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Diurnal rhythm in the counts and types of milk somatic cells, neutrophil phagocytosis and plasma cortisol levels in Karan Fries cows during different seasons and parity

Mohanned Naif Alhussien and Ajay Kumar Dang

Lactation and Immuno-Physiology Laboratory, ICAR- National Dairy Research Institute, Karnal, India

ABSTRACT

Chances of mammary infections are comparatively higher in high producing cows during harsh environmental conditions and are usually characterised by changes occurring in various somatic cells secreted in the milk and their activities. The present study was conducted to record diurnal rhythmicity in milk somatic cell counts (SCC), neutrophil: macrophage (N: M) ratio, phagocytic activity (PA) of milk neutrophils and plasma cortisol concentrations during different seasons and parity in high producing Karan Fries cows. Values of milk SCC, N: M ratio and plasma cortisol levels were lowest during thermoneutral (TN), intermediate in winter and highest during the summer season. Diurnal rhythm in the milk SCC and N: M ratio was noticed in the summer while plasma cortisol exhibited diurnal rhythm in both winter and summer seasons. Milk SCC, N: M ratio and plasma cortisol increased in multiparous cows, but diurnal variation was noticed only in the N: M ratio and plasma cortisol in cows having more than four parity. Phagocytic activity of milk neutrophils was highest during TN, intermediate in winter and lowest during the summer season. Phagocytic activity was higher and similar in cows up to fourth parity but decreased in subsequent lactation cycles. Diurnal rhythm in the PA was noticed in winter and summer seasons and in cows having more than four parity where morning samples showed higher phagocytosis as compared to the evening samples. These results can be used for immunomodulatory interventions and therapeutic approaches in treating mastitis of crossbred cows reared under tropical conditions.

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KEYWORDS
Milk somatic cell; neutrophil: macrophage ratio; season; parity; cow

1. Introduction

Nearly all the physiological functions of animals are rhythmic including recruitment of various immune cells, the activity of first line of cellular defence, i.e. the neutrophils and secretion patterns of hormones (Lefcourt et al. 1993; Scheiermann et al. 2013). Daily and seasonal rhythms of different cellular activities are coordinated by the master circadian clock located in the hypothalamus. This master clock receives and integrates various environmental and physiological cues that set the master clock at the peripheral tissues level including the mammary gland to consistently maintain its lactation (Casey and Plaut 2012). According to Wang et al. (2015),

CONTACT Ajay Kumar Dang rajadang@rediffmail.com

many circadian genes help in the development of the milk duct, maintenance of polarity and morphology of mammary cells and ultimately the milk composition. Somatic cell counts (SCC) of milk are an important component of the mammary gland and are used universally as an indicator of milk quality and mammary health (Harmon 1994). Out of all somatic cells, milk neutrophils increase significantly in the mammary tissues and secretions when the risk of mammary infection is high (Paape et al. 2003). The phagocytic activity of milk neutrophils is a critical defensive reaction against invading pathogens through which neutrophils continuously try to maintain the health conditions of the mammary gland (Paape et al. 2002; Rainard and Riollet 2003). Mammary gland immunity and the incidence of mastitis are profoundly affected by daily and seasonal fluctuations in environmental conditions (Quist et al. 2008), and the number of lactation, i.e. parity (Mehrzad et al. 2009). Recently the diurnal variation in milk somatic and differential cell counts have been studied extensively in Murrah buffaloes (Bombade et al. 2017). However, information about the diurnal variation in the mammary immunity of crossbred cows reared under tropical environmental conditions is still lacking. Therefore, the present study was undertaken to investigate the diurnal variation in the number of somatic cells coming in milk and to investigate which immune cell exhibits higher daily rhythm in their percentage. Further does this diurnal variation affect the phagocytic ability of milk neutrophils and whether any changes in the morning and evening plasma cortisol levels add to this effect?

2. Materials and methods

2.1. Ethical approval

The current study was undertaken after getting the necessary approval from the institute's animal ethics committee.

2.2. Location and climatic condition of the study area

The present study was conducted in the Karan Fries (*Bos indicus* × *Bos taurus*) cows maintained at the livestock research centre of the National Dairy Research Institute. This institute is located in Karnal city at an altitude of 250 m above mean sea level in the Indo-Gangetic plains of 29°43″N attitude and longitude 77°2″E. Minimum temperature usually falls to around 4 °C in winter and maximum can sometimes be 45 °C on a particular day in summer. Annual rainfall is about 70 cm, and most of the rainfalls occur in the month of July–August. Relative humidity of this farm varies from 40 to 90%, and vapour pressure ranges from 7.0 to 25 mm Hg. The animals are exposed to both extreme climatic conditions due to the wide range of variation in various meteorological factors in this climatic zone. This study was carried out in the year 2016, and the climatic variables recorded during the present study were collected from Central Soil Salinity Research Institute which is located around 5 km from the study area and has been presented in Table 1.

2.3. Study design and selection of experimental animals

Two experiments were carried out in Karan Fries (KF) cows to investigate the diurnal variation of milk SCC, neutrophil: macrophage (N: M) ratio, phagocytic activity of milk neutrophils and plasma cortisol concentrations under the influence of different seasons and parity.

Table 1. Minimum and maximum values of temperature (°C) and relative humidity (%) recorded during the experimental study.

Seasons	Temperature (°C)		Relative humidity (%)	
	Max.	Min.	Max.	Min.
Summer	40.00 ± 5.2^{c}	23.00 ± 3.1^{b}	77.00 ± 4.6^{a}	56.00 ± 4.2^{b}
Thermoneutral	27.00 ± 3.5^{b}	17.00 ± 2.3^{b}	88.00 ± 4.1^{b}	47.00 ± 2.3^{a}
Winter	17.00 ± 2.5^{a}	8.10 ± 2.2^{a}	95.00 ± 3.2^{b}	57.00 ± 4.1^{b}

Notes: Values are expressed as mean ± SE. Values within a column with different superscript (a–c) differ significantly at $p < 0.05$. They are arranged alphabetically from the smallest value to the biggest value.

Experiment 1: This experiment was conducted on 30 healthy KF cows to investigate the diurnal rhythm in various immunity related parameters under the influence of different seasons mainly winter (December, January), thermoneutral (March, April) and summer (May, June). Ten KF cows were followed in each season. All the animals were high producers (>10 kg), in their early lactation (up to 100 days post-partum) and were multiparous (2–4 parity).

Experiment 2: The second experiment was conducted to investigate the diurnal variation in various immunity related parameters in cows having different parity during the thermoneutral (TN) season. This experiment was carried out on 30 healthy crossbred cows (10 in each group) of different parity, i.e. primiparous (parity 1), having 2–4 parity, having parity >4. All the animals were high producers (>10 kg) and in their early lactation.

2.4. Housing and feeding of the experimental animals

All the experimental cows were kept in loose housing system with brick flooring with standard managemental practices followed in the herd as this system facilitates free movement and sufficient exercise to the animals. Although this housing pattern may expose the animals to climatic effects, however, it provides ample air circulation which assists in evaporative cooling. Cows were offered *ad libitum* green maize (Zea mays), and sorghum (Sorghum bicolor) in summer. During the winter season, the animals were fed with oats (Avena spp.), clover (Trifolium spp.), and winter maize (Zea mays). A calculated amount of concentrate mixture based on milk production at the rate of 460 gm/kg of milk produced was also fed at the time of milking. Fresh and clean water made available to all the cows throughout the day.

2.5. Animal milking and samples collection

These experiments were carried out on both blood and milk samples which were collected during the morning milking at 6 am and the evening milking at 6 pm. The lactating KF cows were taken into the milking parlour around 20 min before milking, and the milking process took 4–5 min per cow depending on the milk yield and milk flow rate of an individual animal. All the cows were machine milked at the rate of 50 pulsations per minute. At each milking, the yield was recorded in kg, and composite milk samples represent all four quarters was collected into sterile tubes (200 ml/cow). Blood sampling was done after completion of the milking process, and blood around (9 ml/cow) was collected in sterile heparinised vacutainer tubes from jugular vein puncture with minimum disturbance to the animal. After that, the

samples (blood and milk) were taken to the laboratory in an ice box and were subjected to subsequent processing. In each season, 16 blood and 16 milk samples were collected from each animal in the morning and evening time on a weekly basis.

2.6. SCC and neutrophil: macrophage ratio

SCC of milk samples were measured by two methods. Firstly, they were estimated by Lactoscan milk SCC counter (Milkotronic Ltd. Stara Zagora, Bulgaria), which is based on fluorescent microscope technique of counting cells. Fresh milk (100 µl) was diluted with a double amount of distilled water and mixed with Sofia Green lyophilized dye in a microtube. After that 8 µl was added onto the single lactochip and the chip was loaded into the machine. The dyed cells were filmed by a charge-coupled device (CCD) camera, and the algorithm of analysis of digital images determines the number of the fluorescent cells and counts their concentration in the sample. Secondly, milk smears were prepared on slides to crosscheck the results of SCC obtained from milk SCC counter and also to estimate N: M ratio by the microscopic method using an inverted microscope as described by Dang et al. (2007). Differential cell counting was carried out to determine the presence of different cell types like neutrophils, lymphocytes and macrophages in milk which ultimately help in estimating N: M ratio. The detailed procedure is given as follows:

2.6.1. Slide preparation and microscopic counting

Milk was heated to 40 °C in a water bath for about 15 min before being cooled to 20 °C with continuous stirring. Around 10 µl of milk was spread on 1 cm^2 (1 × 1 cm) area of a microscopic slide and was allowed to dry properly. The smear was then dyed with the May-Grunwald solution for 2–3 min. Excess stain was drained, and the smear was again stained with Giemsa stain for 1–2 min. Excess stain was drained as done in the previous step and the slide was air dried. Milk SCC was then measured microscopically at 40 X, and differential cell counts were carried out at 100 X. After the percentage of various cells was estimated, the percentage of milk neutrophils was divided by macrophages percentage to obtain the N: M ratio.

2.7. Phagocytic activity of milk neutrophils

The *in vitro* phagocytic activity of milk neutrophils was estimated by nitro blue tetrazolium (NBT) assay. For this, neutrophils were isolated by density gradient centrifugation using Histopaque 1119 and Histopaque 1077, and the cells harvested at the interface of both the Histopaque 1119 and Histopaque 1077 layers. The collected neutrophils were washed thrice using phosphate buffered saline (300 × g, 10 min, 4 °C) and suspended in Roswell Park Memorial Institute (RPMI 1640) media for further analysis. The cell suspension was adjusted to 5 × 10^6 live cells/ml using RPMI 1640. About 100 µl of the diluted cell suspension per well in triplicate placed in a tissue culture plate. The cells then allowed to proliferate with Zymosan (650 µg/ml) and NBT (250 µg/ml) concentrations that had been determined previously to provide maximal stimulation of bovine phagocytes (Dang et al. 2013). In all the cases, final culture volume was 200 µl, and the blank wells consisted of 200 µl of culture media along with same concentrations of NBT and zymosan. All cultures were incubated for two h in the CO2 incubator at 5% CO2 and 95% air level. After that, the optical density (OD) was taken at 540 nm using multiwell-scanning spectrophotometer (Microscan MS-5608A).

2.8. Estimation of plasma cortisol hormone

Plasma cortisol was quantified by competitive enzyme immunoassay "Bovine Cortisol Hormone Elisa kit" (Cusabio Biotech co., Ltd). The minimum detectable dose of cortisol hormone was less than 0.049 ng/ml with a detection range of 0.049–200 ng/ml. The intra and inter-assay coefficient of variance (CV) were less than 8% and less than 10%, respectively.

2.9. Statistical analysis

All the data were checked for normality before statistics and has been presented as mean ± SEM. Statistical analysis was done by t-test to investigate the significance variation of various estimated parameters between morning and evening within the same season and parity. One-way ANOVA test was used for within-group analysis and repeated measures two-way ANOVA (mixed model) for between groups' analyses. Correlation analysis was performed by Pearson correlation technique. This followed by Duncan multiple range test (DMRT) comparison tests using SAS software, version 9.1 of SAS system for the window, copyright © (2011), SAS Institute Inc., CARY, NC, USA. The difference at $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Milk SCC, N: M ratio, phagocytic activity of neutrophils and plasma cortisol during different seasons

Diurnal variation of milk SCC, N: M ratio, phagocytic activity of milk neutrophils and plasma cortisol concentrations in KF cows during different seasons (TN, winter, summer) have been presented in Figure 1(a–d), respectively. Milk SCC was similar during the morning session of both TN and winter season and significantly ($p < 0.05$) lower than that recorded during the summer season. However, during the evening samples, the SCC was lowest during TN, intermediate in winter and highest in the summer season. The N: M ratio was low during the TN, increased significantly ($p < 0.05$) in winter and continued to increase in the summer. Milk SCC and N: M ratio were always higher during evening samples as compared to the morning samples in all seasons. Although diurnal rhythm was noticed in milk SCC and N: M ratio in all seasons but it was significantly ($p < 0.05$) different only during the summer season. In the morning samples, there was no difference in the phagocytic activity of milk neutrophils between winter and summer, but it was significantly ($p < 0.05$) lower than that of TN season. However, during the evening sampling, the phagocytic activity was highest in TN, intermediate in winter and lowest in the summer. Phagocytic activity was significantly ($p < 0.05$) higher in the morning samples as compared to the evening samples in both winter and summer, however, there was no difference during the TN season. Plasma cortisol concentrations were similar during the morning samples of both TN and winter but were significantly ($p < 0.05$) lower than that of the summer season. However, during the evening samples, it was revealed that plasma cortisol levels were lowest during TN, intermediate in winter and highest in the summer. Diurnal rhythm in the plasma cortisol levels was recorded during both winter and summer where the plasma cortisol concentrations were significantly ($p < 0.05$) higher in the evening samples as compared to morning samples but no such rhythm could be noticed during the TN season.

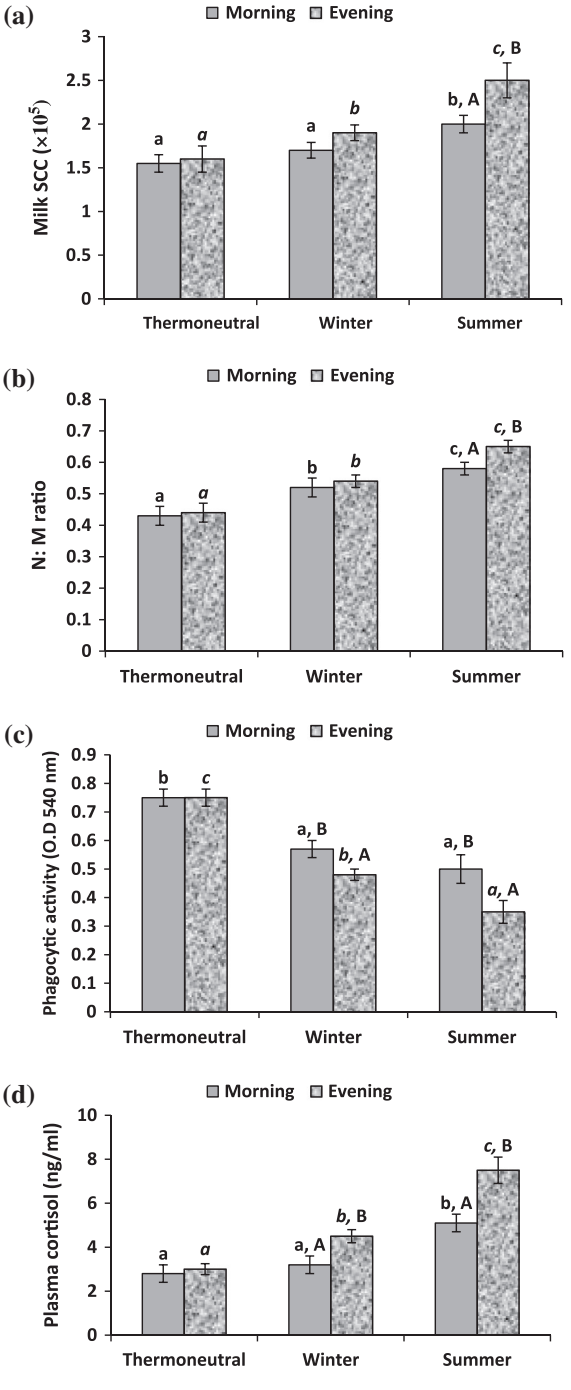

Figure 1. Diurnal variation in milk SCC, neutrophil: macrophage (N: M) ratio, phagocytic activity of milk neutrophils and plasma cortisol levels during different seasons in KF cows.

Note: Bars represent the standard error of the mean. Means with different superscripts (a–c: between morning sessions; *a–c*: between evening sessions; A, B: between morning and evening sessions) differ significantly ($p < 0.05$). They are arranged alphabetically from the smallest value to the biggest value.

3.2. Milk SCC, N: M ratio, phagocytic activity of neutrophils and plasma cortisol in cows having different parity

Diurnal variation of milk SCC, the N: M ratio, phagocytic activity of neutrophils and plasma cortisol concentrations in KF cows during different parity (1, 2–4, >4) have been presented in Figure 2(a–d), respectively. There was no difference in the milk SCC and N: M ratio between primiparous cows and cows having 2–4 parity; however, it increased significantly ($p < 0.05$) in the multiparous cows having more than four parity. Although milk SCC was slightly higher in the evening samples, however, no significant variation could be noticed between morning and evening samples. Significant ($p < 0.05$) diurnal variation in the N: M ratio was observed only in the cows with more than four parity where evening samples had higher levels of N: M ratio. Phagocytic activity was highest in the primiparous cows and remained almost unaltered in the subsequent parity but it declined significantly ($p < 0.05$) in cows having more than four parity. Diurnal variation in the phagocytic activity was noticed only in the multiparous cows having more than four parity where neutrophils isolated from morning samples had higher phagocytic activity as compared to the evening samples. Plasma cortisol concentrations were lowest in the primiparous cows and increased significantly ($p < 0.05$) in the multiparous cows of both the groups. Although diurnal variation was almost undetectable in both primiparous cows and cows in their 2–4 parity, however, a significant ($p < 0.05$) rhythm was noticed in the cows having more than four parity where the cortisol levels were higher in the evening samples as compared to the morning.

3.3. Correlation of plasma cortisol with SCC, N: M ratio and phagocytic activity of milk neutrophils

The relationship among different estimated parameter in KF cows has been presented in (Table 2). Plasma cortisol had a positive correlation with milk SCC ($p < 0.01$), N: M ratio ($p < 0.05$) but negatively correlated with the phagocytic activity of milk neutrophils ($p < 0.01$). The phagocytic activity was negatively correlated with SCC ($p < 0.01$) and N: M ratio. The SCC and N: M ratio had a positive but not significant correlation with each other.

4. Discussion

This study was designed to investigate the diurnal variation in the milk SCC and N: M ratio of milk, the phagocytic activity of milk neutrophils and concentrations of plasma cortisol in KF crossbred cows during different seasons and parity under tropical conditions. Circadian rhythms coordinate changes in various physiological processes and synchronise them with the environment of the cows to support lactation (Plaut and Casey 2012). These circadian rhythms are controlled by molecular circadian clocks located centrally in the hypothalamus and peripherally in the mammary gland. On receiving the environmental and physiological cues, the hypothalamus synchronises the internal physiology by coordinating immunological and endocrine rhythms (Logan and Sarkar 2012). The effect of diurnal rhythm on lactation may be inferred by the external factor (environmental conditions), and internal factor (parity) effects on milk production, which is accompanied by coordinated changes in the immunology of mammary gland essentials for maintaining both mammary health and productivity.

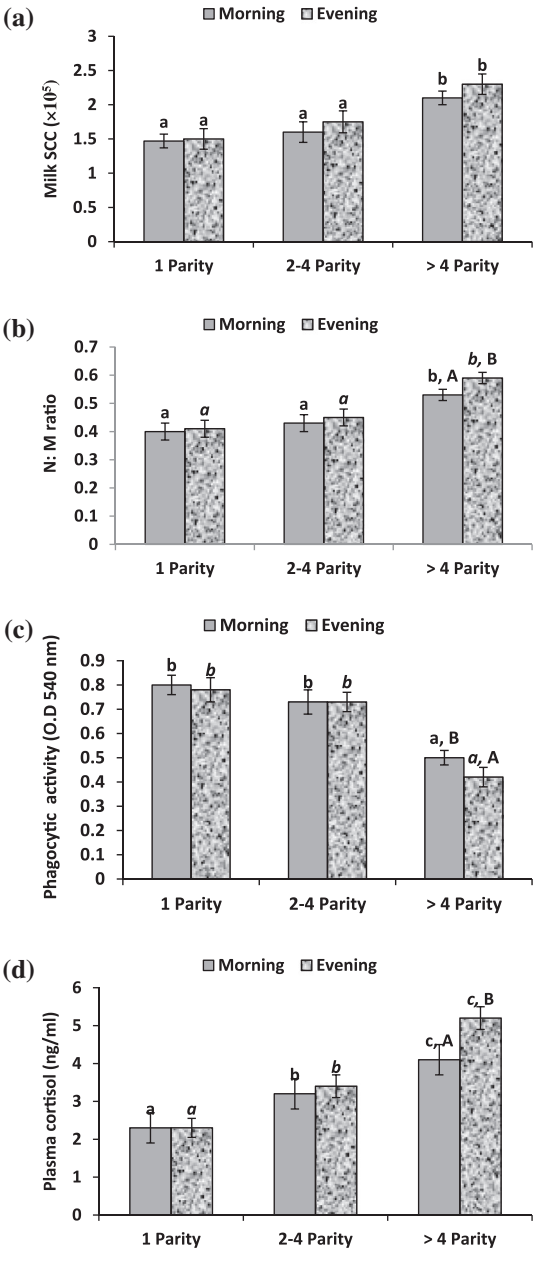

Figure 2. Diurnal variation in milk SCC, neutrophil: macrophage (N: M) ratio, phagocytic activity of milk neutrophils and plasma cortisol levels during different parity (1, 2–4, >4) in KF cows.

Note: Bars represent the standard error of the mean. Means with different superscripts (a–c: between morning sessions; *a–c*: between evening sessions; A, B: between morning and evening sessions) differ significantly ($p < 0.05$). They are arranged alphabetically from the smallest value to the biggest value.

Milk somatic cells consist of epithelial cells that have been shed from the lining of the mammary tissues and leukocytes that have entered the mammary gland in response to injury or infection (Paape et al. 2002; Burvenich et al. 2007). These cells were estimated as they are useful predictors of mammary infection and are being used worldwide to assess all

Table 2. Correlation of plasma cortisol levels with milk somatic cell counts (SCC), neutrophil: macrophage (N:M) ratio and the phagocytic activity of milk neutrophils in morning and evening milk samples collected from crossbred cows under different seasons and parity.

	Plasma cortisol	SCC	N:M ratio	Phagocytic activity
Plasma cortisol	1			
SCC	0.790**	1		
N:M ratio	0.269*	0.154	1	
Phagocytic activity	−0.890**	−0.718**	−0.179	1

Note: Asterisks (** and *) indicate that the values are significant at $p < 0.01$ and $p < 0.05$, respectively.

aspects of quality and hygienic conditions of produced milk (Harmon 1994). Various factors can affect the SCC in cow's milk including species of the animal, breed, environmental and physiological stress, season, parity, day to day variation, diurnal variation, milking interval and management factors (Rupp and Boichard 2000; Li et al. 2014) as also observed during this study. Highest milk SCC was noticed in the summer season which is similar to the results reported by Shock et al. (2015) in the Ontario dairy herds. In clinically healthy mammary gland, macrophages are the prominent cell types representing about 60% of total white blood cells present in the milk followed by neutrophils 20–30% and finally lymphocytes 10–20% (Paape et al. 2002; Alhussien et al. 2015, 2016a).

Whenever the mammary tissues are invaded by various micro-organisms, the neutrophils are recruited in large number, and simultaneously the macrophages proportions are reduced (Rainard and Riollet 2003). Based on this concept the N: M ratio was estimated as it is a reliable indicator of milk quality and reflects the health status of the mammary gland. The N: M ratio was minimum during the TN season indicating the presence of a high macrophages number and lesser neutrophils in normal mammary secretions. The presence of low concentration of neutrophils in the normal mammary gland might be due to the permanent low-grade attraction of blood neutrophils by the constitutive chemotactic agents found in the milk (Manlongat et al. 1998). Higher and significant difference was observed in the milk SCC in the evening samples compared to the morning samples mainly during the summer season. This indicates that cows were more comfortable during early morning because they were taking rest overnight and the temperature is also considerably lower during the early morning periods as compared to the evening which is reflected by lower SCC observed during the morning session as also reported by Quist et al. (2008) in Canadian dairy herd. Moreover, the summer season is more stressful especially that temperature humidity index (THI) increases dramatically and the high yielding KF cows are at a greater risk of developing mammary infections due to the stress of milk production and instability in their innate immunity during this season. Therefore there is a need to continuously mitigate heat stress by providing the animals with sufficient amount of drinkable water, water showers, shade, fans and *ad libitum* green fodder during these extreme environmental conditions (Yadav et al. 2016).

One of the most fundamental defence mechanisms through which neutrophils destroy various invading micro-organisms is phagocytosis (Mehrzad et al. 2009). Interestingly, the phagocytes are reported to exhibit diurnal variation in their phagocytic activity in rodents (Barriga et al. 2001; Hriscu 2004), fish (Roy et al. 2008) and in guinea pigs (Baciu et al. 1994). However, the diurnal rhythmicity in phagocytic activity of milk neutrophils has not been explored in crossbred dairy cows reared under tropical conditions. The phagocytic activity

in the current study was maximum in TN, and the diurnal variation was almost undetectable indicating high immune tolerance and stronger defence mechanism when the animal in the TN zone. Minimum phagocytosis and greater diurnal variation were observed in the summer season. This support the evidence suggesting that higher incidence of mammary infection in dairy cows could be due to high temperature which facilitates the survival and multiplication of pathogens which decrease their immunity (Mukherjee et al. 2015; Das et al. 2016). Our results are in agreement with Hriscu (2004) who reported higher phagocytic activity of neutrophil in rodents during late night and around morning and correlated it with melatonin concentration which peaks during the dark cycle of the day. However, we couldn't estimate and correlate melatonin in the present study.

Cortisol is a well-known marker for physiological and psychological stress, and therefore its use as an index of global stress is well documented (Neary et al. 2002). Cortisol concentrations were low in TN, intermediate in winter and highest in the summer season. Our findings are in agreement with other studies in which researchers have indicated that extreme environmental conditions during summer season induce the release of cortisol (Bouraoui et al. 2002; Alameen and Abdelatif 2012), which plays a significant role in reducing all aspect of neutrophils activity including phagocytosis (Alhussien et al. 2016b). Plasma cortisol displayed a clear diurnal rhythm and evening samples always had higher levels as compared to the morning sample. Our results are in agreement with Hänninen et al. (2006) but are contrary to the results of Lefcourt et al. (1993) who reported higher cortisol levels during morning compared to evening time in dairy cattle kept in the climatic chamber. This may be attributed to different experimental design and environmental conditions of the study area.

Milk SCC recorded during the first lactation was low but then increased in subsequent lactation and attained higher and significant levels in cows having more than four parity. Our results are in agreement with many authors who reported that SCC content increases with parity mainly after 4th parity (Saravanan et al. 2015; Sharma et al. 2016). The N: M ratio followed similar patterns of that of milk SCC, and diurnal variation was more apparent in cows having more than four parity. The change in the percentage of various immune cells present in blood and milk of dairy cows with higher parity suggests that their innate immune response may be functionally impaired and thus may lead to higher incidence of mastitis in older cows especially during early lactation (Rogers et al. 2005; Dang et al. 2010). Our results are in agreement with other authors who also reported higher phagocytic activity in primiparous cows as compared to the multiparous cows (Mehrzad et al. 2009; Dang et al. 2010). The lower phagocytic activity in cows having more than four parity may be due to higher cortisol levels in these animals. Plasma cortisol levels and its diurnal variation were maximum in multiparous cows especially after fourth parity which is in agreement with Burnett et al. (2015). This may be because multiparous animals are under higher stress mainly in their early lactation due to lower immunity and more chances of metabolic disorder.

5. Conclusions

The main finding of this study is that the diurnal variation in milk SCC and N: M ratio is maximum during the summer season and in cows having more than four parity. Out of all the milk cells, neutrophils exhibited maximum diurnal variation in their numbers where their percentage was always higher in the evening samples. Although the number of neutrophils

was more in the summer season and in the multiparous cows, however, their phagocytic activity significantly decreased under such conditions. Plasma cortisol hormone also exhibited diurnal rhythm and had a positive correlation with milk SCC and N: M ratio, but it was negatively correlated with the phagocytic activity of milk neutrophils. This study shows that recording of daily variation in the milk somatic cells and knowledge of the modulation in the activity of various immune cells secreted in the milk during different hours of the day can be used as a useful tool for timely detection and treatment of mastitis. Moreover, these findings draw more attention to the necessity for a careful circadian planning of immunomodulatory interventions and therapeutic approaches as the physiologic oscillations of milk SCC show different patterns in their number and activities during various parts of the day.

Disclosure statement

No potential conflict of interest was reported by the authors.

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