Blood neutrophil dynamics in periparturient Murrah buffaloes A Mohapatra and A K Dang

Article · January 2017

CITATIONS
0

READS
63

2 authors:

Arpita Mohapatra
Central Sheep and Wool Research Institute
16 PUBLICATIONS **43** CITATIONS
SEE PROFILE

Ajay Kumar Dang
ICAR-National Dairy Research Institute
167 PUBLICATIONS **1,258** CITATIONS
SEE PROFILE

Indian J Dairy Sci 70(5):571-576 (2017)

RESEARCH ARTICLE

Blood neutrophil dynamics in periparturient Murrah buffaloes

A Mohapatra and A K Dang

Received: 02 December 2016/Accepted: 02 March 2017

Abstract: Neutrophils play an important role around parturition. To evaluate relationship between cytological changes occurring in blood neutrophils and their functionality during peripartum period, blood samples were collected from 10 Murrah buffaloes (-15 to +15 day) at weekly interval. Total leukocyte count (TLC) and percent neutrophils were significantly higher ($P<0.05$) on the day of calving. Morphological features were assessed using both light and scanning electron microscopy (SEM). Blood neutrophils on different peripartum days showed a multi-lobed and matured nucleus as compared to band shaped nucleus of partum. SEM revealed that blood neutrophils had a more ruffled surface on different peripartum days compared to the neutrophils derived on the day of calving. Phagocytic activity (PA) of neutrophils isolated from blood of peripartum buffaloes were significantly lower ($P<0.05$) on day of calving compared to other peripartum days. Relative messenger RNA (mRNA) expression of Toll like receptors (TLR 2, TLR 4) and interleukin-8 (IL-8) genes in blood neutrophils of Murrah buffaloes was found to be significantly ($P<0.05$) higher on different peripartum days compared to the day of calving. The present study showed that on the day of calving although vast number of neutrophils poured into the blood stream but more of them are immature ones, having poorly developed surface receptors, compromised phagocytic activity and diminished expression of immunologically important neutrophil specific genes.

Keywords: Neutrophils, peripartum period, Murrah buffaloes, SCM, TLR

A Mohapatra (✉)
Arpita Mohapatra, ICAR-Central Sheep and Wool Research Institute, Avikanagar, 304501, Rajasthan, India.
E-mail: arpita.ndri.mohapatra1@gmail.com

A K Dang
Animal Physiology Division, ICAR-National Dairy Research Institute (NDRI), Karnal, Haryana, India 132001

Introduction

Buffaloes are the major milk producers in the Asian countries and contribute significantly to the dairy sector. During peripartum period buffaloes are under myriads of stress. These stressful changes results a reduction in the activity of blood neutrophils, which may invoke infectious outcomes (Mulligan *et al*., 2006). Periparturient immunosuppression contributes significantly to the economic losses in dairy industry. The function of neutrophils is important for protection of early phase infection. During this critical period, various cytokines and hormone *milieu* of blood and extracellular tissue fluid can influence neutrophil development and immunity related activities. The molecular basis of these changes and their physiological benefits or drawbacks occurring during this time are poorly understood. There are a few reports on the immune status of buffaloes particularly in relation to neutrophils (first line of defense) around calving. Therefore, the present study was undertaken to find out the changes occurring in the neutrophil morphology, PA and relative mRNA expression of IL-8, TLR-2 and TLR-4 in the blood neutrophils on various peripartum days of Murrah buffaloes.

Materials and Methods

Selection of animals and blood collection schedule

For the study, 10 healthy Murrah buffaloes approaching parturition were selected from the experimental herd of National Dairy Research Institute (NDRI), Karnal, Haryana, India. All the experimental animals were healthy and free from any anatomical, physiological and infectious disorders. Blood samples were collected from them starting from 15 days pre calving, -7 day, on the day of calving, +7 and on 15 days post calving. All the buffaloes were offered *ad lib* green fodder and calculated amount of concentrate mixture. Fresh tap water was also made available *ad lib* at all times of the day.

Isolation of blood neutrophils

Blood TLC and percent neutrophil were estimated microscopically. The gross morphology was determined by preparing smear stained with Leishman's stain. Isolation of polymorphonuclear

neutrophils (PMN) from peripheral blood was performed using hypotonic lysis of erythrocytes as described by Mehrzad *et al*. (2001, 2004). Briefly, 10 ml of EDTA mixed blood was poured in to Falcon tubes and centrifuged (1000 x g, 15 min., 4ÚC); the plasma layer, buffy coat and top layer of blood-packed cells were discarded. About 2.5 ml of blood-packed cell was lysed by adding 5 ml of diethyl pyro-carbonate (DEPC) treated double distilled water and gently mixed for 45 sec. using a magnetic stirrer. After restoration of the isotonicity by addition of DEPC treated 2.5 ml of 2.7% NaCl with gentle mixing for 60 sec., the suspension was centrifuged (1000 x g, 10 min. and 4ÚC). For the second lysis procedure, after resuspending of the pellets in DEPC treated 2.5ml of Dulbecco's PBS (without $CaCl_2$, $MgCl_2$), 5 ml of DEPC treated double distilled water and gently mixed for 30 sec., and centrifuged (1000 x g, 5 min. 4ÚC). The remaining cell pellet was washed 3 times in DEPC treated PBS (300 x g, 10 min., 4ÚC). Phagocytic activity (PA) was estimated by nitro blue tetrazolium (NBT) reductive assay proposed by Chai *et al*. (2005). Investigation of ultra-structural changes of blood neutrophils was carried out by employing Scanning Electron Microscopy (SEM) as previously described by Tian *et al*. (2005) in goats with minor modifications. Plasma cortisol levels were also estimated by ELISA (Endocrine Technologies, USA) during both the pre- and postpartum days as indicated above.

RNA isolation

RNA was isolated by Trizol method as per Chonczynski and Sacchi (1987) using TRI Reagent from Sigma (St. Louis, Missouri, USA). The RNA pellet was air dried for 15–30 minutes and dissolved in 25 µl of RNA storage solution and stored at "80°C till further use. Quality of RNA was checked by agarose gel electrophoresis using 0.8% gel (in 1× TAE buffer, pH 8.0) of high-quality molecular biology grade agarose (Sigma, USA). Ethidium bromide was used as fluorescence dye at the rate of 0.5 µg/ml of gel, whereas, bromophenol blue was used as tracking dye at the rate of 3 µl mixed with RNA during time of loading of sample into well of the gel. Electrophoresis was carried out at 8 V/cm for half an hour. After completion of electrophoresis, the gel was examined under UV transilluminator. DNase treatment was done by using DNA free Kit (Ambion, UK) according to manufacturers' instructions. Total RNA was quantified, and OD260nm/OD280nm was determined with ND-3300 flurospectrophotomer (NanoDrop Technologies, UT) and purity of RNA was judged on the basis of optical density ratio at 260:280 nm. First strand cDNA was synthesized by RevertAid First Strand cDNA synthesis kit from Fermentas Life Science. PCR was carried out with DreamTaq DNA Polymerase from Fermentas Life Science. Differential m-RNA expressions of neutrophilic genes (IL-8, TLR -2 and TLR- 4) were carried out by using real time PCR. Along with the target genes two housekeeping (glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin) genes were amplified for relative expression measurements. Two housekeeping genes, GAPDH and β-actin, were used in this study. The above housekeeping genes were selected as they had been shown to be the most stably expressed in the neutrophils (Robinson *et al*., 2007). Information regarding specific primers for IL-8, TLR-2, TLR-4, GAPDH and β-actin gene was retrieved from NCBI database and suitable primers were designed using primer 3 web interfaces. Details of primers were given in the table 2. The results indicated that, for our study, β-actin was better than GAPDH. So expression data of β-actin were used for analysis of relative expression data. Relative quantification of a target gene was done by comparing the expression levels of reference gene (β-actin), as per the method of Livak and Schmittgen (2001).

Statistical analysis

Statistical analysis was performed using least square model through SYSTAT software (sigma plot 11.0, Chicago, IL, USA). The means were separated and compared using Tukey test as post hoc test, because this test is able to control the errors of multiple comparisons simultaneously.

Results and Discussion

The number, type and morphological variation of neutrophils in Murrah buffaloes in relation to their function in different peripartum days have been presented in table 2. TLC was highest on the day of calving. Thereafter a significant ($P<0.05$) decrease in TLC was observed at 7 days post calving. Our results are in corroboration with Paape *et al*. (2003). Bone marrow recruits more

Table 1: Primers sequence, accession number, fragment size and annealing temperature used for RT-PCR

Genes	Sequence (5'—3')	Acc no.	Size (bp)	AnnealingTemp (^{0}C)
TLR 2	F GCCTTGACCTGTCCAACAAT	NM174197.2	199	59
	R GACCTGAACCAGGAGGATGA			
IL-8	F TGCTCTCTGCAGCTCTGTGT	EU276073.1	190	59
	R CAGACCTCGTTTCCATTGGT			
β- actin	F TCCCTGGAGAAGAGCTACGA	NM_173979.3	179	59
	R TAGAGGTCCTTGCGGATGTC			
	F GGGTCATCATCTCTGCACCT	NM_001034034.1	176	59
GAPDH	R GGTCATAAGTCCCTCCACGA			

number of leukocytes during acute stress and inflammation. As on the day of calving buffaloes are under nutritional, endocrinological, physiological and psychological stresses, TLC count increases to a peak level. The postpartum decrease in TLC is associated with the increased migration of leucocytes to uterine lumen followed by migration to mammary alveolar lumen (Preiser *et al.*, 2000). Among the leukocytes, neutrophil is the central player which decides animal's susceptibility to infection. The blood neutrophils increased from 31% at 15 days before calving to 39% on the day of calving, which further decreased to 32% at 7 days post-calving.

PA of blood neutrophils isolated during various peripartum days in terms of optical density (O.D) have also been presented in Table 2. O.D of blood neutrophils was minimum on the day of calving and then it started increasing during the postpartum

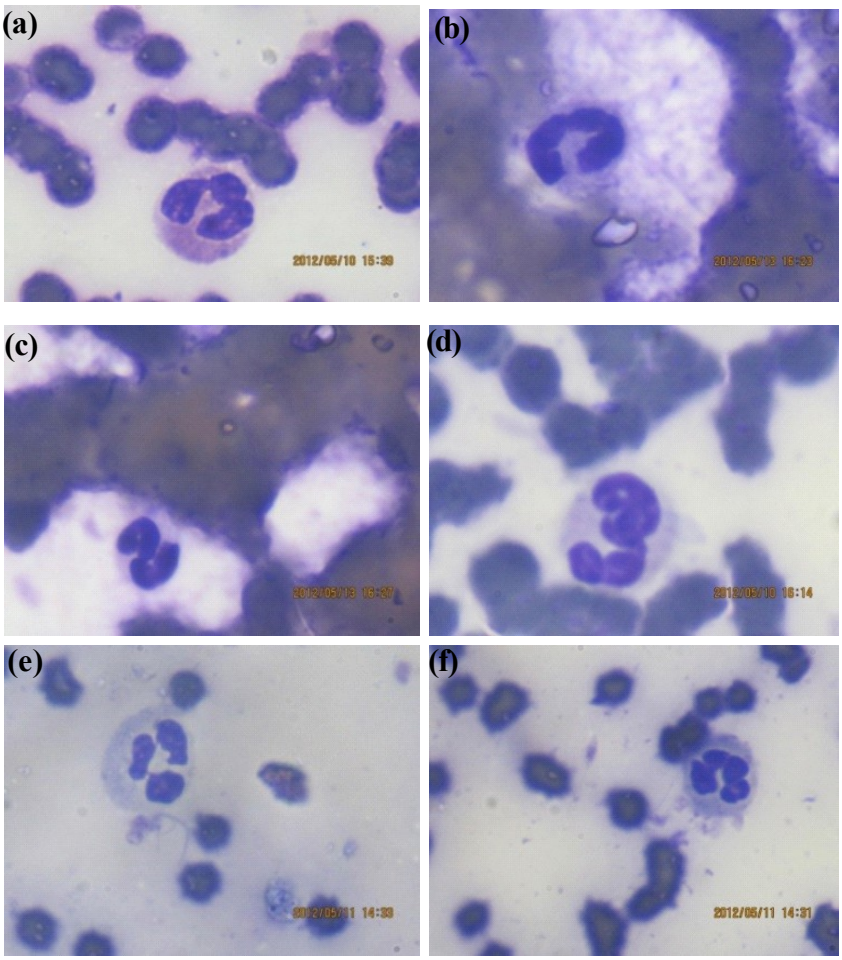
Table 2: Total leucocyte counts (TLC), neutrophil percent and type and optical density (O.D) indicating phagocytic activity of blood neutrophils in different peripartum days of Murrah buffaloes

ParametersUnder Study	Peripartum days				
	-15	-7	0	7	15
*TLC (cells/µl)	7634± 99.08^{a}	7678± 110.17^{a}	8451± 196.80^{b}	7698.5± 172.62^{a}	7407.4± 176.34^{a}
*Total Neutrophils (%)	30.8± 0.86^{a}	34.4± 1.03^{c}	38.6±1.17^{d}	32.8± 1.11^{ab}	30± 1.14^{a}
Band Neutrophils (%)	2±0.91^{a}	5±0.48^{c}	6±0.75^{c}	3±0.87^{b}	1±0.41^{a}
Segmented Neutrophils (%)	98±0.91^{a}	95±0.48^{c}	94±0.75^{c}	97±0.88^{b}	99±0.41^{a}
*Phagocytic Activity (O.D 540 nm)	1.16±0.04^{c}	0.95±0.06^{ab}	0.78±0.08^{a}	1.09± 0.06^{b}	1.24± 0.10^{d}

*Values within a row having different superscript are significantly (P < 0.05) different from each other.

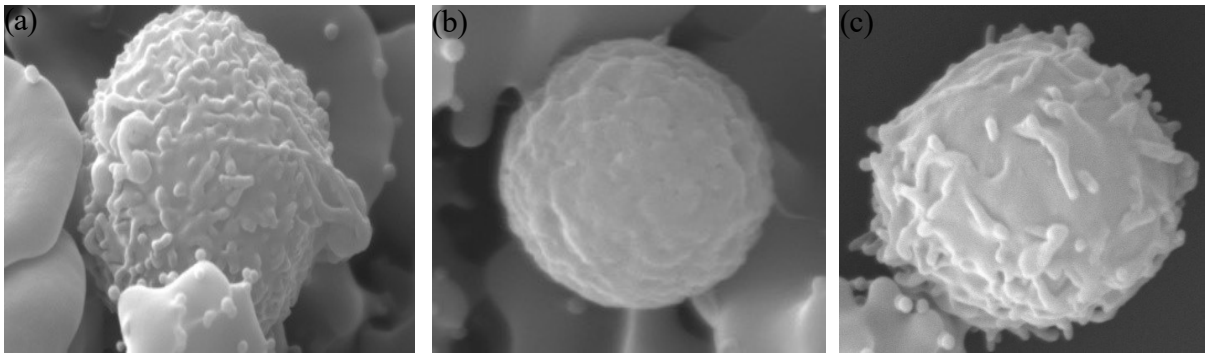
Photographs: Blood smear showing neutrophils (100X) from prepartum Murrah buffaloes at different days

- ❖ Day – 15 (a) showing multilobed nucleus
- ❖ Day -7 (b) showing multilobed but undifferentiated nucleus
- ❖ Day 0 (c, d) showing band shaped immature nucleus
- ❖ Day +7 (e) multilobed and differentiated nucleus
- ❖ Day +15 (f) showing hyper- segmented nucleus/neutrophilic lobes



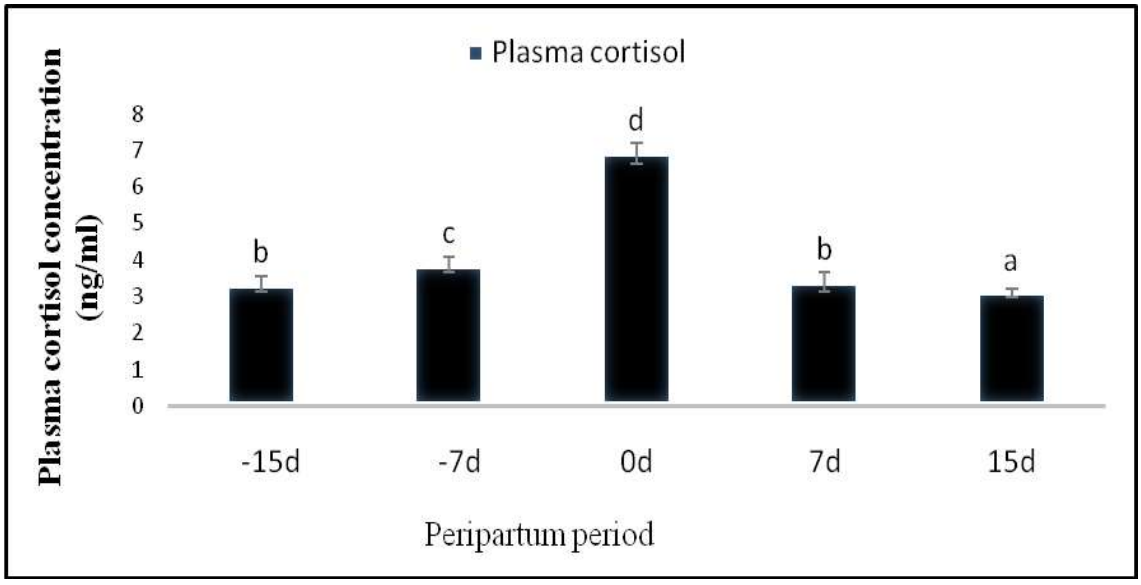
period and attained the prepartum levels. O.D increased significantly (P<0.05) at 7 days postpartum and then did not show any significant pattern thereafter. Only increase in neutrophil number is not sufficient, an increase in neutrophilic activity in terms of phagocytosis and intracellular digestion play pivotal role in fighting against infections. We observed a decrease in the neutrophilic activity of blood neutrophils around parturition in

Plates: SEM of blood neutrophil (N) isolated from Murrah buffaloes from precalving



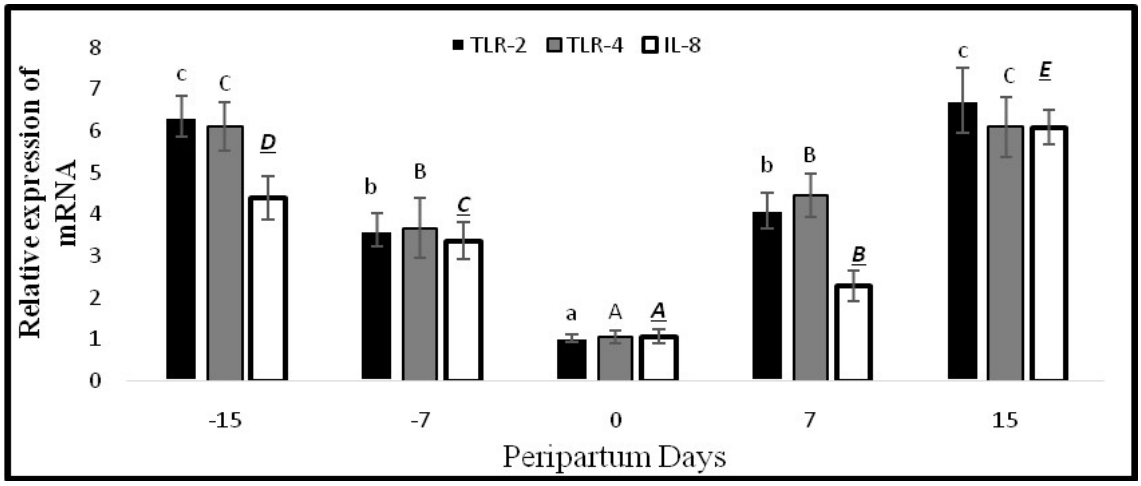
(**a**), on the day of calving (**b**) and post calving (**c**) period (3500 X)

Fig. 1: Plasma cortisol levels (ng/ml) at different peripartum days in Murrah buffaloes



*Figures with different superscripts letters differ significantly (P<0.01) from each other

Fig. 2: Relative mRNA expression of TLR-2, TLR-4 and IL-8 in blood neutrophils of Murrah buffaloes in Periparturient period



* Figures with different superscripts letters differ significantly (P<0.05) from each other.

buffaloes as similar to reported by Meglia *et al*. (2001) in exotic cows and by Pathan *et al.* (2016) in indigenous Sahiwal cows.

Neutrophilic development and migration is associated with altered hormonal levels. Results of plasma cortisol levels at different peripartum days are shown in figure 1. We observed a significant ($P<0.01$) increase in the level of cortisol on the day of calving as compared to different pre- and postpartum days. High levels of cortisol at calving have also been reported by Goff and Horst (1997) to act as powerful immunosuppressive agent. Higher levels of cortisol on partum resulted neutrophilia by an increased output of neutrophils from the bone marrow, hindering neutrophil margination from the blood vessel wall, or by a combination of the two (Meglia *et al*., 2001).

Blood neutrophils observed during -15 day, -7 day, on day of calving, +7 day and +15 day have been presented in photographs 1 to 6 respectively. Photograph 1 and 2 shows the neutrophils morphology at prepartum period. On -15 day they were multilobed and differentiated. Lobes were separated by thick chromatin fibers. On -7day they were multi-lobed but undifferentiated. Photograph 3 and 4 shows neutrophils on the day of calving. These were band cells having "C" shaped and "S" shaped nucleus without differentiation of lobes. Photograph 5 and 6 shows neutrophils isolated from blood samples collected after calving. On +7 day they were multi-lobed and separated by thin chromatin fibers, whereas on +15 day they were hyper-segmented (having more than 3 lobes).

The surface architecture of neutrophils also varied during peripartum period. Ultrastructure changes observed during these days are depicted in plates 1 to 3. Plate 1 shows neutrophil surface on precalving period, plate 2 shows neutrophil on day of calving and plate 3 shows the same on post-calving period. Neutrophilic maturity can be best studied by observing its gross structure and ultrastucture. Matured neutrophils have multilobed/ segmented nucleus, so they are called as "Segs". Light microscopy clearly depicts the presence of immature neutrophils on day of calving. It also emphasizes that periparturient stress begins at about -7 day of calving, reaches its peak at the day of calving and culminates by about +7 day of calving. SEM revealed that blood neutrophils are round in shape. They have ruffled surface on pre and post calving period compared to the neutrophils derived on the day of calving. Ruffledness/ protrusions present on neutrophil surface provide extensive surface area to PMN. The increased surface area is crucial to phagocytic function of PMN because extensive areas of plasma membrane are internalized during phagocytosis to form phagosomes. Neutrophils lack nucleolus; therefore they are incapable to resynthesize their membrane architecture (pseudopods). Neutrophils on the day of calving appeared smoother. These neutrophils have poorly developed micro-ridges and microvilli. This has been reported for the first time in peripartum buffaloes. These plates explain that on the day of calving neutrophils have a compromised phagocytosis compared to that of other peripartum days. The present study clearly depicts that neutrophilic PA is regulated by degree of maturity of neutrophils, as we observed more number of immature or band neutrophils at calving as compared to other days. Therefore, even if there was neutrophilia on day of calving but the PA was very poor.

The expression of IL-8, Toll Like receptor (TLR) 2 and 4 in blood neutrophils were also estimated in the present study and have been presented in Fig. 2. We found a significantly ($P<0.05$) higher expression of TLR2, TLR-4 and IL-8 on 15 day before and 15 day after parturition as compared to the day of calving. TLR receptors detect pathogen by a variety of pattern-recognition systems and are likely to have important roles in the regulation of neutrophil function (Parker *et al*., 2005). They provide cellular signaling during the initiation of the immune response (Janeway *et al*., 2001). Lipopolysaccharide (LPS) is recognized by TLR-4, whereas, bacterial peptides, yeast zymosans of gram positive bacteria are recognized by TLR-2 (Lippolis, 2008). IL-8 is a potential chemoattractant for neutrophil which mediate trans-endothelial migration of neutrophils to tissue spaces to destroy bacterial pathogens (Khoramian *et al*., 2015). All together, they mediate phagocytic activities of neutrophils. There was a decreased expression of the Interleukin-8 (IL-8). According to Wu J *et al*. (2015) IL-8 regulates the recruitment of neutrophils as well as T-lymphocytes to the site of infection. This indicates that the ability of neutrophils to migrate to the site of infection was lower during calving. Down regulation of these receptors as observed in our study may make the animal more susceptible to disease around calving.

According to Mehrzad, 2012 physiological qualities of neutrophils in the blood, such as their rate of diapedesis, phagocytosis, and detoxification mechanisms, represent key components in the development of the inflammation and largely determine the severity of mastitis after bacterial challenge. The results obtained in this study imply that on the day of calving due to the intense stress no doubt a large number of neutrophils were recruited into the blood stream, but many of them cannot exhibit their full potential as they are immature. That results in the poor expression of neutrophil specific genes particularly TLR-2, TLR-4 and IL-8 resulting in reduction of its PA as well as trans-endothelial migration to inflamed tissues. Also due to inefficient lobulation of neutrophilic nucleus, its unique diapedic ability gets reduced. Similarly due to lack of neutrophilic micro-ridges and microvilli PA also get compromised. A state of immunosuppression starts before 7 days of calving in Murrah buffaloes. Neutrophils in and around calving are unable to function properly resulting accumulation of neutrophil in circulation, at the same time higher cortisol level and poor expression of IL-8 receptors reduce their migration to tissue under stress resulting increased susceptibility to postpartum infections.

Conclusions

This study suggests that change in blood neutrophil's percent, change in their morphology and PA can be taken as more reliable parameter for assessing the degree of stress during peripartum period. The higher incidence of disease during peripartum period is partially due to compromised function of neutrophils. Therefore, evaluation of immune activity of neutrophils and altered expression of these immunologically important genes may help us to understand the immune response of Murrah buffaloes during this physiologically critical period. This will help researchers to develop more efficient strategies to combat immunosuppression around this period.

Acknowledgements

The authors would like to thank the Director of NDRI, Karnal for providing the facilities for the execution of this work. This work was supported by the Department of Biotechnology (Grant Number: BT/PR8404/ AAQ/1/548/2013 DATED 20/ 06/2014), Ministry of Science and Technology, Government of India, we are highly thankful to them.

References

Chai EMi, Kim Y, Kim A, Hwang J (2005) Immunomodulating activity of arabinogalactin and fucoidan *in vitro*. J Med Food 8(4): 446-455

Chomczynski P, Sacchi, N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162 (1): 156-159

Pathan MM, Kaur M, Mohanty AK, Dang AK 2016 Effect of milk yield on functional activity of neutrophil in crossbred Karan Fries (Holstein Friesian X Tharparkar) cows around peripartum. Veterinarski Arhiv 86 (3), 295-310

Lippolis JD (2008) Immunological signaling networks: Integrating the body's immune response. J Anim Sci 86(E. Suppl.):E53–E63 doi:10.2527/jas.2007-0620

Janeway CA, Travers P, Walport M, Shlomchik M (2001) Basic concepts in immunology. In: Immunobiology: The immune system in health and disease. 5th (eds), Garland Publishing, New York. 1 – 34

Khoramian B, Vajhi A, Ghasemzadeh-Nava H, Ahrari-Khafi M, Bahonar A (2015) Ultrasonography of the supramammary lymph nodes for diagnosis of bovine chronic subclinical mastitis. IJVR 16: (1) 50, 75-77

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402–408

Meglia GE, Johannisson A, Agenäs S, Holtenius K, Waller KP (2001) Effects of feeding intensity during the dry period on leukocyte and lymphocyte sub-populations, neutrophil function and health in periparturient dairy cows. http://www.medscape.com /medline/ publication. Vet. J 2005 169(3): 376-84

Mehrzad J, Dosogne H, Meyer E, Burvenich C (2001) Local and systemic effects of endotoxin mastitis on the chemiluminescence of milk and blood neutrophils in dairy cows. Vet Res 32: 131–144

Mehrzad J, Duchateau L, Burvenich C (2004) Viability of milk neutrophils and severity of bovine coliform mastitis. J Dairy Sci 87: 4150-4162

Mehrzad J (2012) Molecular aspects of neutrophils as pivotal circulating cellular innate immune systems to protect mammary gland from pathogens. In: Kanwar J.R. (Ed.), Recent Advances in Immunology to Target Cancer, Inflammation and Infections. ISBN: 978-953-51-0592-3. In Tech Publishing Inc. pp. 385–422

Mohapatra A, Swain DK, Sashipal, Pathan MMK, Kaur M, Panigrahy SR, Kapila S, Kapila R, Prasad S, Mohanty AK, Dang AK (2012) Scanning Electron Microscopy and Phagocytic Activity of Blood and Milk Neutrophils Isolated from Early Lactating Buffaloes. Indian J Dairy Sci 65(6): 479-483

Mulligan F, Grady L O, Rice D, Doherty M (2006) Production diseases of the transition cow: Milk fever and subclinical hypocalcaemia. Irish Vet J 59 (12): 697-702

Parker LC, Whyte MK, Dower SK, Sabroe I (2005) The expression and roles of Toll-like receptors in the biology of the human neutrophil. J Leu Bio 77: 886–92

Preisler MT, Weber PS, Tempelman RJ, Erskine RJ, Hunt H, Burton JL (2000) Glucocorticoid receptor down regulation in neutrophils of periparturient cows. Am J Vet Res 61: 14 –19

Robinson TL, Sutherland IA, Sutherland J (2007) Validation of candidate bovine reference genes for use with real-time PCR. Vet Immunol Immunopathol 115: 160–165

Tian SZ, Chang CJ, Chiang CC, Peh HC, Huang MC, Lee JW, Zhao X (2005) Comparison of morphology, viability and function between blood and milk neutrophils from peak lactating goats. Can J Vet Res 69: 39-45

Wu J, Li L, Sun Y, Huang S, Tang J, Yu P, et al. (2015) Altered Molecular Expression of the TLR4/NF-κB Signaling Pathway in Mammary Tissue of Chinese Holstein Cattle with Mastitis. PLoS ONE 10(2): e0118458. doi:10.1371/journal.pone.0118458