Original Research Article

Assessment of Phagocytic Activity of Blood and Milk Neutrophils of Crossbred Cows during Peripartum Period

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ABSTRACT

Six crossbred cows (Holstein Friesian × Kankrej) were investigated in their advance stage of gestation period for Phagocytic Activity (PA) of Blood and Milk. Blood was collected on days -30, -15, -7 and -3 before calving, on the day of calving and on days 3, 7, 15 and 30 after calving while milk collected on the day of calving and 3, 7, 15, 30 day after calving. Highest Neutrophil (%) was observed on 3rd day before calving which was significantly (p<0.05) higher as compared to 30 day before calving. The PA of blood was observed significantly (p<0.05) lower at calving as compared to 15 day before calving. While the PA of milk neutrophil was significantly (p<0.05) higher at calving as compared to 7, 15 and 30 days postpartum. Cortisol level was increased significantly on the day of calving as compared to pre-partum and postpartum days. This study indicated that the phagocytic activity of blood and milk neutrophils get reduced during calving. Increasing level of glucocorticoids during calving is responsible for immunosuppression during peripartum period.

KEYWORDS
Cow, Neutrophil, Phagocytosis

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Introduction

The neutrophil is the most active phagocytic cell in the body. A large number of circulating neutrophils enter into the bovine Mammary gland during inflammation and provide resistance against most mammary pathogens (Straub et al., 1959; Newbould, 1970). Cows vary in their ability to resist mammary infection. As a result from microbial virulence (Newbould, 1967), poor phagocytic activity of neutrophils in milk (Newbould, 1973), physiological stressful situations such as parturition (Newbould, 1973). In this critical period the mammary gland undergoes marked biochemical, cellular and immunomodulatory changes to accommodate involution, to prepare for parturition, to withstand the stress of parturition, to transform colostrum into milk, and then for the attainment of peak milk production. These periods also coincide with impaired lymphocyte and neutrophil function. These immunological dysfunctions not only increase the risks of udder infection, mastitis, retained placenta and metritis but also lead to suppressed growth of mammary cells and subsequent milk production as energy and nutrients go preferentially to immune and homeostatic pathways (Kehrli et al., 1989). Although it is thought that the hormonal and metabolic factors may play a primary role in this physiological immune suppression, it can
further be suppressed by nutritional insults (Grant and Albright, 1995). Therefore, it is important to understand the cellular defence mechanisms operative during peripartum period in the mammary gland in order to find new ways for maintaining the animal healthy and optimizing milk production (Drackley, 1999). Upon The polymorphonuclear neutrophils (PMNs) form the first line of cellular defence against invading pathogens, mainly by the process of phagocytosis (Paape and Wergin, 2003). During periparturient period immune system gets suppressed. At this time the most important factor causing immunosuppression are metabolic stress and stress of parturition resulting from hormonal and metabolic fluctuations, a negative energy balance, and shortage of proteins, minerals and vitamins which are required to meet the demands of the fetus as well as the onset of lactation. Decreased phagocytosis of neutrophils, decreased cytotoxic ability of lymphocytes, as well as depressed activity of their cytokines during parturition make it impossible for the normal, efficient maternal immune recognition and rejection of fetal membranes and finally results in their retention in cows (Mordak and Stewart, 2015).

To achieve maximum production, work should be concentrated on ways of minimizing the negative influence on immune functions and/or ways of stimulating these functions, especially during the periods of immune suppression (Waller, 2000). Therefore, the present study was initiated to evaluate the phagocytic activity of neutrophils during the peripartum period and its relationship with plasma cortisol and prolactin.

**Materials and Methods**

Six crossbred cows (Holstein Fresian × Kankrej) were selected in their advance stage of gestation with different parity from the Livestock Farm Complex (LFC), Department of Livestock Production Management at College of Veterinary Science and A.H., Anand. The study was conducted on selected animals from 30 days before calving to 30 days after calving. The experimental animals were reared in semi-open housing system which is made up of concrete floor under asbestos roofed housing system constructed east west direction and well covered with trees.

From each experimental animal 9 ml Blood was drawn in sterile heparinized vacutainer tube from jugular vein puncture, posing minimum disturbance to the animal during collection on days -30, -15, -7 and -3 before calving, on the day of calving and on days 3, 7, 15 and 30 after calving. Blood samples were obtained from six cows as soon as possible after parturition and always within the first 24 hr. immediately after collection the samples were transported to the laboratory over ice for further processing. The blood sample was used to identify mature and immature neutrophil and neutrophil percentage. Plasma was separated by centrifugation at 3000 rpm for 15 minutes and stored at -20°C in deep freeze until analyzed for cortisol and prolactin. Isolation of PMN from remaining blood pellet was performed by hypotonic lysis of RBC and then centrifugation as described by Mehrzad et al., (2004). From each experimental animal 25ml milk sample was collected on the day of calving and 3, 7, 15, and 30 day after calving. Isolation of PMN from colostrum and milk was performed as per method described by Mehrzad et al., (2001). A semi-quantitative microscopic nitro blue tetrazolium (NBT) assay is used to determine in vitro procedure for measuring phagocytosis of blood neutrophils as described by Chai et al., (2005). Briefly 1 million viable neutrophils were culture with zymosan (650μg/ml) and NBT (250 μg/ml) with RPMI medium in 96 well tissue culture plate. The plate was allowed to incubate at 37°C in a humidified CO2 incubator (95% air and 5% CO2) for 2h.
During the incubation phagocytic cells produce O2- anions. These O2- reduce the yellow colour water soluble NBT to water insoluble blue or purple colour formazan crystals. The optical density (OD) of the reduction product was determined at 540 nm. Significance was tested by employing two ways ANOVA.

**Results and Discussion**

In the present study, we had found that the mean (±SEM) of neutrophil (%) of crossbred cattle was increased significantly (p<0.05) from 30 day before calving to 3 day before calving which is the highest level of neutrophil percent observed during the study. A slight non-significant reduction in neutrophil percent was observed on the day of calving as compared to 3 day before calving. After parturition sudden significant (p<0.05) reduction in neutrophil percent was observed on 3rd day after calving (Table 1). Mature and Immature neutrophils Percentage of crossbred cows during the peripartum period have been presented in Table 2. The number of mature neutrophils was significantly (p<0.05) reduced on the day of calving as compared to pregnancy which was similar with Pomsel (1980) and Kim et al., (2005). Neutrophil per cent had a highly significant (p<0.01) positive correlation with level of cortisol and level of prolactin. So that the increasing level of cortisol around calving might be acting as stimulating agent for the increased neutrophil per cent around calving. Paape et al., (1973) reported that the rapid increase of circulating neutrophils was attributed mainly to an influx of neutrophils from the hematopoietic system and not from a marginal pool of mature leukocytes. A higher level of cortisol around calving is the reason behind an increase in immature neutrophils. Mature neutrophils are more sensitive to cortisol as compared to immature neutrophils and have more number of glucocorticoid receptors as compared to immature
neutrophils. Migration of mature neutrophils from the bone marrow reduces due to reduction in number of adhesion receptors (L-selectin and CD18). Immature neutrophils are less affected due to lower effect of cortisol in response to lower number of glucocorticoid receptors. So, they marginate more from hematopoietic reserve as compared to mature neutrophils (Burton et al., 2005).

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 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. Upon estimation of plasma cortisol it was revealed that the highest cortisol concentration was at calving. Diminished neutrophil functions and compromised host resistance mechanisms during peripartum period in dairy cows have also been observed by Meglia et al., (2001) and Dang et al., (2012). Poor activity of neutrophils at calving may be due to more number of immature neutrophils coming in circulation which have no proper machinery to fight or phagocytose against infection. Reduction in phagocytic activity was significant (p<0.05) on the day of calving as compared to 15 day before calving. This suppression in the PA might be due to a sharp increase in the cortisol levels at calving. In the present study, a significant (p<0.05) negative correlation was observed between PA of blood neutrophils and plasma cortisol levels. There was increased neutrophil numbers during parturition but phagocytic activity remained lower in the present study. Parturition reflex causes higher plasma cortisol level that leads to hyper stimulation of red bone marrow for the faster release of neutrophils. As a result of this, there was release of more number of immature neutrophils and a less number of matured neutrophils. That is why, the phagocytic activity of neutrophils decreased as observed in the present study. According to Meglia et al., (2001), neutrophils and their important functions, like migration and phagocytosis, are impaired when the animal is under stress of calving. During prepartum period (15 day before calving) animals are in dry stage so, there is no stress of milk production but at parturition, animals have to face stress of calving, synthesize colostrum (up to 3 days) and milk. Dang et al., (2014) reported that Changes occurred in the PA of blood neutrophils in primiparous and multiparous crossbred cows during different days of lactation cycle. Phagocytic activity in both the group of cows started decreasing from 15 days prepartum and maximum depression was observed at calving. This indicated that immunosuppression of the neutrophilic activity starts from 2 weeks before parturition and remained low until day of lactation.

Phagocytic activity was maximum in Neutrophils obtained from colostrums. Sugisawa et al., (2001) found that as the immunoglobulins were very high in colostrums as compared to milk, therefore, it increased the PA of colostrum neutrophils by up to 25% and was dose dependent. Further, PA was higher in the colostrums of multiparous animals because they produce more immunoglobulins in their colostrums and PA decreased between day 30 and 60 i.e. when the animal attains peak yield, which might be due to hormonal and metabolic changes such as glucocorticoids, ketone bodies and pregnancy associated glycoprotein (Dang et al., 2009).
### Table 1 Mean (± SE) values of Neutrophil, phagocytic activity (PA) of blood and Milk neutrophil, plasma Cortisol and Prolactin of crossbred cows (n=6) during peripartum period

| Days | Neutrophil (%) | P.A. of Blood Neutrophil | P.A. of Milk Neutrophil | Cortisol (ng/ml) | Prolactin (ng/ml) |
|------|----------------|--------------------------|-------------------------|-----------------|-------------------|
| -30  | 38.34±2.42     | 0.25c±0.01               | -                       | 27.69b±5.22     | 188.97c±24.53     |
| -15  | 41.55b±0.77    | 0.51b±0.15               | -                       | 25.04b±6.20     | 268.44b±27.69     |
| -7   | 42.83b±0.83    | 0.41abc±0.08             | -                       | 26.67b±3.48     | 309.00abc±29.65   |
| -3   | 46.79±2.43     | 0.28abc±0.07             | -                       | 28.60abc±5.15   | 306.75abc±14.75   |
| 0    | 45.69a±2.37    | 0.27abc±0.02             | 1.73a±0.13              | 44.04a±8.87     | 333.28a±4.27      |
| 3    | 40.71b±1.88    | 0.50a±0.08               | 1.31ab±0.14             | 38.61ab±8.73    | 287.43ab±32.05    |
| 7    | 42.65b±1.75    | 0.35abc±0.06             | 0.79c±0.15              | 32.28ab±7.99    | 298.42ab±13.66    |
| 15   | 41.86b±0.90    | 0.26c±0.03               | 0.99bc±0.11             | 22.99b±2.63     | 312.25ab±20.11    |
| 30   | 36.51b±0.34    | 0.36abc±0.04             | 0.70c±0.08              | 35.60b±6.18     | 305.35ab±20.66    |
| Overall Mean | 41.89±1.52 | 0.36±0.07 | 1.10±0.13 | 31.29±5.84 | 289.99±17.13 |
| CV%  | 10.24          | 49.05                    | 28.04                   | 45.72           | 14.47             |
| CD0.05 | 3.94         | -                        | 0.37                    | -               | 48.97             |

### Table 2 Mean (± SE) values of mature and immature neutrophil of crossbred cows (n=6) during peripartum period

|                  | -15       | 0        | 15       | Overall Mean |
|------------------|-----------|----------|----------|--------------|
| Mature Neutrophil (%) | 98.07a±0.15 | 94.67b±0.26 | 99.13a±0.18 | **97.29±0.20** |
| Immature Neutrophil (%) | 2.93b±0.07  | 5.33a±0.18  | 0.87c±0.09 | **3.04±0.11** |

*Values having different superscripts differed significantly (p<0.05) within Row
Mohapatra, (2012) also reported that phagocytic activity of milk neutrophil was higher on the day of calving and significantly decrease on 15 day postpartum. The present result also collaborated with this study. Phagocytic activity was generally lower in milk than in blood neutrophils (Vishnoi et al., 2007). The reason for this is that as milk neutrophils have reduced glycogen stores compared to that of blood, this might limit the availability of energy. Milk neutrophils also have reduced abilities to produce Reactive Oxygen Species (ROS) as compared to that of blood (Dosogne et al., 2001). The decreased bactericidal activity of milk PMN in comparison with blood PMN could also be explained by a lower super oxide production measured by chemiluminescence's (Dulin et al., 1988) and a lower H$_2$O$_2$ production measured by flow cytometry (Salgar et al., 1991). An increased concentration of neutrophils in the first day colostrums provides immunity to the mammary gland. Migration of polymorphonuclear neutrophil leukocytes (PMN) into the mammary gland provides the first line of defense against invading mastitis pathogens (Craven and Williams, 1985; Tizard, 2000). Neutrophils travel from blood to the mammary gland in response to a variety of inflammatory mediators, such as cytokines, complement and prostaglandins (Zecconi and Smith, 2000; Janeway et al., 2001).

Cortisol is the most important glucocorticoid and is produced by the adrenal gland. It is released in response to stress and a low level of blood glucocorticoids. Its primary functions are to suppress the immune system and increase blood sugar through gluconeogenesis. The cows during peripartum period are under various types of stressful conditions during stress of providing nutrition to its growing calf, stress of labour, to synthesize colostrum and then milk. A social stress of being isolated is also there. Overall effects of stress cause increased cortisol level, produced neutrophilia with decreased functional capacity of neutrophils which leads to immunosuppression and ultimately the cows become more prone to mastitis and other infections (Kehrli et al., 1991). High levels of cortisol at calving have also been reported (Goff and Horst, 1997) to act as powerful immunosuppressive agent. The result of present study also collaborated with the findings of Prakash and Madan (1985) in buffaloes, Goff and Horst, (1997) in cows and Khan and Ludri (2002) in crossbred goats. The value of plasma cortisol concentration observed in our study was similar to that reported by Pal (1996) in crossbred KF cows. Mukherjee et al., (2015) observed increase in prolactin in peripheral circulation during the day of parturition. Peak value of prolactin attained at parturition and returned to basal levels within 2 weeks. Similar results were observed during present study. Kodagali et al., (1980) reported that the prolactin concentration varied with each stage of lactation; being high in early, middle in mid lactation, and low in late lactation in Gir cows. The present study also accordance with Akasha et al., (1987), where serum prolactin concentration declined as lactation progressed.

In conclusion, blood phagocytic activity (PA) suppressed on the day of calving due to immunosuppression even after neutrophil percent observed higher. While milk PA observed higher on the day of calving because of their immunoglobulin and hormonal changes. Cortisol and prolactin level highest observed on the day of calving but the effect of prolactin on blood neutrophilic activity was non-significant.

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