Veterinary Immunology and Immunopathology 191 (2017) 14–21

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Integrated effect of seasons and lactation stages on the plasma inflammatory cytokines, function and receptor expression of milk neutrophils in Sahiwal (*Bos indicus*) cows

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ARTICLE INFO

Keywords:
Mammary gland
Phagocytosis
Cortisol
Interleukin
Chemokine receptor
Toll-like receptor

ABSTRACT

Mastitis is a highly prevalent and one of the costliest diseases of dairy cows affecting the mammary gland. Milk neutrophils present in the mammary gland serve as an integral part of the mammary immunity, and their performance is influenced by different environmental conditions and lactation stages. To investigate the combined effects of seasons and lactation stages on the mammary immunity, milk and blood samples were collected from three groups of high producing indigenous Sahiwal cows. Function and receptor expression of milk neutrophils together with cortisol and inflammatory interleukins concentration in blood were studied. The first group of cows started their lactation in winter and completed their lactation in hot-humid season; the second group started their lactation in hot-dry season and completed it in winter. The third group started their lactation in hot-humid and completed by the hot-dry season. Plasma cortisol levels were very high during early lactation in all seasons. An inverse relationship was observed between cortisol levels and glucocorticoid receptor. Elevated phagocytic activity and plasma interleukin-2 levels were seen in winter and during mid lactation of all seasons. A positive correlation was noticed between plasma IL-8, the percentage of milk neutrophils and expression of chemokine receptors (CXCR1 and CXCR2). The highest expression of toll-like receptors (TLR2 and TLR4) and chemokine receptors was in hot-humid season. Reduction in the phagocytic activity of neutrophils, pro-inflammatory cytokines and elevated levels of cortisol in cows which started their lactation and attained peak lactation during hot-humid season indicated more stress in them. Integrated influence of both seasons and lactation stages on the activity of milk neutrophils along with plasma interleukins and cortisol levels may be used to develop suitable managemental strategies to improve mammary health and increase milk production in indigenous dairy breeds experiencing harsh environmental conditions.

1. Introduction

Mastitis is a serious health problem and is common in high yielding dairy cows causing enormous economic losses. Seasons and lactation stages are two important factors which contribute to the incidence of this disease. In dairy cows, mammary gland immunity and the endocrine profile change with seasons. This change enables the cows to adjust their physiology, maintain full lactation and also minimise the incidence of disease (Ebling and Barrett, 2008). Seasonal occurrence of mastitis with increased temperature humidity index (THI), precipitation and hot weather condition is well documented (Ranjan et al., 2011; Moosavi et al., 2014; Zeinhom et al., 2016).

Mammary neutrophils are the first cells to counteract against invading pathogens. They play a significant role in preventing the occurrence and progression of subclinical and clinical microbial infections in the mammary gland during stressful conditions in Tharparkar cows reared under semi-arid conditions (Alhussien et al., 2016a). Studies have been carried out to explore the effects of the season in Zebu breed (Swain et al., 2016) and lactation stage in Karan Fries crossbred (Holstein Friesian × Tharparkar) cows (Mukherjee et al., 2015b) on the competence of neutrophils. But, the combined effect of both stage of lactation and season on mammary health has been missing.

Sahiwal cows are the highest milk producers among the indigenous dairy breeds and have a well developed udder. They have played a valuable role in crossbred programs of Asia, Australia, Europe and in several ecological zones of Africa as they have a greater heat tolerance as well as tick and parasite resistant (Glass et al., 2005; Glass and Jensen, 2007). Being one of the high producing indigenous dairy breeds also make them more prone to mammary infections under harsh environmental conditions (Khate and Yadav, 2010). We believe that in

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http://dx.doi.org/10.1016/j.vetimm.2017.07.010
Received 10 April 2017; Received in revised form 5 July 2017; Accepted 25 July 2017

these native cows the activity of milk neutrophils and expression of genes involved in its activation, migration and phagocytosis as well as plasma hormones and cytokines may show different patterns throughout the entire lactation under the same season as well as during different seasons. These changes may influence the incidence of mastitis and ultimately milk production in these cows. To prove this, we designed our experiment in which the impact of each season could be studied separately throughout the lactation cycle. Studying the integrated effect of season and lactation stage on the competence of the milk neutrophils will help in better understanding and monitoring of udder health and milk quality during the change in season and also guide us to develop effective control methods and appropriate detection techniques for subclinical and clinical mastitis in high producing cows.

2. Material and methods

2.1. Geographical location and climatic condition of study area

National Dairy Research Institute (NDRI) cattle yard is located in the northern part of Karnal city in Haryana, India at 250 m above mean sea level, in the Indo-Gangetic plains on 29°43″ N altitude and longitude 77°2″ E. Temperature goes even up to 46 °C on a single day in summer and falls to around 3 °C in winter. The relative humidity (RH) ranges from 30 to 85% at this place. This study was carried out for about (1.5 years) *i.e.* from November 2014 to June 2016 at the Livestock Research Centre (LRC) of NDRI, Karnal. Maximum temperature humidity index was recorded during hot humid (HH) season (severe stress) followed by hot dry (HD) season (mild stress) and finally winter (W) season (comfortable). Calculation of THI was done according to the formula of National Research Council (1971):

$$THI = (Tdb + Twb) \times 0.72 + 40.6$$

Where THI is temperature humidity index, Tdb is dry-bulb temperature (°C), and Twb is wet-bulb temperature (°C).

The meteorological variables data during the study period were gathered from Central Soil Salinity Research Institute which is located about 5 km from the study area and has been presented in Table 1. All the climatic variables were recorded twice daily at 7.30 A.M. and 2.30 P.M; however, we are showing the average value of each meteorological variable during each season.

2.2. Experimental design

Clearance of this experimental study was taken from the Animal Ethics Committee of the Institute according to the article 13 of the CPCSEA rules, laid down by the Government of India. Initially, a total of thirty healthy Sahiwal cows (10 in each group) in their third parity were selected from the LRC of NDRI and followed for all seasons. However, some of the experimental cows were later excluded from this study due to the incidence of subclinical mastitis (SCM) and clinical mastitis (CM) in them. Finally, eighteen cows were followed throughout their lactation cycle with six cows always maintained in a group during each season. The first group started their lactation in W season (December, January, February) and completed it in HH season (Fig. 1a), the second group started their lactation in HD season (April, May, June) and completed it in W season (Fig. 1b). The third group started their lactation in HH season (July–September) and completed it in HD season (Fig. 1c). None of the cows were included in more than one group on subsequent lactations, and always new animals were recruited for the study to maintain similar parity.

2.3. Sampling and management of the animals

All the cows were housed in open air stalls with asbestos roof. These cows were fed with *ad-libitum* green fodder (Maize, Jowar, Cowpea, Berseem and Oat) and also a measured amount of concentrates diet (20% crude protein and 70% total digestible nutrient) according to National Research Council (2001) recommendation. Fresh and clean drinking water was provided to them throughout the day. All the cows were high yielding (> 12 kg/day) and were machined milked thrice daily. Milk and blood sampling was always done during afternoon milking (between 1:30 PM and 2:30 PM). All cows were screened for mastitis by performing routine California mastitis test and by monitoring their milk somatic cell counts (SCC) throughout the study period. Only cows which were healthy and did not display any SCM symptoms as well as maintained their milk SCC below (2.5×10^5) were included in the study. In each stage of lactation of a particular season, three composite milk samples (representing all four quarters) were collected from each cow with an interval of 30 days into sterile tubes (200 ml/cow). Similarly, blood (7 ml/animal) was drawn from these cows in sterile heparinized vacutainer tubes from the jugular vein puncture with minimum disturbance to them. Plasma was separated and stored for further analysis at −80 °C.

2.4. Evaluation of SCC and differential leukocyte count (DLC) of milk

Somatic and differential cell count of milk samples was done microscopically (Dang et al., 2010), and also cross checked using a somatic cell counter (Milkotronic Ltd, Stara Zagora, Bulgaria). Differential cell counting of milk was carried out to determine the percentage of the major immune cells secreted in the milk like lymphocytes, neutrophils and macrophages.

2.5. Neutrophils isolation and phagocytic activity (PA)

Isolation of milk neutrophils was performed as per the method described by Mehrzad et al. (2001) for milk samples with minor modifications. Briefly, the collected milk was filtered by a nylon filter (40 μm pore size) and then diluted to 50% with cold phosphate buffered saline (PBS) (volume/volume). Around 45 ml of milk was centrifuged (600 × *g*, 15 min, 4 °C) for the removal of fat. The cell pellet then was washed twice with cold PBS (300 × *g*, 10 min, 4 °C and 200 × *g*, 15 min, 4 °C). After that, the cells were resuspended in 3 ml of Dulbecco's PBS containing 0.5 mg/ml gelatin. Using a 15 ml Falcon tube, 3 ml of Histopaque 1119 (Sigma, Germany) was taken, and 3 ml of Histopaque 1077 (Sigma, Germany) was layered over it slowly. Three

Table 1
Meteorological variables data recorded from November-2014 to June-2016.

Year	Season	Temperature (°C)				RH (%)	THI
		Max	Min	Dry bulb	Wet bulb		
2014	Winter	18.00 ± 1.93[a]	07.10 ± 0.73[a]	17.96 ± 1.89[a]	14.23 ± 0.80[a]	57.67 ± 1.20[b]	63.78[a]
2015	Hot Dry	37.10 ± 2.04[c]	21.90 ± 2.17[b]	24.93 ± 1.82[b]	20.36 ± 1.62[b]	32.00 ± 5.29[a]	73.21[b]
	Hot Humid	33.43 ± 0.40[b]	24.13 ± 0.83[b]	29.86 ± 1.21[c]	26.16 ± 0.31[c]	68.67 ± 3.18[c]	80.94[c]
	Winter	19.57 ± 1.17[a]	06.60 ± 0.81[a]	18.76 ± 1.15[a]	14.96 ± 0.64[a]	53.33 ± 3.84[b]	64.89[a]
2016	Hot Dry	38.23 ± 0.32[c]	22.27 ± 2.02[b]	24.60 ± 1.26[b]	22.40 ± 1.17[b]	36.67 ± 3.28[a]	74.44[b]

Values are expressed as mean ± SE. [a,b,c]Values within a column with different superscript letters differ significantly at $P < 0.05$.