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Effect of milk yield on functional activity of neutrophil in crossbred Karan Fries (Holstein Friesian x Tharparkar) cows around peripartum

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ABSTRACT

To study the immunological activities of neutrophils, blood samples were collected from 6 high yielding (HY) and 6 low yielding (LY) Karan Fries (KF) cows on -15,-7, -5, -3, -2, -1 days prepartum, at calving and on 1, 2, 3, 5, 7 and 15 days postpartum. Plasma cortisol levels, phagocytic activity (PA), enzyme (Elastase, Collagenase and Cathepsin G) levels and expression of TLR-2, TLR-4 and IL-8 were also studied. Both HY KF and LY KF cows were found to have increased blood Total leukocyte counts (TLC) and neutrophil percent at calving, but HY KF cows had significantly ($P<0.05$) higher levels than LY KF cows. The number of band neutrophils were also significantly ($P<0.05$) higher in HY cows. Significant ($P<0.05$) immunosuppression in relation to PA was found for HY as compared to LY KF cows throughout the peripartum period, with the lowest immunosuppression at calving in both groups of cows. Cortisol levels were significantly ($P<0.01$) higher during calving and negatively correlated with neutrophilic functions. The difference between the two groups also remained significant ($P<0.05$) as higher level of cortisol were found in HY KF cows. Elastase, collagenase and cathepsin were significantly ($P<0.05$) decreased during parturition. Elastase was reduced approximately 2.5 times on the day of calving in LY KF cows, but no such major reduction was observed for HY KF cows. Collagenase and cathepsin levels were significantly ($P<0.05$) higher in LY cows. Expression of the TLR-2 gene was significantly ($P<0.05$) lower in HY cows during the whole peripartum period than in LY cows. Expression of the TLR-4 gene was significantly ($P<0.05$) lower on days 15 pre- and post-caving in HY cows. IL-8 differed significantly ($P<0.05$) only during the prepartum days. Lower neutrophilic function in cross bred cows with high production potential, provides lower disease resistance and makes the cows more susceptible to peripartum infection.

Key words: cortisol, enzymes, gene expression, neutrophil, phagocytic activity

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Introduction

The period between late pregnancy and early lactation is generally accepted as the most critical period with respect to health in a dairy cow. During the periparturient period, the dairy cow experiences a natural state of immunosuppression, which is associated with a high susceptibility to infectious diseases as a consequence of the higher demand for nutrients and energy (negative energy balance) (SORDILLO and AITKEN, 2009). It is important to note that cows with higher production potential are more susceptible to infection compared to low producers.

Parturition in dairy animals is associated with high cortisol levels, impairment of polymorphonulcear neutrophil leucocytes (PMNL) phagocytosis and oxidative burst activity, (HOEBEN et al., 2000) and decreased ability to fight bacterial infections. Neutrophil, the first line of cellular defense, is a major component of the immune axis which forms an integral part of the innate immune system, and mediates the destruction of bacterial pathogens by phagocytosis, through a cascade of proteases (elastase, collagenase and cathepsin G), antimicrobial peptides, and free radicals (SEGAL, 2005). The primary role of neutrophils is participation in inflammatory response, by producing cytokines, eccosanoids and cell signaling molecules. Neutrophils play a vital role in the onset of disease around parturition (KEHRLI et al., 1989). Hence, reduced neutrophilic respiratory burst and phagocytic activity around parturition (HOEBEN et al., 2000) leads to postpartum diseases, such as mastitis, metritis and retained placenta (KEHRLI and HARP, 2001). Due to these diseases, dairy industries face heavy economical losses every year, globally.

Neutrophilic TLR-2 and TLR-4 recognize gram positive and gram negative bacteria respectively, and ilicit inflammatory responses through a chain of biochemical reactions, leading to secretion of IL-8. Higher plasma cortisol concentration affects neutrophil signaling and functioning around parturition in cattle, due to the higher expression of glucocorticoid receptors on blood neutrophils (BURTON et al., 2005). The immune mechanism behind this has not been completely explained in cows around parturition (NANDA et al., 2003). The complex interplay between all these factors, leads to neutrophil activity, causing host cell protection (SERHAN and SAVILL, 2005).

Karan Fries (KF) is a well-recognized cross breed of cattle and contributes significantly to milk production in India. Unfortunately, scanty information is available on the immune status of high and low yielding cross bred KF cows around parturition (DANG et al., 2009; 2013). Also, there are no reports on the enzymatic activity and differential expression of neutrophil genes related to defense mechanisms during the peripartum period. In the light of this overview, the present study was undertaken to investigate the effect of milk yield on neutrophil activities in Karan Fries cows around parturition.

Materials and methods

Selection of animals. Twelve crossbred KF cows in advance stages 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 the production potential of their previous lactation. HY KF (n = 6) were producing on average 5154.52 ± 93.81 liters per lactation, whereas LY KF (n = 6) were producing on average 3967.61 ± 96.18 liters per lactation. All the KF cows were offered *ad libidum* green fodder and a calculated amount of concentrate mixture. Fresh tap water was also made available *ad libidum* at all times of the day. All the experimental cows were healthy and free from any anatomical, physiological or infectious disorders.

Collection of samples and analysis. Blood samples were collected from all the KF cows 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 count (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., 2013), and plasma cortisol levels were also estimated by ELISA (Endocrine Technologies, USA) during both the pre- and post-partum days as indicated above. The minimum detectible concentration of cortisol was estimated by this assay to be 0.1 ng/mL. Coefficient of Variation (CV) was calculated from the calculated concentrations. Inter-assay % CV was found to be 2.63 and intra-assay % CV was found to be 0.06.

The activities of the 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 on -7, -3 days prepartum, on the day of calving and 3, 7 days postpartum. Neutrophils from peripheral blood were isolated using hypotonic lysis of erythrocytes (VISHNOI et al., 2007). For preparing lysate of neutrophils, the isolated neutrophils were dissolved in 1 mL PBS. Glass beads were added to the neutrophil suspension and it was shaken for 25 second using a Bead beater (Unigenetics Instrument Pvt. Ltd., India). It was placed on ice for 1 minute, then shaken again for 25 second. It was centrifuged at 1000 × g for 10 minutes. Supernatant was taken in 2 mL eppendorf tubes and stored at -20 ºC until further examination. CV percentage was calculated from the calculated concentrations. Inter-assay % CV was found to be 5.38, 4.64, 5.03 and intra-assay % CV was found to be 3.77, 1.08 and 2.69 for Elastase, Collagenase and Cathepsin G respectively.

Relative expression of neutrophil genes. All solutions were prepared using DEPC treated RNase free plastic ware and water. Total RNA from the blood neutrophils was extracted using the Trizol method as per CHONCZYNSKI and SACCHI (1987). The RNA pellet was air dried for 15-30 min, dissolved in 25 µL of RNA storage solution and stored at

-80 °C until further use. The 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 the fluorescent dye, at the rate of 0.5 µg/mL of gel, whereas, bromophenol blue was used as the tracking dye, at the rate of 3 µL mixed with RNA during the loading of the sample into the well of gel. Electrophoresis was carried out at 8 V/cm for half an hour. After completion of electrophoresis, the gel was examined under a UV transilluminator. DNase treatment was done using a DNA free Kit (Ambion, UK) according to the manufacturer's instructions. Total RNA was quantified, and OD_{260nm}/OD_{280nm} was determined with an ND-3300 flurospectrophotomer (NanoDrop Technologies, UT) and purity of RNA was judged on the basis of the optical density ratio at 260:280 nm. Reverse transcription was performed from 1 µg of RNA using a Novagen first strand cDNA synthesis kit (La Jolla, CA).

Table 1. Specifications for qPCR

Genes	Sequence (5′→3′)	Acc. no.	Size (bp)	Annealing Temp (°C)
TLR 2	**F** GCCTTGACCTGTCCAACAAT **R** GACCTGAACCAGGAGGATGA	NM174197.2	199	59
TLR 4	**F** GGCATCATCTTCATCGTCCT **R** CTGGACTCTGGGGTTTACCA	AY634630.1	178	59
IL-8	**F** TGCTCTCTGCAGCTCTGTGT **R** CAGACCTCGTTTCCATTGGT	EU276073.1	190	59
β- actin	**F** TCCCTGGAGAAGAGCTACGA **R** TAGAGGTCCTTGCGGATGTC	NM_173979.3	179	59
GAPDH	**F** GGGTCATCATCTCTGCACCT **R** GGTCATAAGTCCCTCCACGA	NM_001034034.1	176	59

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 a Roche Light Cycler-480, Germany. These 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 the gene was retrieved from the NCBI database, and suitable primers were designed using primer-3 web interfaces. Details of primer specification are given in 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. The PCR conditions were 300 s at 95 °C, 45 cycles of 20 s at 95 °C, 20s at the appropriate annealing temperature (Table 1), and 20 s at 72 °C. A melting curve for each qPCR with a single peak at the correct melting temperature was indicative of the reliable and desired PCR product. mRNA abundance on day 0 (the day of parturition) was taken as the calibrator regarding which relative expressions were seen. Calculation was done using the $2^{-\Delta\Delta Ct}$ method (LIVAK and SCHMITTGEN, 2001).

Statistical analysis. Statistical analysis was performed using the least square model by means of 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 a 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 the Tukey test as the post hoc test, because this test is able to control the errors of multiple comparisons simultaneously. Further, the effect of different treatments 15 days prepartum was not used as a covariate for subsequent analysis, as our main interest was to differentiate the effect of two different treatments.

Results

Total Leukocyte count (TLC) was measured from the blood of HY and LY KF cows during pre- and post-partum periods, and shown in Table 2. The highest (P<0.001) TLC was found on the day of calving in both the groups, but the levels were found to be significantly (P<0.05) higher in HY KF cows compared to LY KF cows. TLC was reduced significantly (P<0.01) on days 3, 5 and 7 after calving, compared to the day of calving in both HY and LY KF cows. The difference in TLC between the day of calving and 15 days after calving was highly significant (P<0.001). Between HY and LY KF cows, significant changes in TLC were recorded on all postpartum days except days 7 and 15. The overall mean of TLC remained significantly (P<0.05) higher for HY than LY KF cows.

Table 2. Blood Total Leukocyte Counts (TLC $\times 10^3/\mu L$) in high yielding (HY) and low yielding (LY) KF cows during pre and postpartum period

	Pre-partum days							Overall Mean ± SE
	-15	-7	-5	-3	-2	-1	0	
HY	7.73 ± 0.14^{ABC}	7.51 ± 0.23^{AB}	7.78 ± 0.18^{ABC}	7.97 ± 0.19^{BCE}	8.59 ± 0.10^{D}*	8.74 ± 0.15^{DF}*	9.20 ± 0.10^{D}*	8.22 ± 0.16*
LY	7.36 ± 0.11^{ad}	7.27 ± 0.12^{ad}	7.40 ± 0.11^{ad}	7.72 ± 0.11^{ab}	7.81 ± 0.11^{ab}	8.30 ± 0.13^{bc}	8.61 ± 0.13^{c}	7.78 ± 0.12
	Postpartum days							
	0	1	2	3	5	7	15	
HY	9.20 ± 0.10^{D}*	9.09 ± 0.12^{DEF}*	8.52 ± 0.12^{CF}	8.32 ± 0.15^{ACE}	7.87 ± 0.19^{ABE}	7.67 ± 0.21^{ABF}	7.21 ± 0.22^{A}	8.27 ± 0.16*
LY	8.61 ± 0.13^{c}	8.37 ± 0.10^{bc}	8.16 ± 0.14^{bce}	7.90 ± 0.17^{bcd}	7.53 ± 0.16^{ade}	7.33 ± 0.18^{ad}	7.15 ± 0.17^{a}	7.86 ± 0.15

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

Table 3. Blood neutrophil counts (%) in high yielding (HY) and low yielding (LY) KF cows during pre and postpartum period

	Pre-partum days							Overall Mean ± SE
	-15	-7	-5	-3	-2	-1	0	
HY	31.17 ± 1.01^{A}	31.83 ± 0.79^{AC}*	31.67 ± 0.56^{AC}	32.00 ± 0.82^{AC}	32.00 ± 0.45^{AC}	33.13 ± 0.71^{AC}	39.00 ± 0.58^{B}*	33.14 ± 0.70*
LY	29.33 ± 1.02^{a}	28.83 ± 0.31^{a}	30.17 ± 0.48^{ad}	31.50 ± 0.43^{ab}	31.33 ± 0.42^{ab}	34.33 ± 0.67^{bc}	36.17 ± 0.48c	31.67 ± 0.54
	Postpartum days							
	0	1	2	3	5	7	15	
HY	39.00 ± 0.58^{B}*	35.00 ± 0.73^{C}	34.50 ± 1.02^{AC}	34.33 ± 0.67^{AC}*	33.83 ± 1.01^{AC}*	33.00 ± 0.73^{AC}	32.17 ± 1.14^{AC}	34.55 ± 0.84*
LY	36.17 ± 0.48^{c}	35.00 ± 0.73^{ce}	33.17 ± 0.60^{bcd}	31.50 ± 0.62^{ab}	31.83 ± 0.48^{abe}	31.50 ± 0.62^{ab}	30.33 ± 0.42^{ad}	32.79 ± 0.56

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

The neutrophil percentage was significantly (P<0.05) higher in HY compared to LY KF cows during the peripartum period. However, the blood neutrophil percentage was found highest (P<0.001) on the day of calving in both groups. After parturition, a significant (P<0.01) reductions in neutrophil counts were observed on day 1 and day 3 in HY and LY KF cows respectively (Table 3). We also observed an increase in the percentage of band neutrophils and a decrease in the segmented neutrophil percentage on the day of caving in both groups of cows. However, the percentage of immature or band neutrophils was significantly (P<0.05) higher in HY than LY KF cows.

The neutrophil PA was estimated in both groups of cows during the peripartum period (-15 to +15 days). The PA was represented in terms of optical density due to the formation of formazan crystals (Table 4). The lowest neutrophilic PA was observed on the day of calving in both HY and LY KF cows. A significantly (P<0.05) lower PA was found in HY as compared to LY KF cows throughout the peripartum period. The PA of blood neutrophils remained suppressed until day 1 and day 2 after calving in HY and LY KF cows respectively. However, a significant (P<0.05) increase in PA was observed on days 3 and 5 postpartum in HY and LY cows, respectively, A highly significant (P<0.001) PA was observed on day 15 after calving, as compared to day 1 and day 2 after calving in HY and LY KF cows respectively.

Table 4. Optical Density of formazan crystals of blood neutrophils isolated from high yielding (HY) and low yielding (LY) KF cows during Pre- and postpartum period

	Pre-partum days							Overall Mean ± SE
	-15	-7	-5	-3	-2	-1	0	
HY	0.22 ± 0.02^{ABE}	0.23 ± 0.01^{AB}	0.24 ± 0.02^{AB}	0.21 ± 0.01^{ABE}	0.19 ± 0.03^{BCF}	0.15 ± 0.01^{CDF}	0.10 ± 0.02^{CDF}*,**	0.19 ± 0.01*
LY	0.21 ± 0.01^{acd}	0.25 ± 0.04^{abc}	0.27 ± 0.02^{ab}	0.22 ± 0.04^{ac}	0.21 ± 0.03^{cd}	0.15 ± 0.01^{de}	0.14 ± 0.02^{e}**	0.22 ± 0.02
	Postpartum days							
	0	1	2	3	5	7	15	
HY	0.10 ± 0.02^{CDF}*	0.09 ± 0.03^{D}	0.14 ± 0.02^{CDF}	0.16 ± 0.01^{EF}	0.17 ± 0.03^{BEF}*	0.24 ± 0.01^{AB}	0.28 ± 0.02^{A}*,**	0.16 ± 0.02*
LY	0.14 ± 0.02^{e}	0.11 ± 0.01^{e}	0.11 ± 0.02^{e}	0.14 ± 0.01^{e}	0.24 ± 0.02^{ac}	0.26 ± 0.03^{ac}	0.31 ± 0.01^{b}**	0.19 ± 0.02

Values within a row having different superscript are significantly (P<0.05) different from each other whereas, ** indicate difference was highly significant (P<0.001). Values within a column having (*) as superscript are significantly (P<0.05) different from each other

Plasma cortisol levels were measured by ELISA during the peripartum period (-15 to +15 days) and are depicted in Table 5. Levels of plasma cortisol were found to be significantly (P<0.001) higher in HY than LY KF cows throughout the peripartum period. A steady rise in plasma cortisol was observed in both groups of animals, with a peak on the day of calving. The plasma cortisol level observed on the day of calving was approximately four and three times higher for HY and LY KF cows respectively, as compared to the level of cortisol observed on day 15 pre-partum. After parturition, a significant (P<0.001) decline in cortisol levels was observed on day 1 after calving in both groups of cows. The cortisol level of LY cows obtained 15 day postpartum reached the normal or initial level of day 15 pre-partum, but in HY cows, the cortisol level was still higher than the initial level.

Table 5. Plasma cortisol levels (ng/mL) in high yielding (HY) and low yielding (LY)KF cows during pre and postpartum period

	Pre-partum days							Overall Mean ± SE
	-15	-7	-5	-3	-2	-1	0	
HY	5.97 ± 0.89^{A}	7.37 ± 0.78^{AB}	9.06 ± 0.38^{AB}	9.68 ± 0.82^{AB}	10.60 ± 0.46^{AB}	12.32 ± 0.84^{B}	18.03 ± 1.34^{C}*.**	10.43 ± 0.78*
LY	5.45 ± 0.60^{a}	9.52 ± 1.31^{a}	8.44 ± 0.77^{a}	10.11 ± 0.17^{a}	9.75 ± 0.63^{a}	9.87 ± 0.15^{a}	14.56 ± 0.72^{b}**	9.67 ± 0.60
	Postpartum days							
	0	1	2	3	5	7	15	
HY	18.0 ± 2.34^{C}*.**	9.28 ± 1.32^{AB}*	8.29 ± 1.24^{AB}	7.37 ± 1.24^{AB}	7.34 ± 0.65^{AB}	7.41 ± 0.93^{AB}	7.16 ± 1.43^{AB}	9.27 ± 1.31*
LY	14.56 ± 0.72^{b}	5.47 ± 0.55^{a}	8.13 ± 1.11^{a}	9.32 ± 1.75^{a}	6.53 ± 0.72^{a}	5.39 ± 0.51^{a}	5.21 ± 0.60^{a}	7.59 ± 0.84

Values within a row having different superscript are significantly (P<0.05) different from each other whereas, ** indicate difference was highly significant (P<0.001). Values within a column having (*) as superscript are significantly (P<0.05) different from each other

Table 6. Levels of elastase, collagenase and cathepsin G enzymes (ng/mL) of blood neutrophils isolated from high yielding (HY) and low yielding (LY)KF cows during peripartum period

Enzymes (ng/mL)		Peripartum days					Overall Mean ± SE
		-7	-3	0	3	7	
Elastase 2	HY	16.35 ± 0.98^{A}*	25.75 ± 2.43^{B}*	11.54 ± 0.78^{C}*	20.03 ± 4.52^{B}*	20.68 ± 2.21^{B}	18.87 ± 2.18*
	LY	36.16 ± 7.12^{a}	36.39 ± 5.09^{a}	13.23 ± 1.51^{b}	39.25 ± 4.10^{a}	24.94 ± 4.55^{c}	29.99 ± 4.47
Collagenase	HY	5.95 ± 0.73^{A}*	9.61 ± 0.95^{BD}	2.36 ± 0.27^{C}*	7.87 ± 0.73^{AD}	10.48 ± 0.74^{B}*	7.25 ± 0.68
	LY	6.94 ± 0.33^{a}	8.85 ± 0.89^{a}	3.23 ± 0.44^{c}	7.45 ± 0.82^{a}	12.23 ± 0.63^{b}	7.74 ± 0.62
Cahepsin G	HY	2.96 ± 0.36^{A}*	4.29 ± 0.37^{B}*	3.54 ± 0.64^{A}*	12.26 ± 0.33^{C}	18.56 ± 0.48^{D}*	8.32 ± 0.44*
	LY	4.15 ± 0.91^{a}	8.44 ± 0.74^{b}	6.35 ± 0.54^{c}	13.02 ± 0.98^{d}	15.69 ± 1.00e	9.53 ± 0.83

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

Table 7. Relative expression of TLR-2, TLR-4 and IL-8 genes of blood neutrophils isolated from high yielding (HY) and low yielding (LY)KF cows during peripartum period

Genes		Peripartum days				
		-15	-7	0	7	15
TLR-2	HY	$2.13 \pm 0.23^{AB*}$	$1.82 \pm 0.18^{B*}$	1.01 ± 0.12^{C}	$2.11 \pm 0.63^{B*}$	$3.59 \pm 0.24^{A*}$
	LY	3.25 ± 0.44^{a}	2.40 ± 0.45^{ab}	1.05 ± 0.07^{c}	3.42 ± 0.59^{a}	4.05 ± 0.34^{a}
TLR-4	HY	$3.37 \pm 0.57^{A*}$	2.37 ± 0.32^{AB}	1.06 ± 0.24^{C}	1.69 ± 0.45^{C}	$3.39 \pm 0.42^{A*}$
	LY	5.17 ± 0.74^{a}	3.37 ± 0.62^{ac}	1.08 ± 0.31^{b}	2.16 ± 0.40^{c}	5.21 ± 0.57^{a}
IL-8	HY	$2.12 \pm 0.51^{AB*}$	$1.48 \pm 0.05^{AB*}$	1.07 ± 0.25^{C}	1.66 ± 0.38^{AB}	2.90 ± 0.74^{A}
	LY	3.04 ± 0.28^{a}	2.02 ± 0.26^{b}	1.09 ± 0.39^{c}	1.28 ± 0.15^{c}	3.48 ± 0.45^{a}

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

Table 8. Correlation coefficient between Cortisol, PA, Elastase, Collagenase and Cathepsin G in Karan Fries cows

	Cortisol	PA	Elastase	Collagenase	Cathepsin G
Cortisol	1	-0.167*	-0.216*	-0.501*	-0.472*
PA		1	0.270*	0.145	0.138
Elastase			1	0.166	0.240*
Collagenase				1	0.535
Cathepsin G					1

The values containing * as a superscript indicate significant (P<0.05) orrelation between the pairs. Negative Values indicate negative correlation

Neutrophil enzymes that are important in combating infection, such as elastase, cathepsin G and collagenase, were estimated by ELISA in both groups of KF cows and are presented in Table 6. Initial pre-partum levels of Elastase 2 observed on day 7 pre-partum were half the level in HY than in LY KF cows. Elastase 2 was reduced by approximately 2.5 times on the day of calving in LY KF cows, but no such major reduction was observed for HY KF cows. The level of elastase 2 on the day of calving was significantly (P<0.05) lower for HY as compared to LY KF cows. After calving, a rapid increase in elastase 2 was observed on day 3 postpartum in both group of cows. The levels of elastase 2

observed during whole peripartum period remained significantly (P<0.05) lower for HY as compared to LY cows.

Collagenase levels were significantly (P<0.05) increased on day 3, as compared to day 7 pre-partum in HY KF cows. A significant (P<0.05) reduction in collagenase levels wase found on the day of calving as compared to all pre-partum days in both HY and LY KF cows. After parturition, increases in collagenase levels were significant (P<0.05) up to day 7 as compared to the day of calving in both groups. Collagenase levels in LY KF cows remained significantly (P<0.05) higher than in HY KF cows on most days of the peripartum period, except day 3 before and day 3 after parturition.

The lowest level of cathepsin G was also observed on the day of calving in both HY and LY KF cows. Between HY and LY KF cows, significant (P<0.05) differences were observed throughout the peripartum period, except on day 3 after parturition. The overall mean of cathepsin G during the peripartum period remained significantly (P<0.05) lower for HY KF cows.

The results for the relative expression of the important neutrophil genes TLR-2, TLR-4 and IL-8 are presented in Table 7. Significantly (P<0.05) lower expressions 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 KF cows. Expression of these genes remained lower in HY KF cows as compared to LY KF cows. Between HY and LY KF cows, a significant (P<0.05) difference was found in expression of the TLR-2 gene throughout the peripartum period, whereas, TLR-4 gene expression differed significantly (P<0.05) only on day 15 before and day 15 after parturition. Expression of the IL-8 gene differed significantly (P<0.05) between HY and LY KF cows on all pre-partum days.

Discussion

White blood cells are involved in defense against pathogens. The blood TLC and neutrophil counts reflect the immune status of the animal, here HY and LY KF cows. The pattern of TLC and neutrophil counts in both groups of cows observed during the peripartum period is in accordance with the study by MEGLIA et al. (2001) in exotic cows. An increase in TLC around calving is coupled with a rise in cortisol levels in both groups. It is believed that TLC increases around calving as a result of the antipartum rise in cortisol levels. However, TLC decreases during the postpartum period and this is coupled with the migration and recruitment of blood neutrophils towards uterine lumen and mammary tissues (PREISLER et al., 2000). The study also signifies that higher cortisol stimulates the release of a larger amount of neutrophils around calving.

Neutrophils, the first line defense, migrate first from the blood into an inflamed area for phagocytosis and intracellular killing. The killing of engulfing bacteria is performed through two distinct mechanisms: the respiratory burst and by digestion

through lysosomal enzymes (JAIN, 1986). According to PAAPE et al. (2003) only matured segmented neutrophils have the complete machinery for phagocytosis. However, we observed significantly higher numbers of immature neutrophils at calving in both groups of cows. Cortisol causes a reduction in expression of the adhesion receptors (L-selectin and CD18) on neutrophils that are responsible for migration. Also, mature neutrophils are more sensitive to cortisol as compared to immature neutrophils, due to the higher number of glucocorticoid receptors (BURTON et al., 2005). The larger number of band neutrophils in high producing cows as compared to low producing cows may be due to the stress on the mammary tissues to produce more milk. The rapid increase in circulating neutrophils was attributed mainly to an influx of neutrophils from the hematopoietic system, and not from a marginal pool of mature leukocytes. The higher level of cortisol around calving is the reason behind the increase in immature neutrophils. Therefore, the migration of mature neutrophils from bone marrow is reduced, and immature neutrophils marginate more from hematopoietic reserves (PAAPE et al., 2003; BURTON et al., 2005). The higher number of immature (band) neutrophils in HY as compared to LY KF cows could be explained by the higher cortisol level resulting from the greater stress on the mammary tissues to produce more milk in HY KF cows. The rapid increase in circulating neutrophils was attributed mainly to an influx of neutrophils from the hematopoietic system and not from a marginal pool of mature leukocytes.

In vitro analysis of neutrophil function in terms of phagocytic activity (PA) provides a very effective tool for the study of natural disease resistance. We observed decreased PA of blood neutrophils around calving in both groups and found similar results to those reported by MEGLIA et al. (2001) and DANG et al. (2013). However, PA in HY KF cows were observed to be more diminished than in LY KF cows. This could be explained by the poor activity of neutrophils due to higher numbers of immature neutrophils in circulation, which have no proper machinery to fight against infection. We also found a significant negative correlation ($r = 0.3$) between the PA of blood neutrophils and plasma cortisol levels. There were increased neutrophil numbers during parturition, yet phagocytic activity remained lower. The PA was lowest on the day of calving, and this suppression might be due to a sharp increase in cortisol levels during parturition. Cortisol release during parturition causes hyper stimulation of red bone marrow for the faster release of neutrophils. As a result, there is a rapid release of immature neutrophils (PAAPE et al., 2003). During the pre-partum period (15 days before calving) the animals are in dry stage so, there is no stress from milk production, but at parturition, the animals have to face the stress of calving, synthesize colostrum (up to 3 days) and milk. Further, milk production potential is higher in high producing than low producing cows; therefore, they exhibited more stress and lower PA compared to low yielding cows.

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 regulates immune activity. Cortisol is released in response to stress and a low level of blood glucose. Glucocorticoid suppresses the immune system, increases blood sugar through gluconeogenesis, and aids in metabolism of protein, fat and carbohydrates. We observed a significantly higher level of cortisol at calving as compared to 15 days before and 15 days after calving in both groups. A similar observation was reported in cattle (PRAKASH and MADAN, 1984; 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 these authors. We also found a significantly higher level of cortisol in HY than LY KF cows. The cows are under various types of stress during the peripartum period such as the stress of providing nutrition to the growing calf, the stress of labor, and the need to synthesize colostrum and then milk. The social stress of being isolated also exists. The overall effects of stress increased cortisol levels, produced neutrophilia with decreased functional capacity of neutrophils, immunosuppression and ultimately the high producing cows become more prone to mastitis and other infections (KEHRLI et al., 1991). In agreement with our findings, the high levels of cortisol at calving have also been reported to act as a powerful immunosuppressive agent (GOFF and HORST, 1997).

Neutrophils mediate phagocytosis through a complex cascade of enzymes and their interrelated pathways. The enzymes that play a crucial role in immune response are collagenases, elastase and cathepsin G, and these enzymes are estimated in HY and LY KF cows to learn about the effects of production pressure on immunity. The enzymes elastase and cathepsin G are found in azzurophilic or primary granules, whereas collagenases are part of specific granules present in the neutrophils. These enzymes increase in quantity during diseased conditions and provide immunity to animals (HADDADI et al., 2006). The release of enzymes is specifically regulated by the cytokine network and their signaling to neutrophils via cytokine receptors. The timely and net release of these enzymes determines the ultimate fate of neutrophil activity, in terms of phagocytosis and resolution of inflammatory cascades. Neutrophils release elastase and cathepsin, which are serine proteases, to bring about the destruction of pathogens during inflammation (BELAAOUAJ et al., 2000). During calving, animals have a minimum concentration of these enzymes due to the higher number of immature neutrophils under the influence of increased cortisol. This effect is more pronounced in HY KF cows. Thus, HY KF cows are more susceptible to postpartum diseases (mastitis, matritis etc.) compared to LY KF cows. 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 an important role in the regulation of neutrophil function (PARKER et al., 2005). Our study is the first to indicate the down regulation of the blood PMN expression of TLR-2, TLR-4 and IL-8 genes during the peripartum period in cows. During the periparturient

period animals experience a negative energy balance (NEB) from 3 days before to 3 days after calving (INGVARTSEN and ANDERSEN, 2000). The higher level of cortisol helps to provide the necessary energy 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 inhibit them (LEE and HWANG, 2006). Decreased expression of these genes during calving might be due to the increased levels of unsaturated fatty acids.

IL-8, which is considered to be the central regulator of neutrophil signaling (BURTON et al., 2005) was also down regulated, similar to TLR 2 and TLR 4. It is a potential chemoattractant for neutrophils 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 15 days before and 15 days after parturition, as compared to the day of calving. This indicates that the neutrophils of high producing cows have a lower ability to migrate to the site of infection than of low producing cows. This finding demonstrates that the neutrophils of cows with higher production potential are less immunocompetent than cows with low production potential. We observed a significantly higher expression of TLR-2, TLR-4 and IL-8 on day 15 before and after calving as compared to the day of calving.

Conclusions

There is general immunosuppression during the peripartum period. During parturition there is stress and a negative energy balance, and hence cortisol is released in response. Under the influence of cortisol, TLC is increased but there is a release of a higher number of immature incompetent neutrophils in the circulation. The relatively higher circulating concentration of cortisol is the determining factor for the decreased functional activity of neutrophils, which is evident from the lower PA, and decreased levels of elastase, collagenase and cathepsin G, and expression of TLR-2, TLR-4 and IL-8. This makes the animal more susceptible to postpartum diseases and this effect is more pronounced in HY than LY KF cows. Altogether, these findings lead us to draw the conclusion that the LY cow neutrophils are more potent compared to the HY cow neutrophils. These results will help in understanding the physiology of neutrophils at calving, and help to develop strategies to improve immune functions around this period. Also, further studies are required to employ genetic and proteomic tools to discover the exact mechanism of neutrophil action in cows.

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PATHAN, M. M., M. KAUR, A. K. MOHANTY, A. K. DANG: Utjecaj prinosa mlijeka na funkcionalnu aktivnost neutrofila u krava križanki Karan Fries (Holstein Friesian x Tharparkar) tijekom peripartusnog razdoblja. Vet. arhiv 86, 295-310, 2016.

SAŽETAK

S ciljem istraživanja imunološke aktivnosti neutrofila, od 6 visokoproizvodnih (VP) i 6 niskoproizvodnih (NP) Karan Fries (KF) krava prikupljeni su uzorci krvi -15, -7, -5, -3, -2, -1 dana prije partusa, zatim tijekom partusa, te 1, 2, 3, 5, 7 i 15 dana nakon partusa. Analizirana je razina kortizola u plazmi, aktivnost fagocita (AF), razine enzima (elastaze, kolagenaze, katepsina G) te ekspresija TLR-2, TLR-4 i IL-8. Kod obje skupine krava, VP KF i NP KF, utvrđeno je povećanje ukupnog broja leukocita (UBL) i postotka neutrofila pri teljenju, ali razine su kod VP KF krava bile signifikantno više ($P<0,05$) nego kod NP KF krava. Broj nesegmentiranih neutrofila bio je također signifikantno ($P<0,05$) viši kod VP krava. Signifikantna ($P<0,05$) imunosupresija u odnosu na AF utvrđena je kod VP u usporedbi sa NP KF kravama tijekom peripartusnog razdoblja, s najnižom razinom imunosupresije pri teljenju obje skupine krava. Razine kortizola bile su signifikantno ($P<0,01$) više tijekom teljenja i negativno povezane s funkcijom neutrofila. Razlike između skupina također su ostale signifikantne ($P<0,05$) i u slučaju više razine kortizola utvrđene kod VP KF krava. Elastaza, kolagenaza i katepsin signifikantno su ($P<0,05$) opadali tijekom teljenja. Elastaza je smanjena za približno 2,5 puta na dan teljenja NP KF krava dok kod VP KF krava takvo jako smanjenje nije opaženo. Razine kolagenaze i katepsina bile su signifikantno ($P<0,05$) više kod NP krava. U odnosu na NP krave, ekspresije TLR-2 gena kod VP krava bile su signifikantno ($P<0,05$) niže tijekom cijelog peripartusnog razdoblja. Ekspresija TLR-4 gena bila je signifikantno ($P<0,05$) niža 15. dan prije i poslije teljenja VP krava. IL-8 je bio signifikantno različit ($P<0,05$) samo tijekom prepartusnog razdoblja. Snižena funkcija neutrofila kod krava križanki s visokim proizvodnim potencijalom doprinosi njihovoj manjoj otpornosti na bolesti i čini ih osjetljivijima na infekcije tijekom peripartusnog razdoblja.

Ključne riječi: kortizol, enzimi, ekspresija gena, neutrofili, aktivnost fagocita

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