doi: 10.17221/63/2015-CJAS

Immunological attributes of blood and milk neutrophils isolated from crossbred cows during different physiological conditions

M. Alhussien[1], P. Manjari[2], A.A. Sheikh[2], S. Mohammed Seman[2], S. Reddi[2], A.K. Mohanty[3], J. Mukherjee[4], A.K. Dang[2]

[1]Animal Production Division, Aleppo University, Aleppo, Syria
[2]Lactation and Immuno-Physiology Laboratory, ICAR, National Dairy Research Institute, Karnal, India
[3]Animal Biotechnology Centre, ICAR, National Dairy Research Institute, Karnal, India
[4]Department of Veterinary Physiology, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata, India

ABSTRACT: The goal of this work was to understand how polymorphonuclear leukocytes (PMN), the first line of cellular defence, integrate and prioritize multiple chemotactic signals to navigate during periods of demand like subclinical mastitis (SCM), clinical mastitis (CM), pregnancy (P), and calving (C). For this, blood and milk samples were collected from five groups of crossbred cows (healthy, with SCM, CM, pregnant, and newly calved). Maximum viability was seen in the PMN of healthy cows, whereas minimum viability was observed in CM cows. Phagocytic activity (PA) of blood and milk PMN decreased significantly ($P < 0.05$) at C and in CM cows. Chemotactic activity of blood PMN was minimum in C followed by CM, SCM, and P cows. PA was found to be negatively correlated with the plasma cortisol levels and inverse relationship was observed between the plasma interleukin-8 (IL-8) levels and the chemotactic activity of neutrophils. There was a significantly ($P < 0.05$) higher expression of *CXCR1* and *IL-8* on both blood and milk PMN of CM cows followed by C and SCM cows. Minimum expression of selectin (*CD-62L*) was seen on blood PMN isolated around calving, whereas maximum expression of integrin (*CD-11b*) was in CM cows. Healthy and P cows showed the highest expression of *CD-62L* on blood PMN but its expression remained unaltered in milk PMN. Irrespective of physiological stage of the cows, immune suppression was found to be always cortisol dependent. Observing the neutrophil activity and mRNA expression of genes isolated from cow neutrophils can be used as indicators to assess the health/physiological status promptly for immediate therapeutic or management-related actions.

Keywords: phagocytic; chemotactic activity; cortisol; *IL-8*; *CXCR1*; selectin; integrin

INTRODUCTION

Neutrophils or polymorphonuclear leukocytes (PMN) are terminally differentiated cellular components of the innate immune system that constitute about 35–75% of the population of peripheral leukocytes. Neutrophils are the first line of immunity defence against most pathogens and are equipped with an array of defence molecules. The role of neutrophils in initial inflammatory response is well documented (Tizard 2000). When immunity of an animal goes down it signals the body to release inflammatory mediators. These chemical mediators or messengers are responsible for massive neutrophil migration from circulation to the site of inflammation. The ability of neutrophils to transmigrate into areas of inflammation is dependent upon recognition of inflammatory me-

Supported by the Department of Biotechnology (Grant No. BT/PR8404/AAQ/1/548/2013 dated 24/06/2014), Ministry of Science and Technology, Government of India.

doi: 10.17221/63/2015-CJAS

diators by cytokine, chemokine, and complement receptors. The majority of the research in cattle has investigated the role of neutrophils following mastitis (Paape et al. 2003) and/or calving (Meglia et al. 2005) in individual studies. However, there is no documentation and comparison between the roles played by neutrophils during different physiological stages. We hypothesize that both the neutrophils number, activity and expression of genes involved in neutrophil migration and phagocytosis will differ between various stressful conditions experienced by animals. Therefore studying the activity of neutrophils with the type of stress may provide insight into the immune function of that animal and provide possible targets for generating new strategies to either prevent or treat diseases in cattle. To test this hypothesis, neutrophils were collected during various physiological and pathophysiological conditions like calving, pregnancy, and mastitis and the activity and expression of genes involved in neutrophil adhesion, migration, and chemotaxis was studied.

MATERIAL AND METHODS

Selection of experimental animals. For the present investigation, a total of thirty Karan Fries cows of different physiological conditions were selected (healthy (n = 6), subclinical mastitis (SCM; n = 6), clinical mastitis (CM; n = 6), calving (C; n = 6), and pregnant (P; n = 6)). Karan Fries was developed in India at the National Dairy Research Institute at Karnal, Haryana by crossing Holstein Friesian with Tharparkar, an Indian milch breed. All the cows were in their early lactation (40–60 days) maintained in the Livestock Research Centre of National Dairy Research Institute. These cows were multiparous and were fed according to the feeding standards followed at the institute and *ad libitum* water was provided to them throughout the day. The animals with CM were screened by a varying degree of clinical symptoms exhibited, e.g. swelling of udder, fever, anorexia, and clinical changes in physical characters of milk (consistency, colour, etc.) with a confirmation by high milk somatic cell counts (SCC) ($7.5 \pm 0.54 \times 10^5$ cells/ml compared to $1.6 \pm 0.38 \times 10^5$ cells/ml in healthy cows). Animals with SCM were evaluated by Bromothymol blue (BTB) card test and Modified California mastitis test by commercially available kit Masti Check (Avantor Performance Materials India Ltd., New Delhi, India) as per manufacturer's protocol together with milk SCC ($4.6 \pm 0.39 \times 10^5$ cells/ml in SCM). In the case of P animals, blood and milk samples were collected on day 60 of pregnancy. All C cows were sampled within 6–12 h of calving.

Blood samples (15 ml/animal) were taken into sterile heparinized vacutainer tubes from jugular vein puncture, posing minimum disturbance to animals. About 500 ml milk samples representing milk from all four quarters were also collected through hand milking. Immediately after collection both blood and milk samples were transported to the laboratory in ice for further processing. A part of blood sample (5 ml) was centrifuged at 3000 rpm for 30 min for separation of plasma and plasma samples were stored at –20°C for further processing. All the procedures followed the guidelines for animal experiments outlined by the Institutional Animal Ethics Committee.

Evaluation of total leukocyte counts (TLC) and differential leukocyte counts (DLC) of blood and SCC and DLC of milk. TLC were enumerated by a hemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) as per standard hematological procedure. SCC of milk samples were done microscopically (Olympus iX51; Olympus, Tokyo, Japan) using the method of Dang et al. 2008. Blood and milk DLC were evaluated microscopically.

Isolation of blood and milk neutrophils. Both blood and milk neutrophils were isolated within 2 h post sampling using the method of Mehrzad et al. 2002 and > 95% neutrophils were obtained. Trypan blue exclusion method was used to determine the proportion of viable cells in the isolated neutrophils.

Phagocytic and chemotactic activity of blood and milk neutrophils. *In vitro* phagocytic activity (PA) of blood and milk neutrophils was estimated by calorimetric nitro blue tetrazolium (NBT) assay as described by Chai et al. 2005. Chemotaxis of blood and milk neutrophils was carried out using Dunn chemotaxis chamber (DCC100) as per method given by Zicha et al. 1997. Isolated neutrophils were diluted with 500 µl RPMI medium. Cells were seeded onto a sterile coverslip and allowed to settle prior to assembling the chemotaxis chamber. The outer annular well was filled with chemoattractant (lipopolysaccharide 50 µg/ml of RPMI) medium and the inner well was filled with control medium (RPMI media). The cover slip was inverted onto the chamber. The chamber was

doi: 10.17221/63/2015-CJAS

adjusted microscopically to observe the migration of cells across the outer edge of bridge. Time-lapse option was selected to capture images after the fixed time interval.

Estimation of plasma cortisol, IL-8, and progesterone levels. Plasma cortisol, IL-8, and progesterone levels were quantified by commercially available ELISA kit specific for bovines. Cows with progesterone levels higher than 2.0 ng/ml were considered pregnant which was further confirmed by rectal palpation on day 60 post artificial insemination.

Expression of CXCR1, CXCR2, IL-8, CD-62L, and CD-11b on blood and milk neutrophils. Total RNA extraction from isolated blood and milk neutrophils was performed using TRIzols Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Integrity of the RNA was evaluated after running the agarose gel electrophoresis (1.5% agarose). Two distinct intact bands on the gel represent two subunits of RNA, one for 18 S and the other for 28 S, respectively. DNase treatment of RNA samples was performed using a DNA-free Kit (Invitrogen). RNA samples were dissolved in 25–30 µl of RNA storage solution (Invitrogen) and the concentration was determined by the optical density value at 260 nm. The reverse transcription (RT) reaction was carried out by Novagen first strand cDNA synthesis kit (La Jolla, San Diego, USA) according to the manufacturer's protocol. Synthesized cDNA was kept at –20°C (or –70° C for long term use) till use.

Optimization of annealing temperature for all the primers of target (*CXCR1, CXCR2, IL-8, CD-62L*, and *CD-11b*) and housekeeping (*GAPDH*) genes was performed by gradient PCR of 55–60°C temperature range. PCR products were analyzed using agarose gel electrophoresis. The presence of single bands of expected sizes for all the genes at 59°C signifies that the primers were highly specific to their target sequence. The specificity of PCR amplified products was determined by a melting curve analysis as well as sequencing the amplified product.

qPCR was performed by Roche's LightCycler 480 instrument (Roche, Basel, Switzerland). Primers for specific bovine *CXCR1, CXCR2, IL-8, CD-62L*, and *CD-11b* genes (as listed in Table 1) having an equal melting temperature range were designed and synthesized (Sigma-Aldrich, St. Louis, USA). For normalization of qPCR data *GAPDH* was used as reference gene. The reaction condition for each individual gene was optimized using LightCycler 480 SYBR Green I Master (Roche). Before using for qPCR study, First strand cDNA was diluted in a 1 : 1 ratio. The master mix for qPCR for all the samples and genes was prepared as follows: 1 µl template, 5 µl 2X SYBR Green mix, and 0.5 µl of both reverse and forward primer, and 3 µl nuclease free PCR grade water. The four step programme for the LightCycler 480 was as follows: (1) denaturation at 95°C for 5 min; (2) amplification for 45 cycles, denaturation at 95°C for 15 s, annealing at 59°C for 20 s, and extension at 72°C for 20 s; (3) melting curve by 95°C for 10 s, 65°C for 1 min, and 95° C for continuous mode (according to the manufacturer's instructions); and (4) cooling at 40°C. Calculation for crossing points (CP) was automatically performed by the instrument's Second Derivative Max method of absolute quantification.

A five-fold serial dilution was prepared with crude cDNA template of all the genes under study of a known positive sample covering the respective dynamic ranges of detection for both target

Table 1. Details of primers used in the experiments

Genes	Sequence (5'→3')	GenBank Acc. No.	Size (bp)	Basic temperature (°C)
CXCR1	F: AGTCCCCGTGAGATAAGCAC R: CCAGGTTCAGCAGGTAGACA	EF597244.2	163	59
CXCR2	F: CAACACTGACCTGCCCTCTA R: CCAGGTTCAGCAGGTAGACA	DQ328664.1	197	59
IL-8	F: TGCTCTCTGCAGCTCTGTGT R: CAGACCTCGTTTCCATTGGT	EU276073.1	190	59
CD-11b	F: CAAACTGGCAGAAAGCAACA R: TCCAGGAAGACTCTGGAGGA	NM_175781.1	183	59
CD-62L	F: CCGATTGCTGGACTTACCAT R: CCAAGTCCACACCCCTTCTA	NM_174182.1	194	59
GAPDH	F: GGGTCATCATCTCTGCACCT R: GGTCATAAGTCCCTCCACGA	NM_001034034	176	59

doi: 10.17221/63/2015-CJAS

and housekeeping genes. Standard curve of "log concentration of template vs CP value" was plotted and the efficiency of the reaction was calculated by the instrument's absolute quantification second derivative max method.

The relative expression of each gene was analyzed using the relative quantification method described by Pfaffl 2001 with the relative ratio given as

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Cp_{\text{target}}\,(\text{control} - \text{sample})}}{(E_{\text{ref}})^{\Delta Cp_{\text{ref}}\,(\text{control} - \text{sample})}}$$

where:

E_{target} = Real Time PCR efficiency of the target gene transcript

E_{ref} = Real Time PCR efficiency of the reference gene transcript

$\Delta Cp_{\text{target}}$ = crossing point deviation of control sample and experimental sample with target gene

ΔCp_{ref} = crossing point deviation of control sample and experimental sample with reference gene

Normal healthy cows acted as calibrator for the other cow groups (pregnant, calving, and mastitic). The mRNA abundance of the target gene was calibrated with that of the reference gene (*GAPDH*) and expressed as fold of induction over the control at each time point.

Statistical analysis. All analyses were done using one way ANOVA by the SYSTAT software package (Version 12, 2007) considering group as factor. The relative expression ratio of the target genes was tested and analyzed for significance by Relative Expression Software Tool REST (Version 2.0.13, 2009) (Pfaffl et al. 2002).

RESULTS

Milk SCC, blood TLC, and blood and milk DLC. Differential leukocyte populations in blood and milk of normal and mastitic crossbred cows under different physiological conditions are presented in Table 2. Maximum TLC was observed in CM cows followed by blood samples collected from C and SCM cows. Maximum milk SCC was observed in CM cows followed by SCM cows and in the first milk, i.e. colostrums collected on the day of calving. Blood TLC and milk SCC were significantly ($P < 0.05$) higher during CM and SCM compared to healthy control. Blood and milk neutrophils (immature band neutrophils) were significantly ($P < 0.05$) higher during calving as well as mastitis. Blood lymphocytes were significantly ($P < 0.05$) lower in CM compared to healthy cows.

Viability of blood and milk neutrophils. Viability of neutrophils was checked by Trypan blue method which indicated that viability was always higher in healthy samples and decreased subsequently in infected samples.

Table 2. Blood TLC and DLC, milk SCC and DLC in healthy, mastitic, pregnant, and newly calved cows

Source	Parameters	Healthy	Subclinical mastitis	Clinical mastitis	Pregnant	Calving
Blood	TLC ($\times 10^3$ /μl)	$7.76^{b} \pm 0.12$	$11.28^{c} \pm 0.19$	$12.97^{e} \pm 0.08$	$7.41^{a} \pm 0.04$	$11.82^{d} \pm 0.05$
	blood neutrophils (%)	$27.07^{a} \pm 0.23$	$32.80^{c} \pm 0.47$	$39.04^{e} \pm 0.53$	$28.73^{b} \pm 0.46$	$35.30^{d} \pm 0.37$
	segmented neutrophils (%)	$98.17^{d} \pm 0.24$	$96.50^{c} \pm 0.17$	$94.17^{b} \pm 0.33$	$98.17^{d} \pm 0.19$	$91.50^{a} \pm 0.25$
	band neutrophils (%)	$1.83^{a} \pm 0.22$	$3.50^{b} \pm 0.20$	$5.83^{c} \pm 0.22$	$2.00^{a} \pm 0.16$	$7.33^{d} \pm 0.32$
	blood lymphocytes (%)	$58.25^{e} \pm 0.25$	$52.08^{c} \pm 0.36$	$45.60^{a} \pm 0.29$	$55.25^{d} \pm 0.19$	$49.27^{b} \pm 0.27$
	blood monocytes (%)	$10.08^{b} \pm 0.13$	$11.07^{c} \pm 0.29$	$10.45^{c} \pm 0.46$	$10.07^{b} \pm 0.12$	$8.16^{a} \pm 0.19$
	viability of neutrophils (%)	$95.33^{e} \pm 0.28$	$86.00^{c} \pm 0.20$	$80.00^{a} \pm 0.31$	$90.50^{d} \pm 0.30$	$83.33^{b} \pm 0.22$
Milk	SCC ($\times 10^5$ cells/ml)	$1.60^{a} \pm 0.38$	$4.60^{d} \pm 0.39$	$7.50^{e} \pm 0.54$	$2.00^{b} \pm 0.19$	$4.00^{c} \pm 0.08$
	milk neutrophils (%)	$19.27^{a} \pm 0.24$	$43.12^{c} \pm 0.37$	$75.83^{e} \pm 0.40$	$22.00^{b} \pm 0.42$	$67.83^{d} \pm 0.59$
	segmented neutrophils (%)	$98.00^{d} \pm 0.19$	$96.00^{c} \pm 0.18$	$93.00^{a} \pm 0.24$	$96.33^{c} \pm 0.25$	$95.00^{b} \pm 0.26$
	band neutrophils (%)	$2.00^{a} \pm 0.18$	$4.00^{b} \pm 0.19$	$7.00^{d} \pm 0.24$	$3.66^{b} \pm 0.25$	$5.00^{c} \pm 0.19$
	milk lymphocytes (%)	$14.88^{e} \pm 0.24$	$11.43^{c} \pm 0.24$	$7.80^{a} \pm 0.39$	$12.00^{d} \pm 0.24$	$10.82^{b} \pm 0.16$
	milk macrophages (%)	$65.53^{e} \pm 0.48$	$45.45^{c} \pm 0.47$	$16.95^{a} \pm 0.36$	$59.07^{d} \pm 0.43$	$19.32^{b} \pm 0.38$
	viability of neutrophils (%)	$92.53^{e} \pm 0.31$	$80.40^{b} \pm 0.36$	$71.47^{a} \pm 0.63$	$85.22^{d} \pm 0.45$	$82.22^{c} \pm 0.61$

TLC = total leukocyte counts, DLC = differential leukocyte counts, SCC = somatic cell counts

values are expressed as mean ± SE; values lacking a common letter within a row differ significantly ($P < 0.05$)

doi: 10.17221/63/2015-CJAS

Phagocytic activity (PA) of blood and milk neutrophils and plasma cortisol level. PA of blood and milk neutrophils along with plasma cortisol levels are presented in Figure 1. PA of blood neutrophils was significantly ($P < 0.05$) higher than of milk neutrophils except at calving where milk neutrophils showed higher activity than blood ones. The highest PA of blood and milk neutrophils was found in healthy cows, however it decreased significantly ($P < 0.05$) during SCM and CM. The lowest PA of blood neutrophils was at calving. Upon estimation of plasma cortisol it was revealed that the highest cortisol concentration was at calving. A significant ($P < 0.05$) increase in plasma cortisol was found both during SCM and CM compared to healthy and P animals.

Chemotactic activity (CHA) of blood and milk neutrophils and plasma IL-8 levels. CHA of blood and milk neutrophils together with plasma IL-8 levels are presented in Figure 2. CHA of blood neutrophils was significantly ($P < 0.05$) higher than of milk neutrophils except at calving where milk neutrophils showed higher activity than those of blood. CHA of blood and milk neutrophils was the highest in healthy animals and decreased significantly ($P < 0.05$) during SCM and CM as well as at calving. A significant ($P < 0.05$) increase in plasma IL-8 concentration was detected at calving and CM compared to healthy control cows. CHA of both blood and milk neutrophils was negatively correlated with plasma IL-8 concentration during mastitis and calving.

Expression of CXCR1, CXCR2, IL-8, CD-62L, and CD-11b on blood and milk neutrophils. Relative mRNA expression profile of *CXCR1, CXCR2, IL-8, CD-62L,* and *CD-11b* genes is presented in Figure 3. Relative mRNA expression of *CXCR1* and *CXCR2* genes was significantly ($P < 0.05$) higher in CM and newly calved animals compared to control but remained unaltered in P with a non-significant difference. A significant ($P < 0.05$) increase in *IL-8* mRNA expression was observed during SCM and CM and remained unaltered around P and non-significantly increased in blood neutrophils isolated at calving. In blood neutrophils, relative mRNA expression of *CD-62L* decreased significantly ($P < 0.05$) during mastitis and calving compared to healthy animals but this expression remained unaltered in milk neutrophils. Relative mRNA expression of *CD-11b* gene was significantly ($P < 0.05$) higher in CM and SCM compared to control. It remained unaltered in pregnancy but there was a significant increase around calving.

DISCUSSION

Milk SCC, blood TLC, and blood and milk DLC. In the present study, we have investigated activation, adhesion, and migration of neutrophils during mild and severe stressful conditions by studying their number, activity, and mRNA expression of various receptors and genes. We have found that neutrophils are the predominant cell type in mammary tissues and in mammary secretions during early inflammation and can constitute even > 70% of total mammary gland leukocytes in the case of mastitis. Kehrli and Shuster (1994) stated that high SCC in milk are not the cause of mastitis; instead,

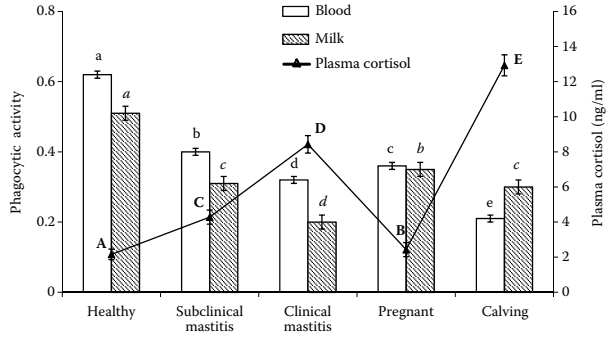

Figure 1. Phagocytic activity of blood and milk neutrophils along with plasma cortisol values in healthy, mastitic, pregnant, and newly calved crossbred cows

means with different letters differ significantly ($P < 0.05$) ($^{a–e}$blood neutrophils, $^{a–d}$milk neutrophils, $^{A–E}$plasma cortisol)

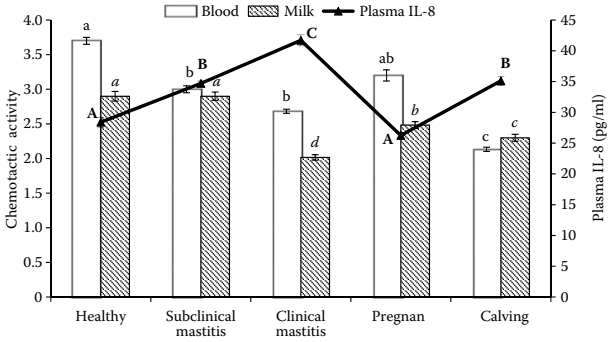

Figure 2. Chemotactic activity of blood and milk neutrophils along with plasma IL-8 in healthy, mastitic, pregnant, and newly calved crossbred cows

means with different letters differ significantly ($P < 0.05$) ($^{a–c}$blood neutrophils, $^{a–d}$milk neutrophils, $^{A–C}$plasma IL-8)

doi: 10.17221/63/2015-CJAS

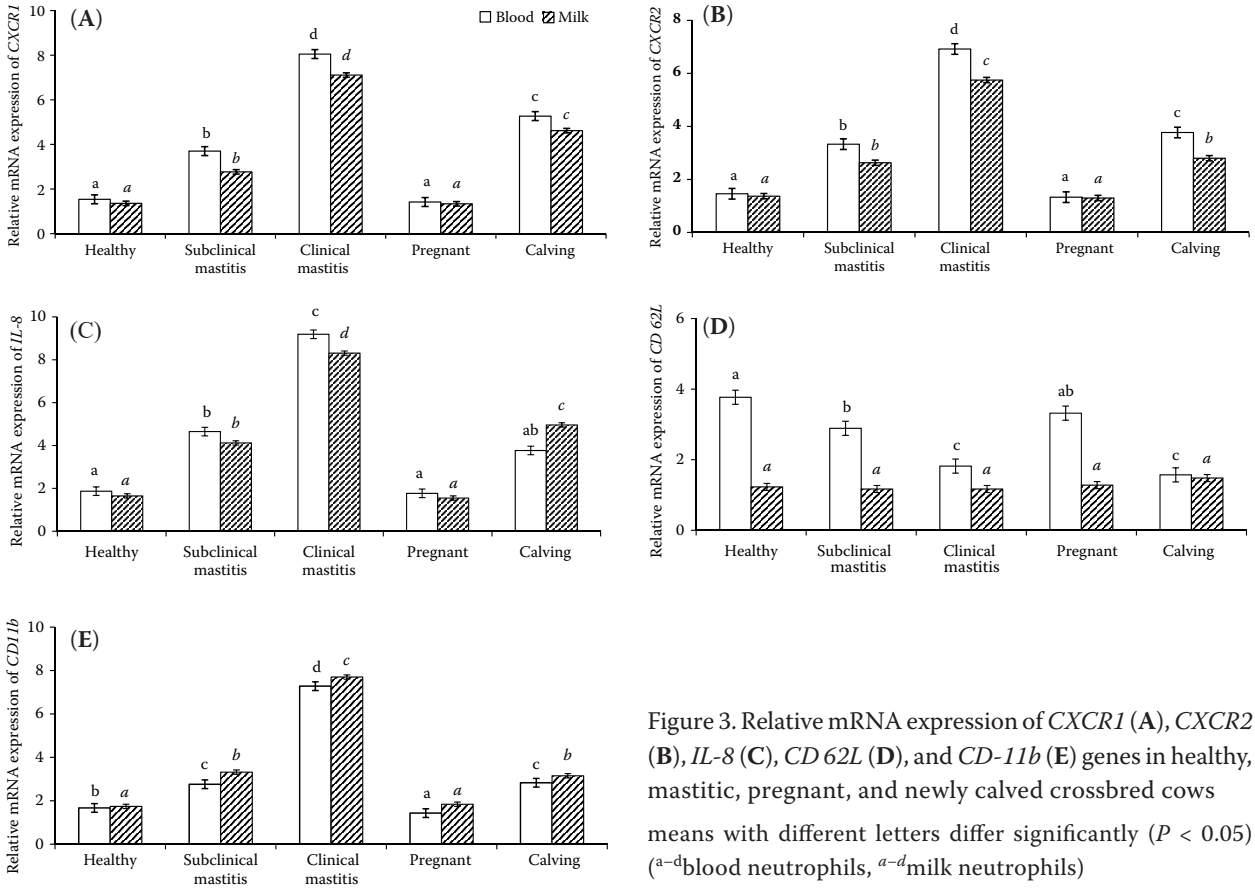

Figure 3. Relative mRNA expression of *CXCR1* (**A**), *CXCR2* (**B**), *IL-8* (**C**), *CD 62L* (**D**), and *CD-11b* (**E**) genes in healthy, mastitic, pregnant, and newly calved crossbred cows

means with different letters differ significantly ($P < 0.05$) ($^{a–d}$blood neutrophils, $^{a–d}$milk neutrophils)

they are a necessary and correlated response to combat microbes in the mammary gland. Values of higher colostrum SCC are in agreement with our earlier studies (Dang et al. 2008). Less viability in both blood and milk neutrophils means poor phagocytic activity by the neutrophils in infected cows. In the present investigation, higher blood TLC and lower blood lymphocytes were found in periparturient cows, which may be due to stress of calving, in accordance with the earlier reports of Meglia et al. 2005.

Phagocytic activity of blood and milk neutrophils and plasma cortisol level. The main function of neutrophils is phagocytosis and intracellular killing. Neutrophils engulf bacteria by two distinct mechanisms, the respiratory burst and digestion by lysosomal enzymes. The ability of neutrophils to phagocytose foreign particles is an essential mechanism for protection of any tissue against various invaders. That is why observing the *in vitro* PA of neutrophils provides a very effective tool for the study of natural mastitis resistance. In this investigation the capability of blood neutrophils to perform phagocytosis was higher than of milk neutrophils which could be due to higher viability of blood neutrophils as compared to those of milk (Mehrzad et al. 2009). However, the milk neutrophils isolated from colostrum at calving have a higher activity than blood ones due to very high levels of immunoglobulins as revealed in the previous studies (Sugisawa et al. 2003) and confirmed in the present investigation, too. In this study both blood and milk neutrophils function was diminished around calving as well as during mastitis with a concomitant increase of plasma cortisol around these periods. A dramatic impairment in random migration, adhesion, and ROS production of blood PMN was observed during the first week after parturition (Dosogne et al. 2001) and mastitis (Mehrzad et al. 2009) which may be due to hormonal changes (Suriyasathaporn et al. 2000). Immune cells (neutrophils, macrophages, and lymphocytes) in bovine exhibit relatively abundant levels of glucocorticoids receptors in their cytoplasm and, through these receptors the glucocorticoids tend to exert multiple effects (Webster et al. 2002). During the peripartum period cortisol is increased which in turn suppresses the genes

doi: 10.17221/63/2015-CJAS

involved in producing inflammatory, antibody, and cytotoxicity responses in cattle (Nonnecke et al. 1997). This is supported by another report that the incubation of cattle immune cells with a physiologically relevant high concentration of cortisol decreases lymphocyte proliferative response, IL-2 production, and neutrophil functions (Blecha and Baker 1986). The present findings confirmed the pre-existing concept of immune suppression around the periods of parturition as well as during mastitis. On the other hand they also proved that mastitis is mainly manifested due to decreased phagocytic activity of blood neutrophils once they cross the blood mammary barrier and become milk neutrophils. However, a similar magnitude of *in vitro* phagocytosis was observed in milk neutrophils around pregnancy and a higher activity around calving but, in all the cases this immune suppression is cortisol dependent.

Chemotactic activity of blood and milk neutrophils and plasma IL-8 levels. Neutrophil chemotaxis is a complex process which results in the attachment of neutrophils to the walls of blood vessels, transmigration through the vasculature endothelium, and migration into the tissue site. Chemoattractants like IL-8 help regulate and control migration by inducing leukocyte migration *in vitro* and to mediate inflammation *in vivo*. Their roles in recruiting neutrophils during inflammation lead us to estimate IL-8 in plasma during inflammation of the mammary gland (mastitis). IL-8 can be produced from a variety of cell types including leukocytes (Qi and Kreutzer 1995) and epithelial cells (Elner et al. 1990) during mastitis. However, IL-8 was not found to be involved in *in vitro* chemotaxis of neutrophils induced by non mastitic mammary secretions (Ayoub et al. 1996). This may partly be due to the inflammatory environment of the gland allowing for the production of other factors which inhibit T-cell migration like the transforming growth factor β, IL-10 (Kasama et al. 1994), and IL-4 (Jinquan et al. 1995). In the present investigation we found decreased CHA of milk neutrophils compared to blood neutrophils except at calving. It was also found that there was an inverse relationship between plasma IL-8 concentration with CHA of both blood and milk neutrophils.

Expression of CXCR1, CXCR2, IL-8, CD-62L, and CD-11b on blood and milk neutrophils. Human and bovine neutrophils express several chemokine receptors, including *CXCR1* and *CXCR2*, which are members of the seven-transmembrane G-protein coupled receptor family. The expression of *CXCR1* observed in this study was greater in cows infected with mastitis and newly calved cows. An up-regulation of *CXCR1* was observed when the quarters of Holstein cows were inoculated with *S. chromogenes* (Verbeke et al. 2015). *CXCR1* is a receptor specific for IL-8 and is expressed on the surface of neutrophils and strongly associated with the inflammatory response to Gram-negative bacteria infections (Rainard and Riollet 2006). The receptor-ligand interaction between *CXCR1* and IL-8 induces conformational changes in neutrophils that permit their chemotaxis to the site of infection where they release their antimicrobial components (Olson and Ley 2002). Chemotactic migration, recruitment, and infiltration of neutrophils from the blood stream during inflammation are associated with the activation of *CXCR2*. Interleukin-8, also known as neutrophil chemotactic factor, is an immune related chemokine released in response to cell stressors such as reactive oxygen species, bacterial fragments, and pro-inflammatory cytokines (DeForge et al. 1993). The expression of *IL-8* was greater in the blood neutrophils of calving and CM cows indicating that the recruitment of blood neutrophils was more towards the stressful sites. A positive association between the *IL-8* gene expression level and the incidence and severity of mastitis has been found (Galvao et al. 2011). IL-8 activates two receptors – *CXCR1* and *CXCR2*, the former seemingly activating the respiratory burst, whereas the latter primarily inducing survival from spontaneous apoptosis (Glynn et al. 2002). A significant association between *CXCR2* and percentages of SCM in Holsteins has also been detected.

In the present investigation, the expression of selectin was found to be greater in the blood neutrophils of healthy cows, but it decreased further in SCM and CM cases. This indicates that in healthy cows blood neutrophils were more sensitive to adhesion. A lower expression of selectin on mastitis neutrophils indicates an impairment of migration towards the mastitic agent and more time taken in the resolution of an infection. There is a down regulation of selectin on peripheral neutrophils but supplementation of vitamins enhanced L-selectin levels in mastitic cows (Mukherjee et al. 2010). Neutrophils respond to an infection through selec-

doi: 10.17221/63/2015-CJAS

tins, integrins, and chemokines. These neutrophils initially bind the blood vessel through selectins, whereas integrins cause the neutrophils to decelerate from rolling to a stable arrest. Maximum and significant expression of *CD-11b* (integrin) was observed in CM cows indicating that they acquire increased adhesive ability to endothelium. A progressive decrease of *CD-62L* (selectin) expression was observed and continuous rise of *CD-11b* after infusion of lipopolysaccharides to the mammary gland (Diez-Fraille et al. 2004).

The current study was designed to evaluate the functions of blood neutrophils isolated from dairy cows undergoing various degree of stress. We were able to evaluate the functions of neutrophils collected from blood samples taken during subclinical mastitis, clinical mastitis, calving, and in pregnancy. Our combined neutrophil activity and gene expression data substantiate the already available information that during periods of stress the activity of the first line of defence – the neutrophils, is impaired. We also found a higher expression of chemokine receptors, neutrophil chemoattractants, and integrins both in blood and milk neutrophils during severe stressful conditions like clinical mastitis as compared to other mild physiological stressors like pregnancy. These results may provide prospective ways to enhance or regulate neutrophil function and potentially increase an animal's resistance to mastitis and other inflammatory diseases.

Acknowledgement. M. Alhussien is thankful to the Syrian Arab Republic for giving permission to carry out this research in India.

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Received: 2015–09–05
Accepted after corrections: 2016–02–23

Corresponding Author

Mohanned Alhussien, M.Sc., Animal Production Division, Aleppo University, Aleppo, Syria
Phone: +91 01 842 259 084, e-mail: mohannad-alhussien@hotmail.com