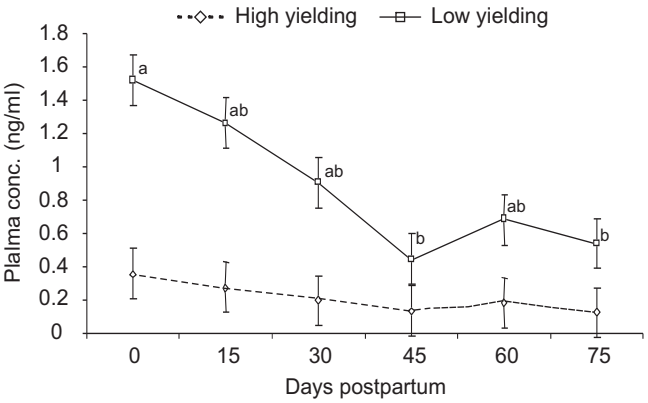

Fig. 2. Plasma concentration of TNF-α in high and low yielding Karan Fries cows during early lactation period.

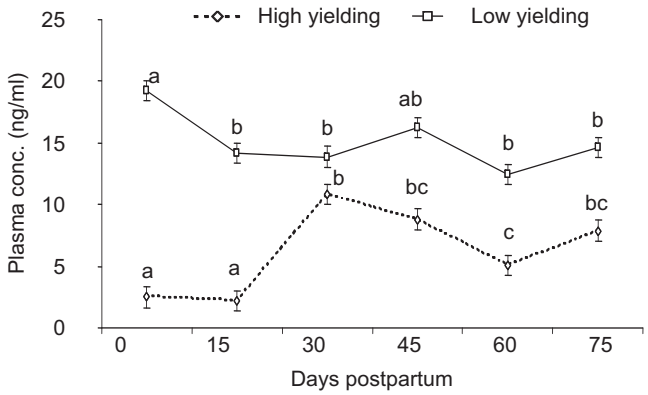

Fig. 3. Plasma concentration of IL-6 in high and low yielding Karan Fries cows during early lactation period.

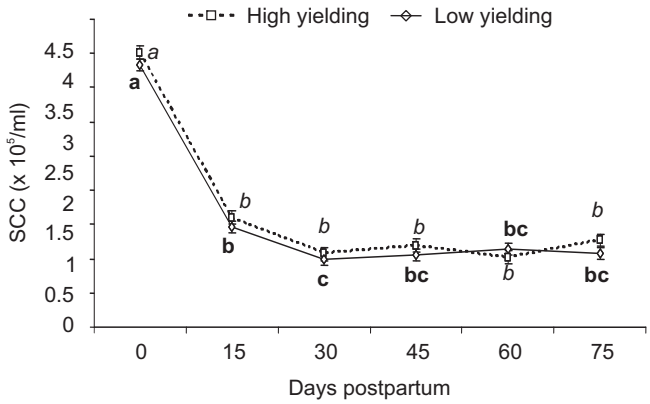

Fig. 4. Milk SCC in high and low yielding Karan Fries cows during early lactation period.

Diminished leukocyte functional capabilities are reported to be influenced by the lactational stage and stress factors such as parturition, onset of lactation, increased level of steroid hormones, change in feeding and management regimes (Mallard *et al.* 1998). Comparatively lower PA of blood neutrophils in high producing cows may be due to

stress on the cow to produce more milk. Earlier studies have also reported that selection of dairy cows with superior milk production traits has resulted in a steady increase in the incidence of clinical mastitis (Owen *et al.* 2000). Nonnecke *et al.* (2003) postulated that the metabolic demands associated with lactogenesis may impact negatively on the leukocyte function during the periparturient period. Blood PMN functions required for killing the pathogens and initiation of phagocytosis such as chemotaxis and diapedesis (Shuster *et al.* 1996) are also compromised during early lactation. This suppression in polymorphonuclear chemi-luminescence (PMNCL) was found associated with the sudden changes in concentrations of ketone bodies (Suriyasathaporn *et al.* 1999), glucocorticosteroids (Guidry *et al.* 1976) and pregnancy and lactation-associated molecules (Hoeben *et al.* 2000). These changes probably contribute for lowering the PA of neutrophils during period immediately after calving in cows.

Plasma TNF-α concentrations were significantly (P<0.01) higher for low yielding KF cows than high yielding cows. It also differed significantly (P<0.05) for different fortnights of early lactation (Fig. 2). Mean plasma concentration of TNF-α in high yielding KF cows was 0.21 ± 0.08 ng/ml, whereas, in low yielding group concentration was 0.89 ± 0.08 ng/ml with the highest value obtained at the day of calving. TNF-α is a pleiotropic inflammatory cytokine which plays an important role in diverse cellular events, such as the production of other cytokines, cell proliferation, differentiation and apoptosis (Beyaert and Fiers 1994). Cytokine TNF-α is produced mainly from Th1 cell which is indicative of cell mediated immunity (Mosmann *et al.*1986). This may result in increased cellular immunity in low yielders through Th1 system when compared to high yielders, thereby making low yielders more immune to infections.

Plasma IL-6 concentrations were significantly (P<0.01) higher for low yielding KF cows when compared to high yielding cows. There were significant (P<0.05) differences in IL-6 concentrations between different intervals of early lactation. Plasma IL-6 concentrations were highest on the day of calving in low yielding cows whereas it was lowest in high yielding cows during the same period. Hagiwara *et al.* (2001) also found higher concentration of IL-6 in high producing exotic cows. In low yielding animals, IL-6 concentrations were relatively higher than high producers. According to Mosmann *et al.* (1986) Th2 cell induces antibody production by producing mainly IL-4, IL-5, IL-6, IL-10 and IL-13. This may be indicative of higher T cellular activity in low producing cows when compare to high producers, making them more immune against infections. Ishikawa *et al.* (2004) found that IL-6 is a predominant cytokine in Th2 during transition period and reported lower levels of IL-6 in HF cows with retained placenta. They also showed that the peripheral blood IL-6 level decreased until 8 days post partum. However, Hagiwara *et al.* (2001) reported no significant differences in the IL-6 concentrations between the stages (early, mid and late) of lactation of exotic cows. Aitken *et al.* (2009), however, reported that expression of the pro-inflammatory cytokines, IL-1β, IL-6 and IL-8 did not change significantly during the periparturient period of Holstein cows.

In both the groups, PA of blood neutrophils was negatively correlated with plasma concentration of TNF-α (P<0.01) and milk SCC (P<0.05). In high yielding group, SCC was positively correlated (P<0.05) with plasma concentration of TNF-α, whereas, in low yielding group SCC was positively correlated (P<0.05) with plasma concentration of IL-6. The results obtained in our study could not be compared as no information is available regarding these relationship based on milk production potential of cows.

Phagocytic activity of blood neutrophils of mastitic cows along with plasma concentration of TNF-α, IL-6 and milk SCC for consecutive 5 days of mammary infection is presented in Table 1. TNF-α and IL-6 concentrations were significantly (P<0.01) higher during the first day of mastitis which later declined continuously up to fifth day of mastitis and these values were higher than uninfected cows during early lactation period. Similarly SCC values were highest on the first day (16.9×10^5/ml) of clinical mastitis which declined continuously up to fifth day (6.36×10^5/ml) of mastitis and were still higher than SCC of normal milk (high yielding, 1.69×10^5/ml; low yielding, 1.59×10^5/ml). PA of blood neutrophils was lowest at first day which later increased significantly (P<0.01) up to fifth day of clinical onset of mastitis. PA of blood neutrophils which was significantly impaired due to mastitis increased towards normalcy after treatment of the cows. On comparison between IL-6, TNF-α, SCC and PA of blood neutrophils during different days of

Table 1. Phagocytic activity of blood neutrophils, SCC and plasma concentration of TNF-a and IL-6 in mastitic crossbred cows

Parameters	Days from the onset of mastitis					
	1	2	3	4	5	SEM
PA (O.D at 540 nm)	0.15^{a}	0.18^{c}	0.26^{d}	0.28^{bd}	0.31^{b}	0.008
IL-6 (ng/ml)	26.20^{a}	19.90^{c}	17.70^{bc}	15.64^{bc}	13.91^{b}	0.760
TNF-α (ng/ml)	2.09^{a}	1.52^{cd}	1.48^{d}	0.90^{b}	0.59^{b}	0.085
SCC ($\times 10^5$/ml)	16.90^{ac}	13.56^{c}	9.65^{bc}	7.46^{b}	6.36^{b}	0.941

Values are expressed as mean ± SEM and the values within a row bearing different letters are significantly (P<0.01) different.

mastitis, it was found that there was a significant positive correlation (P<0.05) between plasma concentration of TNF-α, IL-6 and SCC; whereas, these factors were negatively correlated (P<0.05) with PA of blood neutrophils.

Plasma IL-6 concentration for first day of clinical mastitis was higher in KF cows which later declined up to fifth day, similar results were reported by Hagiwara *et al.* (2001). Blum *et al.* (2000) reported that IL-6 and TNF- α were the pro-inflammatory cytokines incriminated in the development of signs of acute septic shock in coliform mastitis. One of the mechanisms of increased milk PMN viability during mastitis can be the antiapoptotic effect of cytokines, for example, tumor necrosis factor-α, interleukin-1, and interleukin-6 or expression of antiapoptotic and proliferative genes (Long *et al.* 2001). This supports the idea of decreased PMN activity in blood through increased concentration of cytokines during mastitis thereby promoting PMN migration to udder and boost up of milk PMN viability. Therefore it can be used as an appropriate strategy for mastitis prevention and treatment.

This pioneer study quantifies the levels of TNF-α and IL-6 in high and low producing KF cows. From this study it can be concluded that cytokines like TNF-α and IL-6 can be used as an indicator of mastitis/mammary stress in dairy cattle. Also, in high yielding crossbred cows, period immediately after calving and around peak yield is more stressful as compared to low yielders and needs maximum managemental interventions. Therefore, future investigation should be conducted to find exact mechanism for such relationship between cytokines and activity of blood leukocytes and milk SCC in order to understand the complex inflammatory reactions thereby expanding vista for emerging cytokine immunotherapies.

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