Effect of different stages of lactation on erythrocyte osmotic fragility, blood leucocyte entities, and milk somatic cell of Badri cattle

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Abstract

The present investigation was undertaken to study the effect of different stages of lactation on erythrocyte osmotic fragility, blood leucocyte indices and milk somatic cell count in indigenous Badri cattle. Badri is first registered indigenous, dual purpose cattle breed of the state Uttarakhand (India) mainly reared in hilly areas of the state. Osmotic fragility evaluates the stability of erythrocyte membrane to osmotic stress. Leucocytes form defence mechanism against invading micro-organisms. Somatic cell count is an indicator of udder health. The study was carried out in forty healthy Badri cows divided into five groups according to their lactation stages viz. group 1 (0–60 days), group 2 (61–120 days), group 3 (121–180 days), group 4 (181–240 days) and group 5 (to be dried off). Erythrocyte osmotic fragility, blood total leucocyte count (TLC), differential leucocyte count (DLC) and milk somatic cell count (SCC) were estimated. The percent hemolysis of erythrocytes among different groups was significant ($p < 0.05$) at 0.9% and 0.5% saline concentrations. A significant effect *($p < 0.05$)* of lactation stages on blood lymphocytes, neutrophils and milk somatic cell count was observed.

Introduction

Badri, the indigenous cattle of Uttarakhand is the first registered cattle breed of the state on 21st June 2016 and is named as Badri (ICAR-NBAGR, 2016), mainly reared in hilly areas of the state. Unique physical characteristics of Badri cattle are small size, weighing about 200–250 kg, milk yield is about 1.5 (0.5-2.0) kg per day. The average age at first calving is 3–5 years and have 8–10 calving during its lifetime. The breed have short lactation length (208 days) and relatively long dry period 138 days (4–6 months). These are well adapted to the hilly terrain and climatic conditions, comparatively more resistant to diseases (Pundir et al. 2014) and can play significant role in development of Uttarakhand as an organic state (Banga et al. 2005). The livestock owners of the state prefer Badri cattle over other animals in terms of religious value, better adaptability in hilly region, disease resistance, medicinal properties of milk and urine, good manure, requires less external inputs, milk flavour, excellent feed conversion efficiency and draught power (Joshi et al. 2018).

Pregnancy and lactation are the important physiological stages that cause considerable alteration in individual's hematological parameters (Maria and Monika, 2010). Erythrocyte osmotic fragility is defined as degree or proportion of hemolysis which occurs when a blood sample is subjected to osmotic stress by placing in a hypotonic solution. Osmotic fragility is affected by various factors like membrane composition and integrity, cell's size and/or surface area to volume ratio (Fischbach and Dunning, 2008). Increased osmotic fragility is due to the damage of erythrocyte's membrane by oxygen radicals in the form of lipid peroxidation and protein degradation with decreased antioxidant levels caused by imbalance in redox signalling and generation of free radicals at rates that cannot be matched by endogenous antioxidant (glutathione and superoxide dismutase) (Oyagbemi, 2009), which has influence on RBC's membrane integrity and makes cells more fragile and labile to damage. Intrinsic factors including age, species, breed, genes, phenotype, sex, pregnancy and lactation, size and difference in erythrocyte membrane composition can also affect osmotic fragility of erythrocytes (Igbokwe, 2018).

Milk somatic cells are a mixture of milk producing epithelial cells and immune cells, secreted in milk during the normal course of milking and are used as an index for estimating mammary health and milk quality of dairy animals. Milk somatic cell count (SCC) is quantified as the number of cells per ml of milk. When the amount of SCC is around one lakh, it indicates a healthy udder of the animal (Alhussien and Dang, 2018). Somatic cells are primarily milk-secreting epithelial cells that have been shed from the lining of the mammary gland and white blood cells (leucocytes) that have entered the mammary gland in response to infection or injury (Dairyman's digest, 2009). Milk SCC is affected by production level, stages of lactation, parity, effect of body weight and body condition score, season, milking, breed, physiological stage of the animal (Alhussien and Dang, 2018). The present study investigates the effect of different stages of lactation on erythrocyte osmotic fragility, blood leucocyte entities and milk somatic cell count in indigenous Badri cattle.

Material And Methods

The experimental procedures were carried out in accordance with Animal welfare and Ethics Censorship. The animal care protocol was reviewed and approved by Animal ethical committee of GBPUAT, Pantnagar, India **(IAEC/C.V.A.Sc/VPB/388)**.

Experimental design

The study was carried out in forty healthy adult Badri cows, kept at Instructional Dairy Farm (IDF), GBPUAT, Pantnagar, Uttarakhand, India located in the foothills of Himalayas at 29.5°N latitude and 79.3°E longitudes. The animals were distributed in five groups with eight animals in each group according to lactation stages. The groups were as follow: group 1 (0-60 days), group 2 (61-120 days), group 3 (121-180 days), group 4 (181-240 days) and group 5 (to be dried off). The animals were reared under standard environmental conditions with provision of *ad libitum* feed and water supply.

Sample collection

Blood samples were collected during morning hours, from jugular vein after taking all precautionary measures in vials containing EDTA. Samples were transported immediately to the lab and were processed within 12 hours of collection. Milk samples were collected after taking all precautionary measures. All the samples were processed within 24 hours of collection. Milk samples were collected during evening milking time. Prior to milk collection, teats were washed and sterilized using 70% ethyl alcohol, then initial few streaks of milk were discarded and milk samples were collected in sterile vials. Milk samples were immediately transported to laboratory in ice box for analysis.

Determination of erythrocyte osmotic fragility

Erythrocyte osmotic fragility was determined as described by (Novozhilov et al. 2013). Sodium chloride solution (pH 7.4) was prepared at varying concentrations (0.0%, 0.1%, 0.3%, 0.5%, 0.7% and 0.9%) from 1% phosphate buffer. 5 ml of each NaCl concentration was placed in labelled test tubes serially and 0.02

ml (20 µl) of the blood sample was pipetted into each test tube. The content of the tubes was gently mixed by inverting the tubes and allowing them to stand at room temperature (24-26°C) for 30 minutes, thereafter, the tubes were centrifuged at 1500 rpm for 15 min using a centrifuge. The supernatant obtained from each tube was transferred to a clean glass cuvette and the absorbance of the supernatant was measured spectrophotometrically at a wavelength of 540 nm. The percentage haemolysis for each sample was calculated using the following Faulkner and King (1970) formula. Osmotic fragility curve of erythrocytes was determined by plotting the hemolysis percentage against various saline concentrations.

$$\text{Percent hemolysis} = \frac{\text{Optical density of test}}{\text{Optical density of standard}} \times 100$$

Determination of blood leucocyte indices

Blood samples were analysed for total leucocyte count (TLC) and differential leucocyte count (DLC), and absolute leucocyte count (ALC) and neutrophil: lymphocyte (N:L) ratio were calculated as per standard methods described in Jain (1986).

Determination of milk somatic cell count (SCC)

Somatic cell count was determined by the method described by Schalm et al. (1971). Milk samples were thoroughly mixed before testing. 10 µl of milk was spread over 1 cm^2 marked area on a clean glass slide. Thin milk film was left at room temperature until dry. Then slide was put into methanol for 5 minutes and after which it was stained with modified Newman-Lampert stain for 3 minutes. The slide was washed in tap water for 3 times and distilled water for 2 times. Then it was dried at room temperature (Marshall, 1992). Somatic cells were counted under 100 X magnification using oil immersion power.

10 µl or 0.01 ml of milk was spread in 1 cm^2, the possible number of such fields which could be counted in 1 cm^2 was 4000 cells. Milk volume represented by each field was 1/100 × 1/4000 = 1/400000. Hence microscopic factor was 400000. As total number of field counted were 50, therefore, working factor was 400000/5 = 8000.

Milk SCC/ml = 8000 × no. of cells counted in 50 microscopic fields

Statistical analysis

Data were analysed using SPSS statistical software version 26.0 to determine analysis of variance between groups. Groups were subjected to one-way analysis of variance (ANOVA) and Duncan's multiple range test. All data are expressed as mean ± standard error of the mean (SEM). Differences were considered to be statistically significant at $p<0.05$.

Results

The mean ± SE values of erythrocyte osmotic fragility during different stages of lactation are represented in Table 1. Erythrocyte osmotic fragility was recorded against 100 ± 0.00% hemolysis at 0% saline concentration. The percent hemolysis among different groups was significant ($p < 0.05$) at 0.9% and 0.5% saline concentrations. The maximum percent hemolysis was observed in group 2 at all saline concentrations. At 0.9% saline concentration, percent hemolysis was significantly ($p < 0.05$) highest in group 2 and lowest in group 3 than other stages of lactation and to be dried off animals. Whereas, at 0.5% saline concentration, percent hemolysis was significantly ($p < 0.05$) highest in group 2 and lowest in to be dried off animals. As saline concentration is decreased, percent hemolysis of erythrocytes increased in all the groups.

Table 1
Mean ± SE values of percent hemolysis during different stages of lactation and at different concentration of saline (NaCl %) solution in Badri cattle (n = 8 in each group)

Saline concentration	Percent hemolysis				
	Group 1 (0 to 60 days)	Group 2 (61 to 120 days)	Group 3 (121 to 180 days)	Group 4 (181 to 240 days)	Group 5 (To be dried off animals)
0.9%	2.79 ± 0.45^{ab}	4.65 ± 0.75^{b}	2.07 ± 0.45^{a}	2.80 ± 0.62^{ab}	2.64 ± 0.55^{ab}
0.7%	4.44 ± 0.66	6.09 ± 0.81	5.24 ± 0.74	4.83 ± 0.72	3.78 ± 0.66
0.5%	34.13 ± 2.59^{ab}	36.59 ± 2.53^{b}	34.86 ± 3.04^{ab}	33.22 ± 3.25^{ab}	24.51 ± 1.59^{a}
0.3%	83.52 ± 4.02	88.17 ± 6.90	85.40 ± 2.64	80.78 ± 4.08	75.69 ± 2.12
0.1%	91.05 ± 3.81	91.82 ± 1.60	91.45 ± 2.04	90.71 ± 2.93	87.85 ± 3.31
0%	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00

Mean values with different alphabets $^{(a,b)}$ in superscript differ significantly ($p < 0.05$) along the row.

The mean ± SE values of leucocyte entities during different stages of lactation are represented in Table 2. No significant *($p > 0.05$)* effect of stages of lactation on blood total leucocyte count (TLC) was observed in this study. However, it was lower during early lactation than other stages of lactation, but higher than to be dried off animals. Percent lymphocytes and neutrophils showed significant *($p < 0.05$)* variation during different stages of lactation. Lymphocytes percent was highest during early lactation and lowest in to be dried off cows. Neutrophil percent was lower during early lactation stage than all other stages. Absolute leucocyte count (ALC) and neutrophil:lymphocyte ratio showed non-significant *($p > 0.05$)* variation between different stages of lactation.

Table 2
Mean ± SE values of leucocyte entities during different stages of lactation in Badri cattle (n = 8 in each group)

Blood Leukocytes (%)	Stages of Lactation				
	Group 1 (0–60 days)	Group 2 (61–120 days)	Group 3 (121–180 days)	Group 4 (181–240 days)	Group 5 (To be dried off cows)
TLC (10^3/µl)	7.44 ± 0.63	7.95 ± 0.99	7.84 ± 1.06	7.95 ± 1.06	6.31 ± 0.63
DLC -					
L (%)	63.0 ± 4.56^{b}	55.12 ± 3.96^{ab}	50.25 ± 4.71^{ab}	56.75 ± 4.33^{ab}	43.75 ± 1.96^{a}
N (%)	32.0 ± 4.24^{a}	42.0 ± 3.72^{ab}	46.38 ± 5.42^{b}	39.88 ± 4.33^{a}	51.75 ± 1.81^{c}
M (%)	1.0 ± 0.33	1.0 ± 0.27	0.62 ± 0.26	0.75 ± 0.31	0.88 ± 0.51
E (%)	3.75 ± 0.59	2.29 ± 0.54	4.0 ± 0.71	2.5 ± 0.42	3.5 ± 0.60
B (%)	0.25 ± 0.16	0.13 ± 0.13	0.13 ± 0.13	0.13 ± 0.13	0.13 ± 0.13
N:L	0.56 ± 0.12	0.83 ± 0.12	1.06 ± 0.20	0.81 ± 0.20	1.21 ± 0.10
ALC					
L (%)	4.68 ± 0.44	4.04 ± 0.62	3.64 ± 0.22	4.74 ± 0.84	2.76 ± 0.29
N (%)	2.35 ± 0.39	3.32 ± 0.41	3.96 ± 0.86	2.95 ± 0.23	3.26 ± 0.31
M (%)	0.07 ± 0.02	0.08 ± 0.02	0.03 ± 0.01	0.06 ± 0.02	0.05 ± 0.03
E (%)	0.28 ± 0.05	0.23 ± 0.04	0.33 ± 0.07	0.20 ± 0.04	0.22 ± 0.04
B (%)	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01

Mean values with different alphabets $^{(a,b,c)}$ in superscript differ significantly ($p < 0.05$) along the row.

DLC- Differential leucocyte count, ALC – Absolute leucocyte count, L- Lymphocyte, N-Neutrophil, M - Monocyte, E- Eosinophil, B- Basophil, and L:N – Lymphocyte: Neutrophil

The mean ± SE values of milk somatic cell count (SCC) during different stages of lactation are represented in Table 3. A significant *($p < 0.05$)* effect of different stages of lactation on milk somatic cell count was observed in this study. Milk SCC was higher during early lactation, decreased in group 2, again increased in group 3 and then, decreased in group 4. In the cows to be dried off, milk SCC was higher than all lactation stages.

Table 3
Mean ± SE values of milk SCC during different stages of lactation in Badri cattle (n = 8 in each group)

Milk Component	Stages of Lactation				
	Group 1 (0–60 days)	Group 2 (61–120 days)	Group 3 (121–180 days)	Group 4 (181–240 days)	Group 5 (To be dried off cows)
Milk SCC (10^5/µl)	1.14 ± 0.13^{b}	0.52 ± 0.18^{a}	1.14 ± 0.14^{b}	0.86 ± 0.16^{c}	1.52 ± 0.16^{b}

Mean values with different alphabets $^{(a,b,c)}$ in superscript differ significantly ($p < 0.05$) along the row.

Discussion

Lactation being one of the important physiological stage imposes stress on dairy cattle. The erythrocytes of lactating cows were more susceptible to osmotic fragility than erythrocytes of pregnant cows (Kadah et al. 2014). This could be attributed due to hemodilution as a results of increased immature RBCs (reticulocytes) production which can resist osmotic fragility more than mature RBC. Lower resistance to osmotic fragility of erythrocytes in lactating cows could also be due to an imbalance of oxidative stress and lipid peroxidation (Ronchi et al. 2000). There was no significant difference in erythrocyte osmotic fragility between lactating and non-lactating animals (Habibu et al. 2014). Erythrocyte osmotic fragility decreases in late pregnancy due to improved membrane stabilization due to progesterone and increased during early lactation because of decreased cholesterol and triglycerides concentration in the membrane (Igbokwe et al. 2015). Lactation induces stress in dairy animals which then induces generation of free radicals which overwhelm the antioxidant defense mechanisms of the body (Nazifi et al. 2009). Increased free radical generation in the body has been shown to cause lipid peroxidation of cytomembranes, resulting in cell injury and death (William et al. 2008) of erythrocytes (Adenkola and Ayo, 2009). Although the proximate mechanism of haemolysis was not investigated in the present study, it has been seen that free radicals are generated in animals subjected to stress (Tauler et al. 2003) which apparently occurs in animals during lactation. Droge (2002) stated that stress factors may cause damage of erythrocyte membrane by inducing increased generation of oxygen radicals which cause lipid peroxidation and protein degradation.

Similar to our findings, Petrera and Abeni, 2018 found lower TLC during early lactation which further increased towards mid-lactation and this might be due to migration of leucocytes to uterus and mammary gland to fight post-partum diseases i.e. metritis and mastitis, respectively. Neutrophil decrease during early lactation stage could be due to the migration of neutrophils to the tissue to fight infections as this period causes stress and lowers the immune responses (Petrera and Abeni, 2018).

In the present study, elevated milk SCC during early lactation could be due to animal's immune response to calving and onset of lactation, to increase defence mechanism of the mammary gland and assist in

repairment of damaged tissue, an excessive desquamation of epithelial cells lining the alveoli with increased milk yield. Since 25% of epithelial cells and 75% of leucocytes cells normally contributes to milk SCC, which appear in small volume of milk synthesised by the mammary gland. The number of cells increased due to resumption of functional activity of mammary gland after a dormant period of pregnancy.

Conclusion

Lactation being one of the important physiological stages impose stress on lactating animals. During early and mid-lactation, there is increased demand of energy for production which is compensated by mobilization of lipids from adipose tissue. As erythrocyte's membranes are made up of phospholipids, any deficiency of lipids alters the flexibility of lipid bilayer of erythrocytes and make the membranes fragile. Besides this, increased free radicals in circulation also act on erythrocyte's membrane. There was no significant difference in blood total leucocyte count during different stages of lactation. However, differential leucocyte count of lymphocytes and neutrophils showed significant variation between different lactation stages. Milk SCC significantly varied between different stages of lactation as well as to be dried off cows. Milk SCs are an indicator of both resistance and susceptibility of dairy cows to mammary infections. High milk SCC is undesirable from the standpoint of quality but maybe too much low SCC will make the cows more prone to mammary infections. Further, the use of milk SCC as a management tool on a routine basis will help to maximize immunity and improve quality and quantity of milk as well as cow comfort and welfare. So proper feeding and stress management during lactation period is required for maintaining proper health status of dairy cattle.

Declarations

Author contributions All authors contributed to the study's conception and design. Swati Thakur: laboratory work, writing; A. Maiti, S. Dhara and M. Verma: review and editing; R. Huozha, S.K. Rastogi and M. Batra: original draft, methodology; S. Ramanarayanan and B.N. Shahi: data analysis. All authors have read and approved the submitted version.

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Data availability Data generated and analysed during this study are included in the current article.

Statement of animal right Experimental procedures were carried out in accordance with animal ethics. The animal use and care protocol were reviewed and approved by the Animal ethical committee of the GBPUAT, Pantnagar, Uttarakhand, India **(IAEC/C.V.A.Sc/VPB/388)**.

Conflicts of interest The authors declare no conflicts of interest.

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Figures

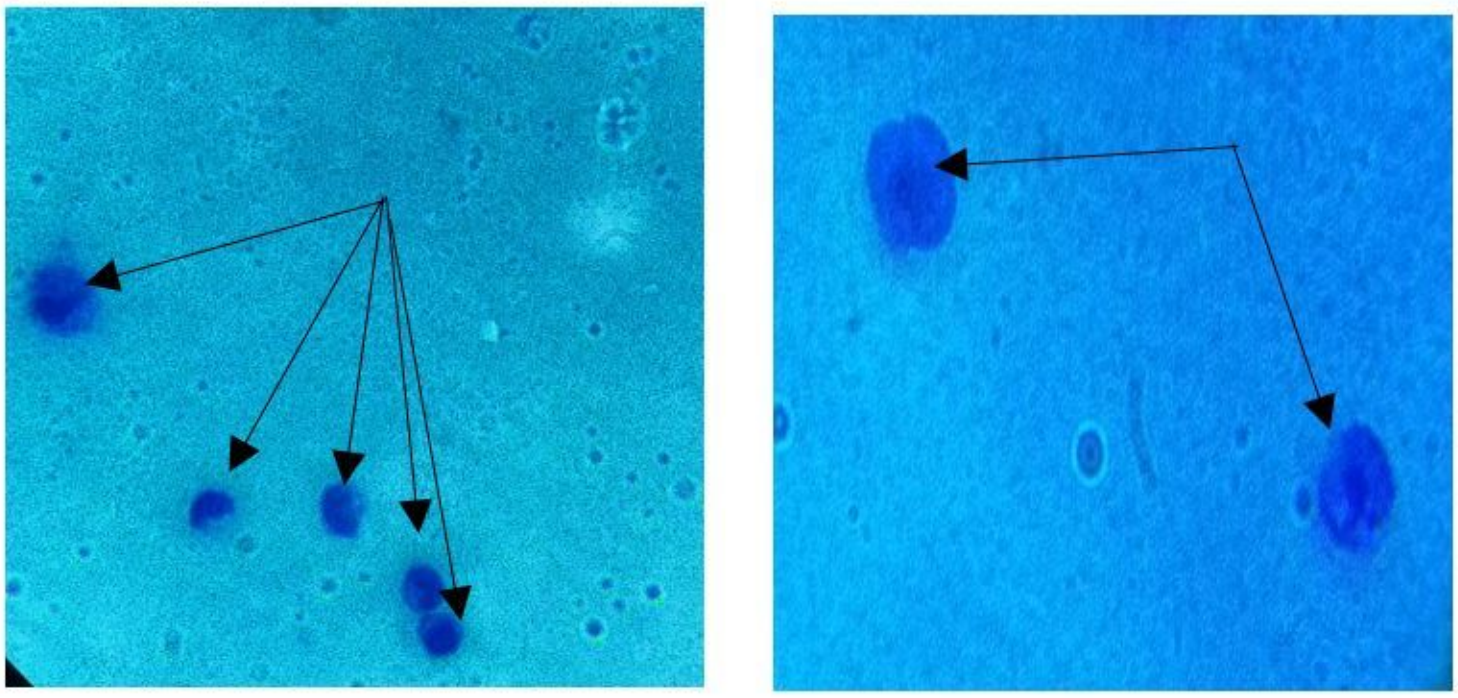

Figure 1

Milk somatic cell count (SCC) (Newman Lampert stain) in Badri cattle