COMPARATIVE EVALUATION OF FUNCTIONAL ACTIVITY OF NEUTROPHIL IN HIGH AND LOW YIELDING MURRAH BUFFALOES DURING PERIPARTUM PERIOD

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ABSTRACT

To evaluate immunologic activities of neutrophils, blood samples were collected from 6 high yielding (HY) and 6 low yielding (LY) Murrah (MU) buffaloes on -15, -7, -5, -3, -2, -1 days prepartum, at calving and on 1, 2, 3, 5, 7 and 15 days postpartum. Blood Total leucocyte counts (TLC) and neutrophil percent increased at calving in both the groups, but the levels were found to be significantly (P<0.05) high in HY MU buffaloes. The number of bend neutrophils were also significantly (P<0.05) higher in HY buffaloes. Significant (P<0.05) immunosuppression in relation to PA was found for HY MU buffaloes throughout the peripartum period with lowest immunosuppression at calving in both the groups. Cortisol levels were significantly (P<0.01) higher during calving and negatively correlated with neutrophilic functions. The difference between two groups also remained significant (P<0.05) as higher level of cortisol found in HY MU buffaloes. Elastase, collagenase and cathepsin were significantly (P<0.05) decreased during parturition. Elastase of HY buffalo neutrophil was reduced 2 times than 1.5 times for LY buffalo at calving. Collagenase and cathepsin levels were significantly (P<0.05) higher in LY buffaloes. At 7 day pre calving and 7 and 15 days post calving, expression of TLR-2 gene were significantly (P<0.05) lower in HY buffaloes. Expression of TLR-4 and IL-8 genes were significantly (P<0.05) lower on days 15 pre and post caving in HY buffaloes. Decreased blood neutrophilic functions in buffaloes having high production potential provides lower disease resistance and make them more susceptible to infection around peripartum.

Keywords: buffaloes, cortisol, gene expression, neutrophil activity, peripartum

INTRODUCTION

Buffaloes are found mostly in the Indian subcontinent and some part of South America, Southern Europe, Middle East and Northern America. Buffalo is the major source of milk production contributing 12.1% in world, 38% in Asia and 55% in India's total milk production (FAO STAT, 2007). Buffaloes are highly resistant to various infections as compared to cows (El-Wishy, 2007). Milk production potential also affects the immunity of animals. Buffaloes with higher production potential are more prone to infection as compared to low producing buffaloes.

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The immunological basis behind this has not been completely elucidated in buffaloes in specific during peripartum period (Nanda *et al*., 2003). Keeping this objective in mind present study was formulated to explore the immune physiological basis of resistance of buffaloes and also to understand the immune related factors which affect the health status of high producing buffalo and make them more prone to infection. For that, blood neutrophil is taken as the cell of interest and one of the major components of immune axis as they are considered as first line of cellular defence which form an integral part of innate immune system. Neutroophils mediate killing of bacterial pathogens by phagocytosis through a cascade of proteases, antimicrobial peptides, and free redicals (Segal, 2005). Eventually, neutrophils undergo apoptosis in the tissues or at the sites of inflammation. Primary role of neutrophils is the participation in inflammatory response by producing cytokines, eccosanoids and cell signalling molecules. The complex interplay between all these leads to neutrophilic activity causing host cell protection (Serhan *et al*., 2005).

The critical period of calving was selected as neutrophils play a vital role in the onset of disease around parturition (Kehrli *et al*., 1989). Phagocytic and respiratory burst activity of neutrophil reduces around parturition (Hoeben *et al*., 2000), which may lead to mastits, metritis and retained placenta like postpartum disease (Kehrli and Harp, 2001). According to Burton *et al*. (2005), blood neutrophils exhibits expression of glucocorticoids receptors and these receptors respond to high plasma cortisol concentrations in bringing out altered neutrophil signalling and functioning around parturition.

Literature lacks information in high and low yielding buffaloes regarding the activity of neutrophils in terms of phagocytosis (Dang *et al*., 2009, 2012). Also, there are no reports on the enzymatic activity and differential expression of neutrophilic genes during calving period and specific to production potential. With this overview, the present study was undertaken to elucidate and compare the neutrophilic activities in both high and low producing Murrah buffaloes around parturition.

MATERIALS AND METHODS

Selection of animals

Twelve Murrah buffaloes in their advance stage of gestation i.e. at 15 days before the expected date of calving were selected from the National Dairy Research Institute experimental herd. They were further divided into two subgroups, high yielding (HY) and low yielding (LY) based on their production potential of previous lactation. HY MU buffaloes (n=6) were producing above 2157±102.45 liter per lactation whereas LY MU buffaloes (n=6) were producing below 1632.53±77.53 liter per lactation. All the buffaloes were offered *adlib* green fodder and calculated amount of concentrate mixture. Fresh tap water was also made available *adlib* at all times of the day. All the experimental buffaloes were healthy and free from any anatomical, physiological and infectious disorders.

Collection of samples and analysis

Blood samples were collected from all the buffaloes during -15, -7, -5, -3, -2 and -1 days prepartum, on the day of calving and 1, 2, 3, 5, 7 and 15 days postpartum. Calving in all the animals occurred within ±5 days of the expected date of calving.

Blood total leukocyte counts (TLC) and differential neutrophil counts were estimated microscopically from all the group of animals. *In*

vitro phagocytic activity of blood neutrophils by nitro blue tetrazolium (NBT) assay (Dang *et al*., 2012) and plasma cortisol levels were also estimated by ELISA (Endocrine Technologies, USA) during both the pre and postpartum days as indicated above. The minimum detectible concentration of cortisol by this assay was estimated to be 0.1 ng/ml. Coefficient of Variation (CV) were calculated from the calculated concentrations. Inter-assay % CV was found to be 2.59 and intra-assay % CV was found to be 0.05.

Activities of enzymes Elastase 2, Collagenase and Cathepsin G were measured by ELISA kits (WEKA MED and Wuhan Eiaab Science Co., Ltd., China) from blood samples collected during -7, -3 days prepartum, on the day of calving and 3 and 7 days postpartum. For preparing lysate of neutrophils, the isolated neutrophils were dissolved in 1 ml PBS. Glass beads were added to neutrophil suspension and shock was given for 25 seconds by Bead beater (Unigenetics Instrument Pvt. Ltd., India). Put it in ice for 1 minute, then again shock was given for 25 seconds. It was centrifuged at 1000 x g for 10 minutes. Supernatant was taken in 2 ml eppendorf tubes and were stored at -20°C till further estimation. Percent CV was calculated from the calculated concentrations. Inter-assay %CV was found to be 6.12, 5.33, 6.21 and intra-assay %CV was found to be 3.52, 2.11 and 1.88 for Elastase, Collagenase and Cathepsin G respectively.

Relative expression of neutrophilic genes

All solutions were prepared using DEPC treated RNase free plastic wares and water. Total RNA from the blood neutrophils was extracted using Trizol method as per Chonczynski and Sacchi, (1987). The RNA pellet was air dried for 15 to 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 1X 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 in to 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 OD_{260nm}/OD_{280nm} 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. Reverse transcription was performed from 1 µg of RNA using Novagen first strand cDNA synthesis kit (La Jolla, CA).

Real Time PCR for TLR-2, TLR-4 and IL-8 and two housekeeping genes (Glyceraldehydes 3-phosphate dehydrogenase - GAPDH and β- actin) was carried out using Roche Light Cycler-480, Germany. The above housekeeping genes were selected as they had been shown to be the most stably expressed in the neutrophils (Robinson *et al*., 2007). The sequence information of gene was retrieved from NCBI database and suitable primers were designed using primer-3 web interfaces. Details of primer specification are given in the Table 1. Broadly for each real-time quantitative PCR (qPCR), 1 µg cDNA was added to a 20 µl mix containing primers, IQ SYBER-green supermix (Bio-Rad) and nuclease free water. PCR conditions were 300s at 95°C, 45 cycles of 20s at 95°C, 20s at appropriate annealing temperature (Table 1),

20s at 72°C. A melting curve for each qPCR with a single peak at the correct melting temperature was indicative of reliable and desired PCR product. mRNA abundance on 0 day (a day of parturition) was taken as calibrator to whom relative expression were seen. Calculation was done using the $2^{-\Delta\Delta Ct}$ method (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 model used for analysis was $Y_{ij} = \mu + Gi + Dj + Ti\,(Dj) + E_{ij,}$ where Y_{ij} was an observation of dependent variable; μ was the population mean for the variable; Gi was the effect of the group; Dj was the effect days; Gi (Dj) was the interaction between the group and days and E_{ij} was the random error associated with observation. The means were separated and compared using Tukey test as post hoc test, because this test is able to control the errors of multiple comparisions simultaneously. Further, the effect of different treatments on 15 days of prepartum was not used as covariate for subsequent analysis as our main interest was to differentiate the effect of two different treatments.

RESULTS

Total leukocyte counts was measured from blood of HY and LY MU buffaloes during pre and postpartum period and shown in Table 2. Highest ($P<0.001$) level of TLC was found on the day of calving in both the groups but the levels were found to be significantly ($P<0.05$) high in HY MU buffaloes as compared to LY MU buffaloes. After calving TLC was reduced significantly ($P<0.01$) on 3, 5 and 7 day after calving as compared to on the day of calving in both HY and LY MU buffaloes. Difference in TLC between the day of calving and 15 days after calving was highly significant ($P<0.001$). Between HY and LY MU buffaloes, significant changes were found in all postpartum days except day 7 and 15 of postpartum. Overall mean of TLC remained significantly ($P<0.05$) higher for HY than LY MU buffaloes.

High yielding MU buffaloes found significantly ($P<0.05$) higher neutrophil count than LY MU buffaloes. However, blood neutrophils was found highest ($P<0.001$) on the day of calving in both the groups. After parturition, significant ($P<0.01$) reduction in neutrophil counts were observed just one day after calving in both the groups (Table 3). We also observed an increase in percentage of band neutrophils and decrease in segmented neutrophil percentage on the day of caving in both the groups of buffaloes. But the percentage of immature or band neutrophils were significantly ($P<0.05$) higher in HY buffaloes then LY buffaloes.

Neutrophil PA was estimated in both the groups of buffaloes during peripartum period (-15 to +15 days). The PA was represented in terms of optical density due to formation of formazan crystals (Table 4). Lowest neutrophilic PA was observed on the day of calving in both HY and LY MU buffaloes but in between two groups, significant ($P<0.05$) immunosuppression was found for HY MU buffaloes as compared to LY MU buffaloes throughout the peripartum period. After calving, sluggish increase in PA was observed upto 3 day postpartum in HY MU buffaloes and upto 5 days postpartum in LY MU buffaloes which became significant ($P<0.001$) on 15 days postpartum as compared to that observed on the day of calving in both HY and LY MU buffaloes.

Plasma cortisol level were measured by

ELISA during peripartum period (-15 to +15 days) and presented in Table 5. Levels of plasma cortisol were always found to be significantly (P<0.001) higher in HY MU buffaloes as compared to LY MU buffaloes during peripartum period. A steady rise in plasma cortisol was observed in both the groups of animals with peak observed on the day of calving. Level of cortisol observed on the day of calving was approximately four and 2.5 times higher for HY and LY MU buffaloes respectively as compared to level of cortisol observed on day 15 preparrtum. After parturition, steady significant (P<0.001) decline in cortisol levels was observed on 1 day after calving in both groups of buffaloes. Thereafter, cortisol levels increased or decreased but the differences were non significant upto 15 day postpartum as compared to 1 day after calving in both HY and LY MU buffaloes.

Neutrophilic enzymes that are important in combating infection like Elastase, cathepsin G and Collagenase were estimated byELISA in both groups of MU buffaloes and depicted in Table 6. Two and 1.5 time reduction in level of elastase 2 was observed on the day of calving in HY and LY MU buffaloes respectively as compared to prepartum level. After calving, rapid increase in elastase 2 was observed on 3 day postpartum in both the groups of buffaloes. The levels of elastase 2 observed during whole peripartum period were remained significantly (P<0.05) higher for HY MU buffaloes as compared to LY MU buffaloes. But on the day of caving the significantly (P<0.05) lower level was observed in HY MU buffaloes than LY MU buffaloes. Levels of collegenase were also reduced up to 2 fold on the day of calving in both groups of buffaloes (Table 6). However, significant (P<0.05) reduction was seen in HY than LY MU buffaloes. Lowest level of cathepsin G was also observed on the day of calving in both HY and LY MU buffaloes during whole peripartum period (Table 6). Between HY and LY MU buffaloes, significant (P<0.05) difference were observed only on the day of calving with significantly lower level in High yielder as compared to low yielder Murrah buffaloes.

The result for the relative expression of the important neutrophilic genes TLR-2, TLR-4 and IL-8 have been presented in Table 7. Significantly (P<0.05) lowest expression of TLR-2, TLR-4 and IL--8 genes were observed on the day of calving as compared to all peripartum days in both HY and LY MU buffaloes. Expression of these genes remained lower in HY MU buffaloes as compared to LY MU buffaloes. Significant (P<0.05) change in expression of TLR-2 gene between HY and LY MU buffaloes found throughout peripartum period except 15 days before calving, whereas, TLR-4 gene expression differed only at 15 days before parturition in between two groups. Expression of IL-8 gene was differed significantly between HY and LY MU buffaloes on 15 days before and after calving.

DISCUSSION

White blood cells are involved in defence against pathogens. The blood TLC and neutrophil counts reflect the immune status of animal in HY and LY MU buffaloes. Values of blood TLC and neutrophil counts observed around the peripartum period in both groups of buffaloes were within the normal range as reported by Abd Ellaha *et al*. (2013) in midterm pregnant buffaloes. An increase in TLC around calving is coupled with rise in cortisol level in both the groups. It is believed that TLC increases around calving as a result of antipartum rise in cortisol level. However, TLC decreases during

postpartum period and it is coupled with migration and recruitment of blood neutrophils towards uterine lumen and mammary tissues (Preisler *et al*., 2000). The study also signifies that higher cortisol stimulates larger amount of neutrophil release around calving.

Neutrophils are known as first line defence, they migrate first from blood into an inflamed area for phagocytosis and intracellular killing by engulfing bacteria with two distinct mechanisms, the respiratory burst and by digestion through lysosomal enzymes (Jain, 1986). Mature segmented neutrophils only have the complete machinery to phagocytose bacteria and so they must be high in circulation (Paape *et al*., 2003) but, we observed significantly higher numbers of band (immature) neutrophils at calving in all buffaloes. Larger number of bend neutrophils in high producing buffaloes as compared to low producing buffaloes may be due to stress on the mammary tissues to produce more milk. 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 (Burton *et al*., 2005). Migration of mature neutrophils from 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 (Paape *et al*., 2003; Burton *et al*., 2005)

In vitro analysis of neutrophil function provides a very effective tool for the study of natural disease resistance. We observed decreased PA of blood neutrophils around calving. Diminished neutrophil functions and compromised host resistance mechanisms during peripartum period in dairy animals have also been observed by Meglia *et al*. (2001) and Dang *et al*. (2012), Poor activity of neutrophils may be due to more numbers of immature neutrophils which are coming in circulation which have no proper machinery to fight or phagocytose against infection as also observed by us from 3 day prepartum to 5 day post partum, whereas, reduction in phagocytic activity was highly significant 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. We also found a significant negative correlation between PA of blood neutrophils and plasma cortisol levels. There were increased neutrophil numbers during parturition yet phagocytic activity remained lower. Parturition reflex causes higher plasma cortisol level that causes hyper stimulation of red bone marrow for the faster release of neutrophils. As a result of this, there is release of more number of immature band neutrophils and a less number of matured segmented neutrophils. That is why the phagocytic activity of neutrophils decreases as evident in our study (Paape *et al*., 2003). 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. Further, milk production potential is higher in high producing buffaloes than low producing; therefore, they exhibited more stress and low PA as compared to low yielding buffaloes.

Glucocorticoids are a class of steroid hormones that bind to the glucocorticoid receptor, and are part of the feedback mechanism in the

immune system that down regulate the immune activity. Cortisol is released in response to stress and a low level of blood glucose. Glucocorticoid suppresses the immune system, increase blood sugar through gluconeogenesis, and aid in metabolism of protein, fat and carbohydrate. We observed a significantly higher level of cortisol at calving as compared to 15 days before and 15 days after calving in both the groups. A similar observation was reported in cattle (Prakash and Madan, 1985; Goff and Horst, 1997) and cross bred goats (Khan and Ludri, 2002). However, the cortisol values reported at calving were more than those reported by above authors. We also found a significantly higher level of cortisol in HY than LY KF cows. The cows during peripartum period are under various types of stressful conditions like stress of providing nutrition to its growing calf, stress of labor, to synthesize colostrum and then milk. A social stress of being isolated is also there. Overall effects of stress increased cortisol level, produced neutrophilia with decreased functional capacity of neutrophils, immunosupression and ultimately the high producing cows become more prone to mastitis and other infections (Kehrli *et al*., 1991). In agreement with our finding high levels of cortisol at calving have also been reported to act as powerful immunosuppressive agent (Goff and Horst, 1997).

Neutrophils mediate phagocytosis through a complex cascade of enzymes and their interrelated pathways. The release of enzymes is specifically regulated by cytokine network and their signaling to neutrrophils via cytokine receptors. Elastase 2, collagenase and cathepsin G are granular enzymes that are stored in neutrophil cytoplasm. The timely and net release of these enzymes determines the ultimate fate of neutrophil activities in terms of phagocytosis and resolution of inflammatory cascades. Granules are stored in neutrophils and are store house of variety of enzymes that are released to extra cellular space during inflammation and midiate pathogen inactivation and killing. Soluble agent like fMLP (N-formyl-methionine-leucine-phenylalanine), chemotaxins and C5a are the regulators of granule release from neutrophils. Neutrophils release elastase and cathepsin, which are serine proteases during inflammation to bring out destruction of pathogens (Belaaouaj *et al*., 2000).

Decreased neutrophil enzyme levels observed in this study may be because at calving more immature neutrophils are released that are poor in synthesis of granular enzyme as well as due to high cortisol are not able to release granular enzyme. During any disease condition the levels of these enzymes increased, which provide immunity to animals (Haddadi *et al*., 2006). During calving when animals go down in concentration of these enzymes due to higher level of cortisol make animal more susceptible to postpartum diseases (mastitis, matritis etc.). Higher levels of these enzymes in LY MU buffaloes then HY MU buffaloes indicate that the LY buffaloes are more resistant as compared to HY buffaloes to postpartum infections. The animals which give more milk found to have fewer amounts of neutrophilic enzymes which make them more prone to diseases.

Detection of pathogens in neutrophils is mediated by a variety of pattern-recognition systems, foremost amongst which are the Toll-like receptors (TLRs), and thus these receptors are likely to have important roles in the regulation of neutrophil function (Parker *et al*., 2005). Our study is the first to indicate a down regulation of blood PMN expression of immune genes due to increased endogenous blood plasma cortisol during peripartum period in buffaloes. We observed a significantly

higher expression of TLR-2, TLR-4 and IL-8 on 15 day before and after calving as compared to the day of calving. During the periparturient period animal experience negative energy balance (NEB) from 3 day before to 3 days after calving. (Ingvartsen and Andersen, 2000). The higher level of cortisol helps to provide energy demand by increasing lypolysis and gluconeogenesis, which results in an increase in the ratio of unsaturated fatty acid to saturated fatty acid. Saturated fatty acid induces the activation of TLR-2 and 4, whereas, unsaturated fatty acids inhibits (Lee and Hwang, 2006). Decreased expression of these genes during calving might be due to increased levels of unsaturated fatty acids.

Similar role is played by IL-8, which is considered as the central regulator of neutrophil signalling (Burton *et al*., 2005). During the study, we estimated the expression of (IL-8). It is a potential chemoattractant factor for neutrophil which mediate transendothelial migration of neutrophils to tissue spaces to destroy bacterial pathogens (Kehrli and Harp, 2001). IL-8 regulates the recruitment of neutrophils as well as T-lymphocytes to the site of infection (Wang *et al*., 2007). Activation of neutrophils during inflammation is a key event which is mediated by IL-8 (Galligan and Coomber, 2000). In our study, there was a significantly ($P<0.05$) higher expression of IL-8 on 15 day before and 15 day after parturition as compared to the day of calving. This indicates that the neutrophils of high producing buffaloes having lower ability to migrate to the site of infection then low producing buffaloes. With this finding the statement comes out that, neutrophils of buffaloes having higher production potential are less immunocompetent then buffaloes with low production potential.

CONCLUSIONS

Present study was planned to evaluate the relative competency of blood neutrophils in high and low producing Murrah buffaloes during peripartum period. The range experiment conducted during the period were mainly confined to understand some of the basic features of blood neutrrophils in terms of their activities and gene expression that are associated with the regulation of immune physiological responses. We observed a decrease in the PA of HY buffalo blood neutrophils as compared to LY buffaloes. The relative higher circulating concentration of cortisol is another determining factor that keeping up lower PA of HY buffalo neutrophils. Higher content of neutrophilic enzymes in the LY buffalo neutrophils strongly supported that immunocompetency of LY buffalo neutrophils is more than that of HY buffaloes. Eventually, we also reported a decrease in the mRNA expression of TLR-2, TLR-4 and IL-8 genes in HY MU buffaloes. Altogether, these findings make us to frame a conclusion that the LY buffalo neutrophils are more potent as compared to the HY buffalo neutrophils. It can be a probable explanation behind the fact that HY buffaloes are less resistant to infections during transition period as compared to the LY buffaloes. This study although carried out on some of the neutrophilic functions, clearly indicated the degree of immune suppression occurring in two different yielders of same species around peripartum. These results will help in understanding the physiology of neutrophils at calving and help to develop strategies to improve the immune functions around this period. Also, further studies are required to employ genetic and proteomic tools to find out the exact mechanism of neutrophil action in buffaloes.

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REFERENCES

Abd Ellaha, M.R., M.I. Hamedb and R.I. Derarc. 2013. Serum biochemical and haematological reference values for midterm pregnant buffaloes. *J. Appl. Anim. Res*., **41**(3): 309-317.

Belaaouaj, A., K.S. Kim and S.D. Shapiro. 2000. Degradation of outer membrane protein in Escherichia coli killing by neutrophil elastase. *Science*, **289**(5482): 1185-1188.

Burton, J.L., S.A. Madsen, L.C. Chang, P.S.D. Weber, K.R. Buckham, R.V. Dorp, M.C. Hickey and E. Bernadette. 2005. Gene expression signatures in neutrophils exposed to glucocorticoids: A new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows. *Vet. Immunol. Immunop*., **105**: 197-219.

Chonczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem*., **162**: 156-159.

Dang, A.K., S. Kapila, M. Purohit and C. Singh. 2009. Changes in colostrums of Murrah buffaloes after milking. *Trop. Anim. Heath Pro*., **41**: 1213-1217.

Dang, A.K., S. Prasad, K. De, S. Pal, J. Mukherjee, I.V.R. Sandeep, G. Mutoni, M.M. Pathan, M. Jamwal, S. Kapila, R. Kapila, H. Kaur, S. Dixit, A.K. Mohanty and B.S. Prakash. 2012. Effect of supplementation of vitamin E, copper and zinc on the in vitro phagocytic activity and lymphocyte proliferation index of peripartum SW *(Bos Indicus)* cows. *J. Anim. Physiol. An. N.*, **97**(2): 315-321.

El-Wishy, A.B. 2007. The postpartum buffalo: I. Endocrinological changes and uterine involution. *Anim. Reprod. Sci*., **97**(3-4): 201-215.

FAOSTAT, 2007. *Statistics Division*, Food and Agriculture Organization, UNO, RRome, Italy.

Galligan, C.L. and B.L. Coomber. 2000. Effects of human IL-8 isoforms on bovine neutrophil function *in vitro*. *Vet. Immunal. Immunop*., **74**: 71-85.

Goff, J.P. and R.L. Horst. 1997. Physiological changes at parturition and their relationship to metabolic disorders. *J. Dairy Sci*., **80**: 1260-1263.

Haddadi, K., C. Prin-Mathieu, F. Moussaoui, G.C. Faure, F. Vangroenweghe, C. Burvenich and Y. Le Roux. 2006. Polymorphonuclear neutrophils and *Escherichia coli* proteases involved in proteolysis of casein during experimental *E. coli* mastitis. *Int. Dairy J*., **16**: 639-647.

Hoeben, D.E., G. Monfardini, C. Opsomer, H. Burvenich, A. Dosogne, De Kruif and J.F. Beckers. 2000. Chemiluminescence of bovine polymorphonuclear leucocytes during the periparturient period and relation with metabolic markers and bovine pregnancy-associated glycoprotein. *J. Dairy Res*., **67**: 249-259.

Ingvartsen, K.L. and J.B. Andersen. 2000. Integration of metabolism and intake regulation: a review focusing on

periparturient animals. *J. Dairy Sci*., **83**: 1573-1597.

Jain, N.C. 1986. In: Schalm's Veterinary Hematology, Lea and Febiger, Philadelphia. 821-837.

Kehrli, M.E. and J.A. Harp. 2001. Immunity in the mammary gland. *Vet. Clin. North Am Food Anim. Pract*., **17**(3): 495-516.

Kehrli, M.E., B.J. Nonnecke and J.A. Roth. 1989. Alterations in bovine lymphocyte function during the periparturient period. *Am. J. Vet. Res*., **50**: 215-220.

Kehrli, M.E., K.A. Weigel, A.E. Freeman, J.R. Thurston and D.H. Kelley. 1991. Bovine sire effects on daughter's *in vitro* blood neutrophil functions, lymphocyte blastogenesis, serum complement and conglutinin levels. *Vet. Immunol. Immunop*., **27**: 303-319.

Khan, J.R. and R.S. Ludri. 2002. Hormone profile of crossbred goats during the periparturient period. *Trop. Anim. Health Prod*., **34**: 151-162.

Lee, J.Y. and D.H. Hwang. 2006. The modulation of inflammatory gene expression by lipids: Mediation through Toll- like Receptors. *Mol. Cells*., **21**: 174-185.

Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}C_T$ Method. *Methods*., **25**(4): 402-408.

Meglia, G.E., A. Johannisson, L. Petersson and K.P. Waller. 2001. Changes in some blood micronutrients, leukocytes and neutrophil expression of adhesion molecules in periparturient dairy cows. *Acta Veterinaria Scandinavia*., **42**: 139-150.

Nanda, A.S., P.S. Brar and S. Prabhakar. 2003. Enhancing reproductive performance in dairy buffalo: major constraints and achievements. *Reprod. Suppl.*, **61**: 27-36.

Paape, M.J., D. Douglas, X.Z. Bannerman and J.W. Lee. 2003. The bovine neutrophils: Structure and function in blood and milk. *Vet. Res*., **34**: 597-627.

Parker, L.C., M.K. Whyte, S.K. Dower and I. Sabroe. 2005. The expression and roles of Toll-like receptors in the biology of the human neutrophil. *J. Leukocyte Biol*., **77**: 886-892.

Prakash, B.S. and M.L. Madan. 1984. Radioimmunoassay of cortisol in peripheral blood plasma of buffaloes puerperium, *Theriogenology*, **22**(3): 241-246.

Preisler, M.T., P.S. Weber, R.J. Tempelman, R.J. Erskine, H. Hunt and J.L. Burton. 2000. Glucocorticoid receptor down-regulation in neutrophils of periparturient cows. *Am. J. Vet. Res*., **61**: 14-19.

Robinson, T.L., I.A. Sutherland and J. Sutherland, 2007. Validation of candidate bovine reference genes for use with real-time PCR. *Vet. Immunol. Immunop*., **115**: 160-165.

Segal, A.W. 2005. How neutrophils kill microbes. *Annu. Rev. Immunol*., **23**: 197-223.

Serhan, C.N. and J. Savill. 2005. Resolution of inflammation: the beginning programs the end. *Immunology*, **6**: 1191-1197.

Wang, X., S. Xui., X. Gao., H. Ren and J. Chen. 2007. Genetic polymorphism of *TLR4* gene and correlation with mastitis in cattle. *J. Genet. Genomics*., **34**(5): 406-412.