RECOVERY TRENDS AND CORRELATION OF NON INVASIVELY ISOLATED MAMMARY EPITHELIAL CELLS WITH MILK YIELD IN SURTI AND MEHSANI BUFFALOES DURING EARLY LACTATION

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ABSTRACT

This study aims to elucidate recovery trends and correlation of non-invasively isolated mammary epithelial cells with milk yield in Surti and Mehsani buffalo breeds during early lactation. Surti and Mehsani buffaloes, 13 each maintained at Livestock Research Station, Navsari and Sardarkrushinagar, Dantiwada, Gujarat, India respectively were selected for the study. Milk samples were collected repeatedly from the same buffaloes at 15th and 60th day postpartum (pp). Buffaloes were categorized for data analysis and comparisons in to four groups *viz*. S15 (Surti buffaloes 15th day pp), S60 (Surti buffaloes 60th day pp), M15 (Mehsani buffaloes 15th day pp) and M60 (Mehsani buffaloes 60th day pp). The total somatic cells were separated from milk samples through centrifugation and primary bovine mammary epithelial cells (pBMEC) were obtained from total somatic cells using antibody mediated magnetic separation method. There was significant decrease in mean somatic cell count (SCC) with advancement of lactation to the tune of 18.72% in Surti and 16.84% in Mehsani buffaloes from day 15 to 60 pp. Comparable recovery of pBMEC (2.13 to 3.45%) from total somatic cells was observed over the advancement of lactation and between breeds. The pBMEC and SCC had exhibited negligible to low and non significant correlations with cumulative milk yield (CMY) and Test day milk yield (TDMY). Thus, it can be concluded that pBMEC can successfully be recovered from somatic cells. pBMEC and SCC both does not proved to be a significant explanatory variable for milk yield in buffalo breeds under study.

Keywords: *Bubalus bubalis*, buffaloes, Mehsani, milk, primary mammary epithelial cells, somatic cells, Surti

INTRODUCTION

Milk is produced in the udder by mammary epithelial cells (MEC). Milk contains MEC, which are gradually exfoliated from the epithelium during lactation. Previously, it was postulated that milk yield depended primarily on the size of the mammary gland (Linzell, 1966; Sorensen *et al*., 1998). However, it has been demonstrated more recently that milk yield is regulated by the quantity of mammary secretory cells and their secretory activity (Capuco *et al*., 2001). Annen *et al*. (2007) supported the hypothesis that increased milk yield during early lactation is associated with an increased accumulation of new MEC during late gestation and increased MEC shedding during

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early lactation. Similar, significant results were reported by Sigl *et al*. (2012) whereby, the ratios of MEC of total milk cells and milk yield were found to be correlated during the experimental timeframe of 153 days (r = 0.79), whereas correlation of milk yield and total somatic milk cells was lower (r = 0.62). The use of purified MEC is advantageous as it can determine the level of exfoliation of MEC from the mammary tissue, indicating the regulation of mammary cell number and the long term effect on milk yield as recently reviewed (Herve *et al*., 2015). Such studies on dynamics of exfoliated MEC numbers in milk of buffaloes are very scarce. Looking to the fact that buffaloes are major contributors of milk, an attempt was made to understand early lactation recovery trends of non-invasively isolated MEC and their correlation with milk yield in Surti and Mehsani buffaloes.

MATERIALS AND METHODS

Animals and sample collection

Surti (Breed Accession number : INDIA_BUFFALO_0400_SURTI_01005) and Mehsani (Breed Accession number : INDIA_BUFFALO_0400_MEHSANA_01004) buffaloes, 13 each maintained at Livestock Research Station, Navsari and Sardarkrushinagar, Dantiwada, Gujarat, India respectively were selected for the study. Milk samples were collected repeatedly from the same buffaloes at 15th and 60th day postpartum (pp). Buffaloes were categorized for data analysis and comparisons in to four groups *viz*. S15 (Surti buffaloes 15th day pp), S60 (Surti buffaloes 60th day pp), M15 (Mehsani buffaloes 15th day pp) and M60 (Mehsani buffaloes 60th day pp).Whole milk sample from each selected animal was collected during milking into a sterile bucket and milk yield was determined using electronic balance (Model-300-302, Preci-Tech, India). Sixteen hundred milliliter of the total milk was filled in autoclaved glass bottles and was subjected for cell isolation.

Experimental design

Milk (1600 ml) was defattened by centrifugation (Model- 5810 R, Eppendorf, Germany) at 1800 g at 4^{o}C for 30 minutes in several 50 ml sterile polypropylene tubes and skim milk was removed by pipetting. Remaining total cell pellets were resuspended in 25 ml of Phosphate Buffered Saline PBS (Cat. No. 10010072, Gibco, U.S.A) and pooled in pairs. After a second centrifugation step (1850 g, 15 minutes at 4^{o}C) the two total cell pellets were resuspended and pooled in 1 ml of PBS containing 1% bovine serum albumin BSA (Cat. No. 15260037, Gibco, U.S.A). A 7 µl aliquot was removed to perform somatic cell count using Neubauer chamber (Model- Double Silverline, Rohem, India). Purification of pBMEC was performed applying an immunomagnetic-bead based separation technique (Sigl *et al*., 2012). Cell suspension was first incubated for 10 minutes on a rotary mixer at 4^{o}C with a primary mouse monoclonal antibody (Cat. No. MA1-19037, Pierce, Thermo Scientific, U.S.A) against cytokeratin 8 antibody (clone C-43), which was specific to bovine epithelial cells. Unbound antibodies were removed from the cell-antibody complex by 8 minutes of centrifugation at 300 g at 4^{o}C. After discarding the supernatant cell-antibody complex were resuspended in 1 ml of 1% BSA-PBS. Twenty five micro liter Dynabeads (Cat. No. 11041, Invitrogen, Norway) were added and the suspension was incubated for 20 minutes on a rotary mixer at 4^{o}C. Antibody-bound cells were collected by placing the sample vials into the DynaMag™ (Cat. No. 12321D, Life Technologies,

U.K) for 2 minutes to attach cells to the wall and thereafter withdrawing of the supernatant. A second washing including a magnetic separation step was performed with 1 ml of 1% BSA-PBS followed by a suspension of pBMEC in 1 ml of 1% BSA-PBS. A 7 µl aliquot was removed to perform pBMEC count using Neubauer chamber (Model-Double Silverline, Rohem, India).

Statistical analysis

The data on various parameters was subjected to statistical analysis using SPSS (Statistical Package for Social Sciences, Version 20.0) software. Descriptive statistics specifying Mean±S.Em, highest value, and lowest value were calculated for each group. One way ANOVA procedure was undertaken to compare means. Post Hoc multiple comparisons were made using Duncan Multiple New Range Test (DMNRT). Independent sample t-test was used for two group comparisons. Bivariate correlations were calculated using Pearson Correlation coefficient. The size of correlation (Very high, high, moderate, low and negligible) was interpreted as per the classification given by Hinkle *et al.* (2003).

RESULTS AND DISCUSSION

Milk SCC depends mainly on immune status of the udder and also on various non genetic factors. The mean SCC, pBMEC (×1000/ml milk) and their correlation with TDMY among different groups have been tabulated in Table 1. The lowest and highest SCC recorded among S15, S60, M15 and M60 group ranged from (184.38 to 300.00), (175.00 to 231.25), (193.00 to 262.50) and (153.00 to 237.00) thousands per ml of milk respectively. The mean difference of SCC differed significantly ($P \leq 0.05$) with advancement of lactation in both Surti and Mehsani buffaloes. However, significant differences between breed were observed only at day 15 pp. In present study, the SCC was found to be higher in Surti buffaloes as compared to Mehsani buffaloes. This may be attributed to agro-climatic differences of higher rainfall and humid conditions during study period in home tract of Surti buffaloes compared to Mehsani buffaloes. The SCC observed in the present study for all the milk samples was less than the standard set by European Union Directives (92/46 CEE and 94/71 CEE) of 400000 somatic cells/ml of raw buffalo milk (Sharma *et al*., 2011)chemical and bacteriological changes in the milk and pathological changes in the glandular tissue of the udder and affects the quality and quantity of milk. The bacterial contamination of milk from the affected cows render it unfit for human consumption and provides a mechanism of spread of diseases like tuberculosis, sore-throat, Q-fever, brucellosis, leptospirosis etc. and has zoonotic importance. Somatic cell count (SCC. Thus, all the milk samples used in present study were expected to be produced from healthy quarters of buffaloes and were used for further down step processing. The overall mean SCC reported in present study for different groups were comparable with the average SCC of $1.99\pm0.03\times10^5$ cells/ml with the range of 1.86×10^5 to 2.12×10^5 cells/ml of milk reported from healthy quarters of buffaloes (Nandi, 2010). Similarly, Silva and Silva (1994) reported that total SCC in milk from normal buffaloes varied from 0.5×10^5 to 3.75×10^5 cells/ml. Mean SCC of $3.21\times10^5\pm0.18$ cells/ml higher than present study had also been reported in milk from normal buffalo (Patil *et al*., 2015). Contrastingly, Kavitha *et al*. (2009) observed lower mean normal SCC of 1.6×10^5 cells/ml in buffalo milk. Dhakal (2006) reported the SCC of milk from clinically

normal Murrah buffaloes to be 1.51×10^5 cells/ml in his study carried out under Nepal and Indian conditions in 240 mammary quarters from 60 buffaloes.

The decrease in mean SCC with advancement of lactation was to the tune of 18.72% in Surti and 16.84% in Mehsani buffaloes from day 15 to 60 pp. The mean SCC was 13.35% higher at day 15 pp in Surti buffaloes as compared to Mehsani buffaloes. The significant decrease in SCC with advancement of lactation in S60 and M60 group compared to S15 and M15 group was in accordance with rapid decrease in SCC reported for early lactating cows (Muggli, 1995). Almost similar reports of 46.08% decrease in SCC from day 15 day pp to 60 days postpartum had also been reported in cows (Sigl *et al*., 2012). Decreased in SCC during first three months of lactation and constant figures thereafter had been reported in Italian buffaloes (Sarubbi *et al*., 2013). Higher SCC during initial phase of the lactation had also been reported (Shook and Schutz, 1994). Similarly, Ceron-Munoz *et al*. (2002) reported that SCC decreased in the second month of lactation and increased thereafter up to the 9th month. In accordance with the present study Rodriguez-Zas *et al*. (2000) had also reported that SCC decreased in the first 60 day of lactation and then increased.

There was no significant difference observed for mean pBMEC obtained from total somatic cells within or between breeds. The mean pBMEC recovery percent from average total somatic cells were 2.13, 2.82, 2.79 and 3.45% in S15, S60, M15 and M60 groups respectively. The difference between two stages of lactation within and between breed was again found to be non-significant. Varying proportion of SCC in the milk had been reported in different species, breeds and at different stages of lactation with or without increased shedding of pBMEC (Boutinaud and Jammes, 2002). The mean pBMEC recovered (×1000/ml milk) from total somatic cells in present study were also comparable in S15 (5.39±0.55), S60 (5.71±0.47), M15 (6.12±0.5) and M60 (6.3±0.29) groups. However, lower pBMEC that ranged from ($1.10\pm0.06\times10^3$ to $1.40\pm0.03\times10^3$ cells/ml milk) at different stage of lactation had also been reported in cows (Sigl *et al*., 2012). Thus, it can be concluded that pBMEC count/ml in buffaloes milk under present study is higher as compared to earlier reported pBMEC count/ml in cow milk.

In present study pBMEC were successfully recovered from milk (1,600 ml) of all the groups using indirect method by antibody mediated magnetic separation. It has been shown in several studies that milk secreting cells can be purified from milk using immuno-magnetic separation (Alcorn *et al*., 2002; Feng *et al*., 2007; Boutinaud *et al*., 2008, 2012). The antibody mediated binding affinity of cytokeratin 8, a unique marker of pBMEC was successfully utilized in present study using primary mouse monoclonal antibody against cytokeratin 8 antibody, clone C-43 and subsequent magnetic separation using Dynabeads PanMouse IgG. Usage of similar and other unique markers found on the surface of milk secreting cells had earlier been also reviewed and supported (Gomm *et al*., 1995).

(Boutinaud *et al*., 2008) capacity for lactose synthesis, and cell death in mammary epithelial cells (MEC had also isolated lower 1.54×10^3 pBMEC/ml in Holstein-Friesian cow's milk around day 162 pp which comprised 2% of total milk cells. The percentages of pBMEC in relation to total milk cells among different groups in present study were steady with non-significant increase as lactation advances from day 15 to 60 pp. Contrary to this, significant differences in

Table 1. Mean somatic cells, primary bovine mammary epithelial cells count *vis-à-vis* their correlation with test day milk yield among different groups.

Cells/ Groups	SCC (×1,000/ml)			pBMEC (×1,000/ml)		
	Mean±S.E	Correlation		Mean ± S.E	Correlation	
		CMY	TDMY		CMY	TDMY
S15	249.04^{c}±9.96	0.27	0.37	5.39±0.55	0.19	0.20
S60	202.41^{ab}±4.83	0.13	0.17	5.71±0.47	0.22	0.18
M15	219.71^{b}±5.65	0.29	0.17	6.12±0.5	0.40	0.33
M60	182.7^{a}±6.42	0.29	0.24	6.30±0.29	0.41	0.46
F-value	16.17**			0.78		

CMY: Cumulative milk yield **highly significant at P≤0.01, N= Number of observations

percentage of pBMEC in relation to total milk cells were reported in cows (Sigl *et al*., 2012) whereby, percentage of pBMEC increased between day 8 pp (2.0±0.2 percent), day 43 pp (5.6±0.8%, P<0.001) and day 57 pp (6.7±1.05%). This may be attributed to species difference in said study. In present study, the mean percent pBMEC recovered from total cells ranged between 2.16 to 3.45% in milk of Surti and Mehsani buffaloes. Thus, in present study pBMEC shed continuously in small proportion with respect to total somatic cells. Similarly, despite the constant discharge of pBMEC, it was well established that fraction of pBMEC of total milk cells was low (Miller *et al*., 1991; Boutinaud and Jammes, 2002).

The correlations of SCC and pBMEC with CMY and TDMY were found to be non significant (P>0.05). However, pBMEC exhibited higher correlation as compared to SCC with CMY and TDMY respectively among all the groups except for Group S15. Highest correlations of SCC and pBMEC with CMY and TDMY were shown by Group M60. The overall correlation between pBMEC with milk yield of Surti and Mehsani buffaloes found in present study was negligible to low and non significant in contrast to previous studies in cows (Sigl *et al*., 2012).

CONCLUSION

The pBMEC can be successfully recovered from milk of Surti and Mehsani buffaloes using antibody mediated magnetic bead separation method. The recovery of SCC differ significantly with advancement of lactation however recovery of pBMEC remain comparable over lactation. The overall correlation results depicted that both SCC and pBMEC were not good indicator of milk yield in Surti and Mehsani buffaloes.

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